THE BIOLOGY, DIVERSITY AND EVOLUTION OF THE BROAD HOST-RANGE, PROMISCUOUS INCQ PLASMIDS, WITH AN EMPHASIS ON THE INCQ2 SUB-FAMILY

By

Douglas Eric Rawlings

Collection of manuscripts presented in partial fulfilment of the requirements for the Degree of Doctor of Science in Microbiology at the University of Stellenbosch

Supervisor: Prof W. H. van Zyl

December 2014

Declaration

I, Douglas Eric Rawlings, hereby declare that the work contained in this collection of manuscripts is original work carried out by myself as well as by several research students and post-doctoral researchers who have worked under my supervision. I have not previously, in entirety or in part, submitted it at any university for a degree.

Signature December 2014

Acknowledgements

I wish to acknowledge the support of many people who have contributed to the studies reported in this collection of published manuscripts.

Firstly, there are the research students and a post-doctoral researcher who worked on the projects. These are in chronological order, Inge-Martine Pretorius (PhD), although most of her work was not plasmid-related and has not been included, Rosemary Dorrington (PhD), Soraya Bardien (BSc Hons), Jurg Rohrer (PhD), Anne-Marie Clennel (BSc Hons), Anthony Smith (PhD), Leonardo van Zyl (MSc), Murray Gardner (PhD), Gwynneth Matcher (post-doctoral fellow) and Wesley Loftie-Eaton (PhD).

Then a special thanks to Dr Shelly Deane who began working with me as a post-doctoral researcher when I was at the University of Cape Town, came with me to the University of Stellenbosch and continued to support me both as a research associate and a laboratory manager for approximately 20 years. Her support contributed substantially to my being able to serve on several senior university committees and in a number of managerial roles.

To my wife, Janet, with whom I celebrated 40 years of married life in December 2013, for many years of faithful support to myself and our three children.

To my late parents Ted and Dorothy who passed away in May 2009 and June 2012 respectively. My father having received very little formal education himself (he joined the air force during the second world war before completing his schooling), nevertheless realised the importance of education and enabled me to receive a university education debt-free.

To my children and their families, Barry and Wendy, Kim and John, Sarah-Jane and our grandchildren, James, Jack, Christopher, Diya and Timothy for the joy that they are to Janet and myself.

To colleagues in the Department of Microbiology at the University of Cape Town (now amalgamated into Molecular and Cell Biology) and the Department of Microbiology at the University of Stellenbosch that provided wonderful environments in which to work. The support received from staff of the Department of Microbiology at the University of Stellenbosch, and for which I had the privilege to serve as Chairperson from July 1998 to December 2011, has been especially appreciated.

To the funders of this work, mainly piggy-backed onto other work supported chiefly by Genmin, Gencor, Billiton, BHP Billiton, the Foundation for Research Development (Pretoria) which became the National Research Foundation, the Universities of Cape Town and Stellenbosch and BioMinE project of the European Framework 6 research support program.

To Professor Emile van Zyl, current Chair of the Department of Microbiology, who besides being a colleague for many years, acted as supervisor and managed the submission of this body of work.

Contents

Gene, **108:** 7-14.

2.5 Rohrer, J. and Rawlings, D.E. 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol., **174:** 6230-6237. 51

2.6 Rohrer, J. and Rawlings, D.E. 1993. Regulation of the mobilization region of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol., **9:** 1051-1059. 59

2.7 Clennel, A-M., Johnston, B. and Rawlings, D.E. 1995. Structure and function of Tn*5467*, a Tn*21*-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid, pTFFC2. Appl. Environ. Microbiol. 61: 4223-4229. 68

2.8 Smith, A.S.G.S. and Rawlings, D.E. 1997. The poison-antidote stability mechanism of the broad-host-range *Thiobacillus ferrooxidans* plasmid, pTF-FC2. Molecular Microbiology **26:** 961-970. 75

2.9 Smith, A.S.G.S. and Rawlings, D.E. 1998. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain and antidote degradation requires the Lon protease. J. Bacteriol. **180:** 5458-5462. 85

2.10 Smith, A.S.G.S. and Rawlings, D.E. 1998. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (*pas*) is essential for plasmid stabilization. J. Bacteriol. **180:** 5463-5465. 90

2.11 Rawlings, D.E. 1999. Proteic toxin-antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2. FEMS Microbiology Letters **176:** 269-277. 93

2.12 Gardner, M.N., Deane, S.M. and Rawlings, D.E. 2001. Isolation of a new broad host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. **183:** 3303-3309. 102

2.13 Rawlings, D.E. and Tietze, E. 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiology and Molecular Biology Reviews **65:** 481-496. 109

2.14 van Zyl, L.J., Deane, S.M and Rawlings, D.E. 2003. Analysis of the mobilization region of the broad host-range IncQ-like plasmid pTC-F14 and its ability to interact with a related plasmid, pTF-FC2. J. Bacteriol. **185:** 6104-6111. 125

2.15 Deane, S.M. and Rawlings, D.E. 2004. Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad-host-range IncQ-like plasmids. J. Bacteriol. **186:** 2123-2133. 133

2.16 Gardner, M.N. and Rawlings D.E. 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiology **150:** 1797-1808. 144

Summary

Plasmids belonging to the IncQ family have an exceptionally broad host-range and are highly mobilizable in the presence of the self-transmissible IncP plasmids. All IncQ plasmids identified to date have certain features in common. The feature that distinguishes them most from all other plasmids is that they have a unique mechanism of replication. Their replicons consist of *repA*, *repB* and *repC* genes encoding a replicase, primase and DNA-binding proteins respectively. All IncQ plasmids contain at least three 22-bp iterons (or 20-bp iterons with 2-bp spacers) that are identical in sequence and to which the RepC DNA-binding protein binds. They replicate by means of a unique strand-displacement mechanism that is considered to place a limit on their size. Replication proceeds by a partially single-stranded intermediate that is believed to result in an increased likelihood of structural instability with an increase in plasmid size. The most compact backbone of IncQ plasmids is approximately 5.9-kb and the largest natural IncQ plasmid reported is 14.2-kb.

Although the mobilization regions of IncQ plasmids are not as unique as the replicons, they are all characterized by the primase of the replicon being fused to the relaxase of the mobilization genes. The remainder of the mobilization genes may vary substantially in number and sequence between plasmids and have been subdivided into at least four distinct lineages.

This dissertation consists of twenty one manuscripts published during the period 1984 to 2012. The focus is almost entirely on the IncQ plasmid subfamily known as IncQ2. Most of the earlier work was on determining the nature and extent of the replicons, mobilization genes and the toxin-antitoxin plasmid stability system. A strong theme in the latter work focussed on using the natural variation among the IncQ2 plasmids as a means to understand IncQ plasmid evolution. The collection of articles comprises a combination of original research and reviews.

Opsomming

Plasmiede wat aan die IncQ familie behoort kom ‘n uitsonderlike wye gasheerselreeks voor en is hoogs mobiliseerbaar deur middel van die selfoordraagbaar IncP plasmiede. Alle IncQ plasmiedes wat tot datum identifiseer is het sekere gemeenskaplike eienskappe. Die eienskap wat hulle van alle ander plasmiedes onderskei is hul unieke dupliseringsmeganisme. Hul dupliseringsmeganisme bestaan uit *repA*, *repB* en *repC* gene wat onderskeidlik ‘n helikase, ‘n ‘primase’ en ‘n DNS-bindingsproteïen enkodeer. Die IncQ plasmiede het ten minste drie 22-bp iterone (of 20-bp iterone met 2-bp skeidingsnukleotiede) met ‘n identiese nukleotiedvolgorde en waaraan die RepC-bindingsproteïen bind. Hulle dupliseer deur middel van ‘n unieke DNA-string-vervangingsmeganisme wat ‘n beperking op hul grootte plaas. Tydens replikasie word ‘n intermediêre struktuur gevorm wat gedeeltelik enkelstring is en dit is blykbaar die rede vir ‘n verhoging in strukturële onstabilitiet as die plasmied groter word. Die kleinste ruggraat onder die IncQ plasmiede is min of meer 5.9-kb en die grootste natuurlike IncQ plasmied wat gerapporteer is, is 14.2-kb.

Alhoewel die mobiliseringsgebied van die IncQ plasmiede nie so duidelik uitkenbaar as die replikons nie, hierdie gebied is gekenmerk deur ‘n ‘primase’ wat aan ‘n ‘relaxase’ in die mobiliseringsgene gekoppel is. Die oorblywende mobiliseringsgene verskil in beide getal en nukleotiedvolgorde tussen plasmiede en is gebruik om die plasmiede in vier duidelike oorsponggroepe in te deel.

Hierdie proefskrif bestaan uit een-en-twintig artikels wat tussen 1984 en 2012 gepubliseer is. Die fokus is hoofsaaklik op die IncQ plasmiedsubfamilie wat as IncQ2 bekend is. Baie van die vroeër werk het oor die aard en omvang van die duplisering en mobiliseringsgene asook die toksien-teentoksien plasmiedstabiliseringsmeganisme hanteer. ‘n Sterk tema in die latere werk was om die natuurlike variasie onder die IncQ2 plasmiede te bestudeer ten einde IncQ plasmiedevolusie te verstaan. Die publikasie versameling bestaan uit ‘n kombinasie van oorspronklike navorsing en oorsigartikels.

Chapter 1. The biology, diversity and evolution of the broad host-range, promiscuous IncQ plasmids.

1.1 Introduction to the IncQ plasmids.

Plasmids belonging to the *Escherichia coli* incompatibility group Q (IncQ) family are fairly small in size (5.1 to 14.2-kb) and capable of replication in a very broad range of hosts including many Gram-negative and Gram-positive bacteria (Rawlings and Tietze 2001, Loftie-Eaton and Rawlings, 2012). The genetic organization of the IncQ plasmids is such that the plasmid backbone can be viewed as consisting of at least two modules, a replicon and a mobilization region. All IncQ plasmids identified to date have this structure.

A feature of the most studied replicon of the IncQ plasmid family (RSF1010, R1162 and related plasmids, Figure 1.1) is that it replicates by a strand-displacement mechanism that, as far as is known, is unique to the IncQ plasmids (Sakai and Komano, 1996). This is such a unique characteristic, that for a plasmid to be considered as belonging to the IncQ-family it would need to possess this replication mechanism. Although there are some features of IncQ family replicons that vary (reviewed in Loftie-Eaton and Rawlings 2012), all IncQ-family replicons encode for at least three proteins and have an *oriV* region with a minimum three 20-bp iterons that are spaced 22-bp apart. The three essential replication proteins are a RepA helicase, a RepB primase and a RepC iteron-binding protein. Replication by the strand-displacement mechanism appears to have allowed for the very broad host-range of IncQ plasmids but also to have placed a size limitation on IncQ plasmids of about 15-kb or less. This is because replication proceeds from two single strand initiation sites, one on each of the strands and in both directions (Tanaka et al., 1994, Zhou and Meyer, 1990). Only one of the strands in each direction is synthesized and there is no lagging-strand, Okazaki fragment synthesis. At the point where the two replication forks pass each other, the replication intermediate will consist of a maximum amount of displaced single-stranded DNA and from that point onwards the amount of single stranded DNA will decrease until both strands have been fully replicated. It is the structural instability that results from having up to half of each strand in a transient single stranded form that is believed to be the reason for the limited size of IncQ plasmids.

All IncQ plasmid replicons that have been identified to date have a primase gene that is required for replication fused to the relaxase gene of a mobilization gene-containing cassette that is located immediately upstream of the replicon. However, the mobilization genes of different IncQ plasmid families are unrelated to each other and at least four lineages of mobilization genes have been identified (Loftie-Eaton and Rawlings, 2012). Based primarily on the lineage of mobilization genes to which the replicon has been fused, the IncQ plasmids have been allocated to four subgroups, IncQ1, IncQ2, IncQ3 and IncQ4. Other plasmid features support this division into four plasmid subgroups as will be described later (reviewed in Loftie-Eaton and Rawlings 2012).

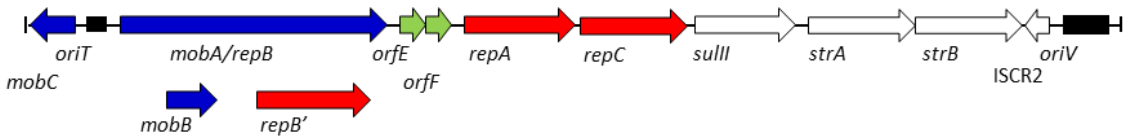


Figure 1.1 The genetic map of the IncQ1 plasmid RSF1010/R300B/R1162 (8.7-kb)

1.2 Structure of this dissertation.

The biology of IncQ plasmids has been reviewed by myself and Erhard Tietze in *Microbiology and Molecular Biology Reviews* (Rawlings and Tietze, 2001), by Richard Meyer in *Plasmid* (Meyer, 2009) and by Wesley Loftie-Eaton and myself in *Plasmid* (Loftie-Eaton and Rawlings 2012). For this reason it was felt that there was little to be gained from again reviewing the IncQ plasmids, particularly as little additional experimental work has been carried out since the 2009 and 2012 reviews.

Instead, the research that comprises this body of this DSc submission will be contextualized and its significance discussed. The final chapter will focus on some interesting issues that have arisen during the course of this work, many of which require further investigation.

1.3 Background to my personal involvement with IncQ plasmids.

In the early 1980's no genetic system existed for the genus of Gram-negative, industrially important biomining-involved bacteria, then known as *Thiobacillus* (now *Acidithiobacillus*). At the time, the most important species among the acidithiobacilli was considered to be *At. ferrooxidans*. This bacterium is an autotrophic, acidophilic, iron- and sulphur-oxidizing bacterium that has a very different physiology from *Escherichia coli*, the heterotrophic and comparatively neutrophilic bacterium for which a genetic system existed. To develop a genetic system one requires a cloning vector, a selectable marker such as a gene for antibiotic or metal ion resistance and a method for introducing DNA into cells of the target species. It was unknown whether cloning vectors, selectable markers and transformation procedures developed for *E. coli* would work in *At. ferrooxidans* especially since their physiologies were so different. It is very difficult to develop a genetic system when there are many unknown variables, any one of which could prevent the successful transformation of the target cells or the identification of transformants. To reduce the number of these unknowns it was decided to identify strains of *At. ferrooxidans* that contained plasmids as these should be a source of replicons that would be expected to be able to function as cloning vectors for the bacterium. A number of strains were screened for plasmids, the plasmids were isolated and subjected to restriction endonuclease site mapping so as to identify restriction endonucleases that cut only once in the plasmid. The first two plasmids isolated and cloned were an approximately 19.6-kb plasmid, named pTF35 (ex *At. ferrooxidans* strain 35) (Rawlings *et al*., 1983) and a 12.2-kb plasmid, pTF-FC2 (the middle-sized of three plasmids from strain, FC that had been selected by growth on Fairview mine arsenopyrite concentrate) (Rawlings *et al*., 1983).

Once pTF-FC2 had been cloned into pBR325 and mapped it became clear that in the case of one of the cloned orientations, if a *Sal*I/*Xho*I deletion was constructed, it would remove the pBR325 replicon plus approximately 300-bp of pTF-FC2 and leave a selectable chloramphenicol resistance gene marker plus the bulk of pTF-FC2. I carried out the cloning, mapping and deletion experiments myself and I will never forget coming into work early in the morning after constructing the deletion and seeing colonies on the plate. Before getting too excited, I prepared a dozen or so colonies for the 'minipreparation' of DNA from the plasmids and carried out the analysis of these plasmids the next day. An agarose gel was prepared in which a restriction endonuclease digest of the original cloned plasmid was compared with the putative deletion variants. It was clear that the deletion had been successfully made. This implied that the deletion plasmid was capable of replication in *E. coli* from a replicon located on the pTF-FC2 plasmid. This experiment was carried out in 1983 and it was a simple finding relative to later work, but even now more than 30 years later, the excitement generated by that result remains with me as one of the most memorable results of my career. The work was published as a note in the Journal of Bacteriology the following year (Rawlings *et al*., 1984). The plasmid was not studied further at the time, but in the late 1980s and 1990s it became the subject of several PhD and one or two MSc theses.

After pTF-FC2 was sequenced (Figure 1.2) it became clear that it was a second member of the IncQ plasmid family that at the time consisted of only the identical plasmids RSF1010 and R300b as well as the almost identical plasmid R1162. Plasmid RSF1010 was the subject of an extensive body of research by several workers including Michael Bagdasarian, Eberhard Scherzinger, Neil Willets and their colleagues, while research on R1162 became the life's work of Richard Meyer. The major difference between the initially discovered IncQ plasmids and pTF-FC2 was that the mobilization genes of pTF-FC2 were distinctly IncP-like and different from RSF1010 and R1162. Almost 15 years later we identified and cloned a second IncQ-family plasmid from a different species of *Acidithiobacillus*, that is, from *At. caldus*. This 14.1-kb plasmid, pTC-F14, though sufficiently related to pTF-FC2 to suggest that they had originated from the same ancestral plasmid was different enough to enable us to ask some important questions regarding the evolution of the IncQ plasmids. This work has formed the subject of several research theses, manuscripts and reviews.

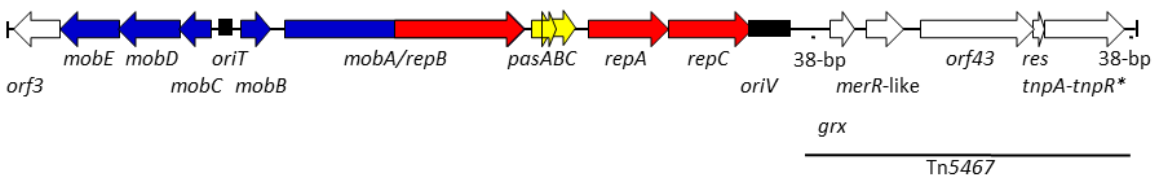


Figure 1.2 The genetic map of the IncQ2 plasmid pTF-FC2 (12.2-kb)

In 2002, the sequences of two related plasmids pRAS3.1 and pRAS3.2 that had been isolated from the fish pathogen *Aeromonas salmonicida* in Norway were published (L'Abee-Lund, T.M., Sørum, H., 2002). These two plasmids clearly belonged to the IncQ2 subgroup and surprisingly most of their features were more closely related to pTF-FC2

than to pTC-F14 even though the latter two plasmids were isolated from different species of the same genus of bacteria (*At. ferrooxidans* and *At. caldus*) and both from South Africa. As these workers had been interested in antibiotic resistance (both plasmids had identical tetracycline resistance genes) and not in the biology of the plasmids, we were provided with samples of both plasmids. Research on the IncQ2 plasmids pTF-FC2, pTC-F14 plus pRAS3.1 and pRAS3.2 constitutes the main body of research for this DSc submission.

1.4 Own contribution to IncQ plasmid biology.

As pointed out above, several researchers have been involved in carrying out research on what have become known as the IncQ1 plasmids. However, all published work on the biology of the IncQ2 plasmid subgroup has originated in my own research group and to date nobody has studied the biology of the more recently identified IncQ3 and IncQ4 subgroups (Loftie-Eaton and Rawlings, 2012).

The work on pTF-FC2 was published during the period 1984 and 1999 and covers research on the replicon (Rawlings *et al*., 1984; Dorrington and Rawlings, 1989, 1990; Dorrington *et al*., 1991), the mobilization genes (Rohrer and Rawlings, 1993 and 1994) and the toxin-antitoxin system (Smith and Rawlings, 1997, 1998a and 1998b) including an invited short review on TA systems (Rawlings 1999). One manuscript was published on the defective transposon present on pTF-FC2 (Clennel *et al*., 1995) that had been allocated the designation Tn*5467* by Esther Lederberg shortly before she stopped carrying out the world-wide task of allocating numbers to transposons.

Much of the work on the identification and study of the replicon, mobilization region and stability system of pTC-F14 had a somewhat different focus. Since pTF-FC2 and pTC-F14 would be expected to have arisen from the same ancestral IncQ2 plasmid, this allowed us a rare opportunity to gain insights into the way in which these plasmids have evolved from their original ancestor. We investigated to what extent their replication (Gardner and Rawlings, 2001 and 2004) and mobilization (van Zyl *et al*., 2003) genes plus the *oriV* and *oriT* sites on which these gene products act, are able to still complement each other. The same applied to the related toxin-antitoxin (TA) stability systems (Deane and Rawlings, 2004), originally denoted as a plasmid addiction *s*ystem (*pas*). Given that this work was spread among several manuscripts an attempt was made to ensure that the evolutionary significance of this work was not missed. To that end a review was written that pulled together mainly the evolutionary aspects of this work (Rawlings, 2005).

Subsequent to this review we established that the pRAS3 plasmids had a completely unrelated toxin-antitoxin system to the *pas* of pTF-FC2 and pTC-F14 but in exactly the same position. This led to the hypothesis that since all three examples of the IncQ2 plasmids had a toxin-antitoxin system immediately upstream of the *repAC* genes, the toxin-antitoxin system was not only a post-segregational stability system but had an additional biological function as a result of its position within the replicon. This led to a rather challenging study in which evidence was obtained that although the absence of a TA system upstream of the *repAC* genes did not affect the maximum plasmid copy number (PCN), its presence resulted in a rapid increase in replication when the copy number fell below a threshold (Matcher and Rawlings, 2009). The autoregulated, strong promoter of the TA genes became derepressed when the copy number dropped too low

and this was responsible for a rapid but transitory increase in *repAC* expression with a corresponding rapid increase in copy number. Should the TA system have been present elsewhere on the plasmid or in the opposite orientation, the rapid kick-start of replication from the strong PA promoter would not have occurred.

The existence of plasmids pRAS3.1 and pRAS3.2 allowed us to address some different and much more precise plasmid evolution questions. These two plasmids have identical backbone sequences with the exception of two small differences. Plasmid pRAS3.1 has five 6-bp repeat sequences in the promoter region of the *mobB* gene and 4 x 22-bp iterons in its *oriV* region whereas pRAS3.2 has only four 6-bp repeats and 3 x 22-bp iterons. It was found that the extra 6-bp repeat upstream of pRAS3.1 increased the expression of *mobB* and the rest of the replication genes resulting in a doubling of the PCN per chromosome from 30 to 59 (Loftie-Eaton and Rawlings 2009) and that the acquisition of the extra 22-bp repeat served to reduce the copy number to about 40. Plasmid pRAS3.1 with its 4 x 22-bp iterons could rapidly displace pRAS3.2 with its 3 x 22-bp iterons when both existed in the same cell. However, pRAS3.1 placed a higher burden on its host than did pRAS3.2 and its host was less competitive. This possibly explains why pRAS3.1 has not completely displaced pRAS3.2 from the environment.

The body of this DSc consists of twenty one original research publications and reviews on IncQ plasmids. A short commentary will be given below each publication.

1.5 Context of and comments on each manuscript included in this work.

1.5.1 The replicon of pTF-FC2

Rawlings, D.E., Pretorius, I-M. and Woods, D.R. 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158: 737-738. (ISI Web of Science Citations 44)

This manuscript created great excitement as it was the first time that any genetic feature from *T. ferrooxidans*, that at the time was considered to be the most important biomining microorganism, was expressed in *E. coli*. The work was published as a note and is a report that the cloned pTF-FC2 was capable of replication in *E. coli* even after the ColEI origin of replication of the pBR325 cloning vector had been deleted.

Dorrington, R.A. and Rawlings, D.E. 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol., 171: 2735-2739. (Citations 25)

This manuscript describes the search for the minimum replicon on the 12.2-kb plasmid pTF-FC2. The initially reported size of 12.4-kb for the plasmid was based on the use of agarose gels to size the restriction endonuclease digestion fragments and after complete sequencing, this turned out to be an overestimate (exact size 12184 bp). We showed that unlike the ColEI-based cloning vector, replication of pTF-FC2 in *E. coli* was *polA* independent. By using this difference the *oriV* region was identified, sequenced and shown to contain the incompatibility determinants (3 x 22-bp iterons). It was during this

work that the similarity of pTF-FC2 with the IncQ plasmids R1162 and RSF1010 was first recognized. The full sequence of none the three plasmids was available at the time.

Dorrington, R.A. and Rawlings, D.E. 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol., 172:5697-5705. (Citations 32)

In this manuscript the basic replicon was found to be on a 3202-bp fragment by making a *Sau*3A partial digest of pTF-FC2, ligating the fragments into pUC19 and selecting for the smallest fragment that could replicate in an *E. coli polA* mutant. In addition it was found that the basic replicon could replicate in *Pseudomonas aeruginosa*. The basic replicon was sequenced, five genes were identified and it was shown that all five were expressed in *E. coli* by identifying a protein product from each using an in vitro transcription-translation system. Two of these were closely related to *repA* and *repC* of RSF1010 (R1162). The other three small genes were located upstream of *repA* and *repC* and were unrelated to any other genes in the data base at that time. Although we had no idea what the function of the small genes was, we showed that if deleted, they negatively affected the stability of the basic replicon in *P. aeruginosa*. Much later we discovered that these were TA post-segregational stability genes and this explained why their removal had reduced stability in *P. aeruginosa* (this was less noticeable in *E. coli*). The *oriV* region was immediately downstream of *repC* in pTF-FC2 whereas in the case of RSF1010/R1162 the *oriV* was dislocated from the *repAC* genes, as the genes for sulphonamide and streptomycin resistance had been inserted between *repC* and *oriV*.

Dorrington, R.A., Bardien, S. and Rawlings, D.E. 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene, 108: 7-14. (Citations 16)

Once we had identified what we thought was the replicon of pTF-FC2 we tried to construct cloning vectors for *At. ferrooxidans* initially using only the basic replicon and the *E. coli* Tn*9* chloramphenicol resistance gene as a selectable marker. We struggled to do this and after much frustration it was found that as soon as the pUC19 cloning vector was deleted, the resulting plasmid became non-viable. Therefore, some function on pUC19 was required (even when in an *E. coli polA* mutant) and this requirement was rendered unnecessary when pTF-FC2 was co-resident. Eventually we realised that another gene immediately upstream of the three small genes of pTF-FC2 was required. This gene was shown to encode a 40-kD protein and using a replication deficient M13 phage we were able to demonstrate that the required gene (*repB*) functioned as a primase. Once this gene was included, the pTF-FC2 replicon was able to function independently of the *ColEI* vector replicon and we were able to construct a series of cloning vectors (not included in this DSc). What this suggested is that even though the ColEI replicon was not able to support replication in an *E. coli polA* host or *P. aeruginosa*, it nevertheless provided a priming site for replication that the host primase was able to recognise. This priming site together with the products of the *repA* (helicase) and *repC* (DNA-binding) genes enabled the partial pTF-FC2 replicon to replicate in the absence of its own *repB* (primase-encoding) gene.

1.5.2 The mobilization region of pTF-FC2

Rohrer, J. and Rawlings, D.E. 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol., 174: 6230-6237. (Citations 47)

A 5317-bp fragment of pTF-FC2 that contained the origin of transfer (*oriT*) and all of the genes required for mobilization between strains of *E. coli* by the self-transmissible IncP plasmids (RP4 or RK2) was identified. The sequence and features encoded on this fragment were unrelated to that of the IncQ plasmids. An ordered set of deletions from both sides of the region required for mobilization identified five genes that were essential for mobilization or affected the mobilization frequency as well as a 184-bp fragment containing the *oriT* to be identified. The genes that were essential for mobilization were designated *mobA*, *mobB* and *mobC*, while the two genes that were not essential, but enhanced the frequency of transfer between *E. coli* hosts, were designated *mobD* and *mobE*. Polypeptides corresponding in size to the predicted products of all five ORFs associated with mobilization could be identified using an *E. coli* transcription-translation system. The predicted amino acid sequences of four of the polypeptides had weak though discernible similarity to four of the polypeptides encoded on the DNA-processing TraI region of RP4. These were MobA/TraI, MobB/TraJ, MobC/TraK and MobD/TraL. No sequence similarity to the pTF-FC2 MobE could be identified on RP4 or any other sequences in available databases.

Rohrer, J. and Rawlings, D.E. 1993. Regulation of the mobilization of the broad-host-range plasmid pTF-FC2. Mol. Microbiol., 9: 1051-1059. (Citations 3, although from a techniques perspective, in my view, one of the better five or six manuscripts of my career).

The five mobilization genes of pTF-FC2 were located on either side of the *oriT* with *mobA* and *mobB* being transcribed in one direction and *mobC*, *mobD* and *mobE* in the opposite direction. Regulated transcription in both directions was therefore required for mobilization. Furthermore, the mobilization region of pTF-FC2 lay upstream of the replicon and the gene for the RepB primase was fused to the gene for the MobA relaxase. This fusion is a common feature of all IncQ plasmids identified to date irrespective of to which of the four families of relaxase the primase has been fused. This means that expression of the genes of the replicon is dependent on expression of the mobilization genes in one of the directions (*mobBA-repB*). The regulation of the bidirectional mobilization genes is therefore of importance to both mobilization and replication. Primer extension studies were carried out to determine the start of transcription in both directions and it was found that the quantity of mobilization gene transcript in the direction away from the replicon (*mobCDE*) was at least 10 times greater than towards the replicon. This difference was even greater when gene fusion studies were used to determine the amount of translation in both directions. Unregulated expression in the *mobCDE* direction was approximately 300-fold greater than in the direction of *mobBA*. It suggested that expression of *mobCDE* was autoregulated with the product of *mobC* being the most effective repressor while the products of the *mobBA* genes had no effect on

regulation. Expression from the weak *mobBA* promoter appeared to be repressed mainly by the product of *mobA* (2-fold)*. The role of integration host factor (IHF) in mobilization was demonstrated as there was both a reduction in mobilization frequency and reduction in expression from the strong *mobCDE* promoter in the absence of IHF. An intrinsic bend in the DNA sequence within the *oriT* region in the vicinity of the putative IHF-binding site was shown. A replication defective M13 phage was used to demonstrate that there was a single strand initiation site in both directions within the *oriT* region.

The working model from these studies was that mobilization is most strongly regulated by controlling expression of the strong *mobCDE* promoter. Regulation from the *mobBA* promoter was less stringent, presumably because transcription in this direction is required for both mobilization and replication. What was still lacking was to determine whether there was any regulation from the products of the *repB*, *repA* or *repC* genes. This study was not carried out for pTF-FC2, though we did investigate this in the case of the related plasmid, pTC-F14 (Gardner and Rawlings, 2004).

Note: When working with pTC-F14, we found that the product of the *repB*, (primase), was a much stronger regulator of the promoter in the *mobBA* direction. The *repB* gene was not present in the experiments described in the Rohrer and Rawlings (1993) manuscript as when the set of ordered deletions was made to identify the minimum mobilization region, the *repB* gene could be deleted without affecting mobilization frequency. That RepB is the major regulator makes sense, as transcription in the *mobBA* direction is required for both mobilization and replication.

1.5.3 The pTF-FC2 toxin-antitoxin system

Smith, A.S.G.S. and Rawlings, D.E. 1997. The poison-antidote stability mechanism of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Molecular Microbiology 26: 961-970. (Citations 27)

As explained earlier, we did not know what the function of the three small genes upstream of the *repAC* genes of pTF-FC2 was. However, we assumed that they were involved in the regulation of replication because in the case of the IncQ plasmids RSF1010/R1162, the small *repEF* genes that are located in the same position were shown to have a regulatory function. Each of the three ORFs was inactivated by introducing a frame-shift mutation. When a mutation was introduced into the first of the three ORFs (at the time termed ORF3), the transformed *E. coli* JM105 cells grew extremely poorly whereas mutations in the second (ORF4) and third ORFs (ORF5) appeared to have little effect on plasmid replication or the host. When a cloned version of ORF3 was expressed *in trans* from a co-resident plasmid, growth of the host cells was restored. The copy number of the ORF3 frame-shift mutant plasmid in the poorly growing host appeared to be one per chromosome, while that of the wild-type, ORF4 or ORF5 mutants was approximately equal at 10-14 per chromosome. Surprisingly, a few of the cells from poorly-growing transformants containing the plasmid with the ORF3 mutation produced large normal-sized colonies. When plasmids from 36 of these normal-sized colonies were isolated, mapped and then sequenced, they were found to have undergone one of two spontaneous deletions. Either the whole of ORF3 and ORF4 and part of ORF5 had been deleted or only the promoter region of the three ORFs immediately upstream of ORF3

had been deleted. When the copy number of the deletions was determined, it was found to be indistinguishable from the wild-type plasmid. This suggested that the three small genes were not involved in plasmid regulation.

Further studies indicated that stability of plasmids with the deletion and frame-shift mutations was greatly reduced in comparison to the wild-type plasmid and furthermore, if the region containing ORF3, ORF4 and ORF5 was cloned into an unstable test plasmid (pOU82), the test plasmid was stabilized. By using an *E. coli* in vitro transcription-translation system we demonstrated that all three ORFs were expressed. By placing each ORF behind an IPTG inducible promoter individually and in combination and by monitoring the growth of host cells following induction we were able to demonstrate that the product of ORF4 was toxic, that ORF3 on its own (but not ORF5 on its own) partly neutralized the toxic effect but that with all three ORFs present, the host cells grew the best. These results were best explained if ORF4 encoded a toxin, ORF3 an antitoxin and ORF5 a protein that enhanced the neutralization of the toxin by the antitoxin. This interpretation also suggested that the reason why the frame-shift mutation in ORF3 made the host cells grow so poorly was that the antitoxin had been inactivated. We realised that we were working with a three-component, plasmid addiction (TA) system that we named *pas*. The *pas* TA proteins were sufficiently unique that only when a large number of gaps were introduced by manually aligning the ParD antitoxin of plasmid RP4/RK2 with the PasA antitoxin of pTF-FC2 could any similarity with previously reported toxin-antitoxin systems be detected. No similarly could be detected between the amino acid sequences of the toxins of the plasmids or any other proteins in public domain databases.

Smith, A.S.G.S. and Rawlings, D.E. 1998a. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. J. Bacteriol. 180: 5458-5462. (Citations 16)

Smith, A.S.G.S. and Rawlings, D.E. 1998b. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (*pas*) is essential for plasmid stabilization. J. Bacteriol. 180: 5463-5465. (Citations 15)

The above two manuscripts appear as back to back notes in the Journal of Bacteriology. They were submitted as a single manuscript as some of the methods, plasmid constructs and the general figure of the *pas* were the same. One of the reviewers suggested that the manuscript be split into two parts and the editor of the journal supported that suggestion. Nevertheless, I will deal with them together as both had to do with aspects of the *pas*.

In the first manuscript we showed that the ability of the toxin-antitoxin system to stabilize pTF-FC2 in *E. coli* was strain dependent with the increase in the level of stabilization being as high as 100-fold in some strains but with no detectable stabilization in others. We were in a good position to address this question because the spontaneous deletions of the *pas* had resulted in plasmid variants with the same copy number as the wild-type plasmid. These deletion plasmids could therefore serve as the necessary controls. Furthermore, we found that this strain variation in the ability of the *pas* to stabilize pTF-FC2 could be correlated with PasB toxicity in that the strains in which pTF-

FC2 was stabilized the most were most sensitive to the toxin and vice versa. *E. coli* has been shown to contain a large number of TA systems (Maisonneuve *et al*., 2011) of various types including at least 10 proteic TA systems. It is possible that there is some variation in these systems between strains and that some cross reaction between the antitoxins of those systems and the *pas* toxin occurs. This is speculative but could explain some of the strain variation observed. Another possibility is that there are strain variations in the (still unknown) target of the *pas* toxin such that the toxin is more effective against some variants of the target than others.

For a TA system to stabilize a plasmid it is necessary that antitoxin should have a half-life that is considerably shorter than the toxin. The final aspect of the first manuscript was to show that the Lon protease of the *E. coli* host was responsible for the turnover of the unstable antitoxin. In the case of a protease-proficient mutant or a Clp protease mutant, the forced loss of a *pas*-containing plasmid resulted in the inhibition of cell growth as would be expected if the antitoxin was degraded faster than the toxin. However, when an *E. coli lon* mutant was used as the plasmid host, the cells were not inhibited after forced plasmid loss. This is what would be predicted if the Lon protease was responsible for antitoxin degradation. Additional evidence was that a *pas*-containing plasmid was more stable in a protease or Clp protease mutant than in a Lon protease mutant.

In the second manuscript it was shown that *pas* expression was autoregulated with the PasA antitoxin functioning as the repressor (25-fold decrease in expression) and that this repression was enhanced in the presence of the PasB toxin (an additional 2-fold decrease). The presence of PasC did not affect *pas* expression. Expression of *pas* from its normal autoregulated promoter resulted in the stabilization of a test plasmid into which the *pas* had been cloned, but expression of the *pas* from a heterologous *tac* promoter did not result in an increase in stability of the test plasmid. Autoregulation appeared to be a requirement for the *pas* to stabilize a test plasmid. The reason it was important to show this is that it is possible that differences in the level of translation of the antitoxin (high-level of translation from a near consensus ribosomal binding site) and toxin (low-level of translation from a weak ribosomal-binding situated within the ORF for the antitoxin and out-of-frame), as well as differences in half-life of the antitoxin (short half-life) and toxin (long half-life) could by themselves be sufficient to increase plasmid stability.

Rawlings, D.E. 1999. Proteic toxin-antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2. FEMS Microbiology Letters 176: 269-277. (Citations 32)

This was an invited mini-review on proteic TA systems. I will not review the contents of the review here. Possibly the most significant part was speculation on how TA systems usually found on the host chromosome may have been acquired by plasmids from the host (or vice versa). The regulatory systems would have had to become modified (at least in some instances) to serve a new purpose. There would also be strong evolutionary pressure for the antitoxin and toxin found on chromosomes and plasmids to diversify as a TA system for which the host already has a functional antitoxin would not be effective at stabilizing a plasmid in that host. This is especially the case for promiscuous, broad host-range plasmids like the IncQ-family plasmids as they are likely to enter many different hosts and should therefore contain a unique TA system if it is to stabilize the plasmid. As

will be pointed out later, this may not be its only or even major purpose, but having a cross-reacting TA system on a host chromosome will affect the efficacy of a TA system whatever its purpose.

1.5.4 The transposon region of pTF-FC2

Clennel, A-M., Johnston, B. and Rawlings, D.E. 1995. Structure and function of Tn*5467*, a Tn*21*-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid, pTFFC2. Appl. Environ. Microbiol. 61: 4223-4229. (Citations 16)

This is not a philosophically sophisticated manuscript and so will be dealt with rather briefly. Plasmid pTF-FC2 was shown to contain an inactive transposon, Tn*5467*, with 38-bp flanking inverted repeats that were 37/38 and 38/38 identical to those of Tn*21*. Tn*5467* had retained only 15% of the *tnpA* gene encoding the transposase and 85% of the *tnpR* gene encoding a resolvase as well as a *res* (resolution) site. As a result the transposon was not capable of transposition into a plasmid trap used to 'catch' the transposon should it have been capable of transposition. However, when the Tn*21 tnpA* and *tnpR* genes were provided in trans, Tn*5467* was capable of transposition into the plasmid trap and the transposition conintegrate was able to be resolved due to the functional 'res' site. As Tn*21* and closely related transposons are widely distributed, it is possible that should pTF-FC2 enter a cell containing such a transposon (or vice versa), Tn*5467* could be transpositionally active.

Of most interest was whether there were any functional genes on the transposon, that may suggest what the presence of the pTF-FC2 could contribute to host fitness and whether any of these genes could possibly serve as a selectable marker for the development of a genetic system for the original plasmid host, *At. ferrooxidans*. Three ORFs were detected, one encoding for a small glutaredoxin-like protein, one for a MerR-like repressor protein and one for a putative 43 KDa protein with clear similarity to large family of membrane translocases. The glutaredoxin-like protein was expressed and active as it was capable of conferring a $TrxA^{+}$ phenotype to an *E. coli trxA* mutant. No product for the *merR*-like gene or the ORF encoding the putative 43 KDa protein that was situated immediately downstream of the *merR* could be detected in *E. coli*. However, in a subsequent project by an honours student it was demonstrated that when a promoterless β-galactosidase reporter gene was fused to the first few amino acids of ORF43, expression of the reporter gene responded to the presence of mercury (unpublished). Thus presumably, a small quantity of the *merR*-like gene product was expressed in *E. coli* in the absence of induction by mercury that was difficult to detect using protein gels. What the exact function of the 43 KDa membrane translocase-like protein is, remains a mystery.

1.5.5 Plasmid pTC-F14 from *Acidithiobacillus* ***caldus***

In 1997, the plasmid pTC-F14 was isolated from a local strain of *Acidithiobacillus caldus* from tanks that were treating an arsenopyrite concentrate at the Fairview mine (Barberton, South Africa). The plasmid was cloned by Dr Shelly Deane who was a post-

doctoral researcher in my laboratory. The bulk of this work was published in the following articles.

Gardner, M.N., Deane, S.M. and Rawlings, D.E. 2001. Isolation of a new broad host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. 183: 3303-3309. (Citations 25)

In this manuscript the isolation and analysis of pTC-F14 replicon was reported. The plasmid was clearly related to pTF-FC2 with the amino acid sequences of the *repA*, *repB* and *repC* gene products being 81, 78 and 74% identical, respectively. Furthermore, pTC-F14 had a *pas* consisting of only two proteins, PasA and PasB (unlike the three of pTF-FC2), that were 81 and 72% identical to the equivalent proteins of pTF-FC2. In spite of these similarities the two plasmids had evolved sufficiently apart for pTF-FC2 and pTC-F14 to be compatible with each other in an *E. coli* host. The sequences of the 22-bp iterons within the *oriV*s of the two plasmids were sufficiently diverged that neither plasmid could support replication from the *oriV* of the other. Unlike the 3 x 22-bp iterons of pTF-FC2, plasmid pTC-F14 had five iterons, with only three being exactly 22-bp in length and of identical sequence. The two flanking iterons were 21-bp and 23-bp long with a single bp deletion from or a single bp insertion into the otherwise sequence-identical iterons. At 12-16 copies per chromosome in both *E. coli* and *At. caldus*, the copy number of pTC-F14 was approximately the same as was found for pTF-FC2 in *E. coli*. The observation that pTC-F14 and pTF-FC2 were related but different opened the possibility of doing some fascinating studies on the evolution of the two plasmids as will be described later.

Rawlings, D.E. and Tietze, E. 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiology and Molecular Biology Reviews 65: 481-496. (Citations 64)

At around about the time that we were part way through working on the replicon of pTC-F14, I felt that there was enough new information and questions on IncQ plasmids besides what was known about the almost identical RSF1010 and R1162 plasmids to put together a review on the topic. Erhard Tietze had been working with IncQ1 plasmids that belonged to different incompatibility groups from RSF1010/R1162 (later designated as IncQ1α plasmids), namely those of the IncQ1β and IncQ1γ groups. Since I was under the impression that not all of this work had been published, I asked him to co-author this review. In the end, he contributed a single paragraph. I will not review the contents of this review here but it concerns many of the insights that had been gained by analysing all of the IncQ plasmids that had been identified at that time, many of them unsequenced (see Table 1 of the manuscript).

van Zyl, L.J., Deane, S.M and Rawlings, D.E. 2003. Analysis of the mobilization region of the broad host-range IncQ-like plasmid pTC-F14 and its ability to interact with a related plasmid, pTF-FC2. J. Bacteriol. 185: 6104-6111. (Citations 10)

This manuscript describes the mobilization region of pTC-F14, which was similar in layout to that of pTF-FC2, with both consisting of five genes and an *oriT*. Although two of the *mob* gene products (MobA and MobB) had fairly high amino acid sequence identities (70-

80%), the products of the three genes located on the opposite side of the *oriT* (MobC, MobD and MobE), had very much lower sequence identities (20-40%). Interestingly two related plasmids, pRAS3.1 and pRAS3.2 isolated in Norway from fish pathogens, were reported to have a mobilization region that is a lot more similar to pTF-FC2 than pTC-F14 with all five *mob* genes of the pRAS3 plasmids being closely related to those of pTF-FC2. What made this surprising was that both pTF-FC2 and pTC-F14 had been isolated in South Africa and from the same *Acidithiobacillus* genus though from different species, yet these two plasmids were more different from each other than pTF-FC2 was from the pRAS3 plasmids.

As was the case for the replicon, at least parts of the mobilization regions of pTF-FC2 and pTC-F14 must have shared the same ancestry. We were therefore in a position to ask what components of the mobilization genes could still cross-complement each other. The mobilization frequency of pTC-F14 between strains of *E. coli* by a chromosomally integrated, self-transferable, RP4 plasmid was 3,500-fold less than for pTF-FC2. However, if both pTF-FC2 and pTC-F14 were co-resident in the same cell, the mobilization frequency of pTC-F14 increased to almost the same as pTF-FC2. By further experimentation it was found that it was the products of the *mobD* and *mobE* genes of pTF-FC2 that were responsible for the increased frequency of transfer of pTC-F14. Thus we had the apparently anomalous situation where the heterologous *mobD* and *mobE* genes of pTF-FC2 were better able to complement the conjugative transfer of pTC-F14 than its own *mobD* and *mobE* genes were. The most likely explanation for this is that the MobD and MobE proteins of pTF-FC2 are better suited to work with the conjugation system of RP4, while the equivalent genes of pTC-F14 had probably evolved to work optimally with an as yet undiscovered self-transmissible plasmid. Furthermore, since the whole of the mobilization regions of pTF-FC2 and the pRAS3 plasmids are highly similar, but differ from pTC-F14 in the region of the *oriT* and *mobCDE* genes, this dissimilar region was most likely acquired by a gene swapping recombination event with the mobilization region of an unknown plasmid.

Additional work demonstrated that a test plasmid containing the *oriT* region of pTC-F14 was able to be mobilized by the mobilization genes of pTF-FC2, but at a frequency of about 200-fold less than the homologous plasmid. However, the reverse was not found as the mobilization of a pTF-FC2 *oriT*-containing test plasmid by pTC-F14 could not be detected. Cross complementation experiments carried out with the cloned *oriT*s supported the findings made with the whole plasmids.

An interesting finding, given that related plasmids may compete for the same replication space, was that no evidence was found that the mobilization of one of the plasmids interfered with the mobilization of the other even though both were being mobilized by the same RP4 conjugation machinery.

Deane, S.M. and Rawlings, D.E. 2004. Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad-host-range IncQ-like plasmids. J. Bacteriol. 186: 2123-2133. (Citations 16)

As stated earlier, it was found that pTC-F14 had a *pas* that consisted of two genes as opposed to the three for pTF-FC2 with amino acid sequence identities of the *pasA* and *pasB* gene products being 81 and 72% respectively. As was the case for the replicon and

mobilization regions, the finding that pTC-F14 had a *pas* related to pTF-FC2 gave us a unique opportunity to address some evolutionary questions. Among these were, which of the two or three component systems was more effective at plasmid stabilization? Were both toxins equally virulent? Could either or both systems lead to the stabilization of the host plasmid in the presence of the other? Was the antitoxin of one system able to neutralize the toxin of the other system and also autoregulate the other operon? Since these two promiscuous plasmids were isolated from two species of bacteria that grow in the same ecological niche, the answers to these questions would be especially relevant should these plasmids ever encounter each other in the same cell.

In short, the three component *pasABC* of pTF-FC2 appeared to have the upper hand on all but one count which was that the toxin of the pTF-FC14 *pasAB* appeared to be considerably more lethal than that of *pasABC*. The *pasABC* was more efficient at stabilizing the same test plasmid with 92% of cells retaining the plasmid after 100 generations compared with 60% for *pasAB* in the same *E. coli* host. However, when *pasC* was inactivated then *pasABC** and *pasAB* were approximately equally efficient at plasmid stabilization. Neither antitoxin was able to neutralize the toxin of the other system but the antitoxin of the *pasABC* was able to repress the synthesis of itself as well as the *pasAB* more efficiently that the *pasAB* antitoxin was able to repress itself. The PasA antitoxin of *pasAB* was unable to repress expression of *pasABC*. Enhancement of repression by PasC was not a reason for the difference. The greater level of repression by the heterologous antitoxin was an unexpected finding and one wonders whether this would have been the case had similar experiments been carried out in non-*E. coli* host cells. The net result of the effective repression of the two component *pasAB* by the *pasABC* antitoxin was that a plasmid with *pasABC* displaced a plasmid with the two component system. This displacement was due to *pasABC* and not due to replicon incompatibility as the stability of pTC-F14 was very close to normal in variants of pTF-FC2 that had undergone a spontaneous deletion of *pasABC*. Furthermore, pTC-F14 with an intact *pasAB* did not displace pTF-FC2 from which *pasABC* had been deleted.

Interestingly a two component TA toxin module that is closely related to the *pas* has been found on plasmid pAM10.6 isolated from *Pseudomonas fluorescens*, though this plasmid does not have an IncQ-like replicon and the TA module was not associated with the replicon (details given in manuscript).

Gardner, M.N. and Rawlings D.E. 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiology 150: 1797-1808. (Citations 6)

Since plasmids pTF-FC2 and pTC-F14 clearly belonged to two incompatibility groups (by this time designated IncQ2α and IncQ2β) and would be expected to have arisen from the same ancestral plasmid, we were in a position to address the question of what changes had taken place for the two plasmids to have evolved to the extent that they were now compatible. By testing the iterons of each plasmid for incompatibility with the other, it was clear that the sequence of the iterons had evolved sufficiently apart for the pTF-FC2 and pTC-F14 to have become compatible. On testing which of the replicon genes from each plasmid could support replication of the other, it was clear that the RepC iteron-binding protein of each plasmid was specific for binding to its own iterons. In contrast, the RepA helicase and RepB primase was not plasmid specific. For example, the *oriV* of

pTC-F14 could be complemented for replication by the *repA* and *repB* genes of pTF-FC2 (or the same genes of the IncQ1γ plasmid, pIE1108) provided that the specific pTC-F14 RepC iteron-binding protein was provided.

Some regulation studies were also carried out. It was found that replication of pTC-F14 was from a 5.7-kb transcript that originated in the region of the *oriT*, immediately upstream of the *mobB* gene and included the region containing the *mobB*, *mobA*, *repB*, *pasA*, *pasB*, *repA* and *repC* genes (Figure 1.3). Studies using β-galactosidase reporter gene fusions to each of the genes indicated the presence of two promoters, one upstream of *mobB* (120-140 Miller units) and a very strong promoter upstream of the autoregulated *pasA* (±1500 Miller units). The *mobB* promoter of pTC-F14 was found to be repressed by the product of the *repB* gene and not by any of the *mob* genes (*mobEDCBA*). Furthermore, this regulation was plasmid specific as when pTF-FC2 or the IncQ1α (RSF1010) plasmids were placed *in trans* to the pTC-F14 *mobB-lacZ* reporter gene fusion, no regulation occurred. The role of the strongly expressed *pasA* promoter will be addressed later.

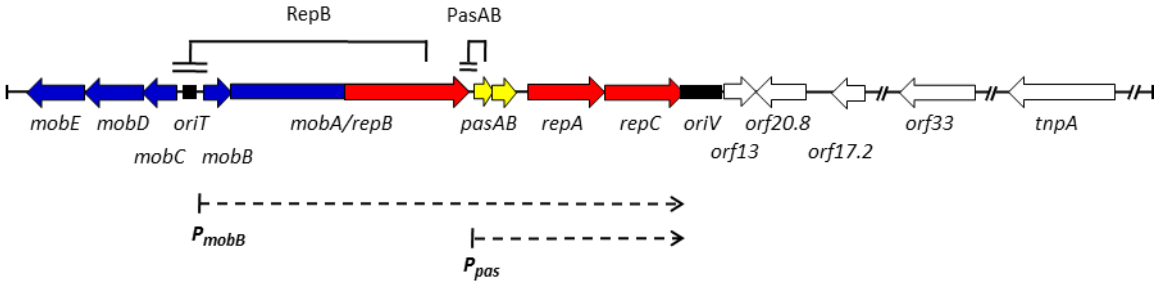


Figure 1.3. A genetic map of pTC-F14 indicating the two major transcripts involved in replication and the major repressors of each transcript.

Although this manuscript focussed on regulation of the pTC-F14 replicon and not the mobilization genes, if we take this work and that of the regulation of the mobilization of pTF-FC2 together, the following model may be proposed. Promoters located in the *oriT* region are responsible for the control of both replication and mobilization. Regulation of replication is mainly from promoter situated in the *oriT* region upstream of the *mobB* gene and that reads in the direction of the replicon. The *repB* gene product is the main regulator (repressor) of transcription in this direction and not the mobilization genes. For mobilization to occur, transcription in both directions is required, one in the direction of *mobC* and the other in the direction of *mobB*. This is because *mob* gene products synthesized from genes on either side of the *oriT* are required. Regulation of mobilization is carried out mainly by controlling transcription of the *mobCDE* genes in the opposite direction to that required for replication. This *mobCDE* promoter is much stronger (300-fold) than in the direction of *mobBA-repB* (Rohrer and Rawlings 1993) and regulated through repression by the *mobC* gene product.

Rawlings, D.E. 2005. The evolution of pTF-FC2 and pTC-F14, two related plasmids of the IncQ-family. Plasmid. 53: 137-147. (Citations 14)

It was my view that the discovery of pTF-FC2 and pTC-F14 had provided a very special opportunity to investigate the evolution of plasmids because the two plasmids were clearly related (similar replicons, mobilization and *pas* genes) but also sufficiently different for several questions regarding evolution to be addressed. Some important observations had been made but these were spread over several manuscripts. I felt that many researchers could not be expected to assemble the information on the evolution of replicons, mobilization and toxin-antitoxin systems to gain an overall picture of the evolution of these important plasmid features. This highly focused review was written in an attempt to integrate the results from several manuscripts.

I will not review this work again here. However, the work was selected by Faculty 1000 member, Peter Williams (Bangor, Wales) who commented on the significance of the work in Faculty 1000 recommendation report (http://f1000.com/prime/1024790# re-commendations).

Matcher, G.M. and Rawlings, D.E. 2009. The effect of the location of the proteic post-segregational stability system within the replicon of plasmid pTF-FC2 on the fine regulation of plasmid replication. Plasmid. 62: 98-107. (Citations 3)

After a short break from working with IncQ plasmids, a post-doctoral researcher joined my laboratory and one of the projects she was asked to work on was to determine the effect of the location of the proteic post-segregational stability system within the replicon of plasmid pTF-FC2 on the fine regulation of plasmid replication. As explained previously, both IncQ2 plasmids pTF-FC2 and pTC-F14 had related toxin-antitoxin genes located within their replicons. In addition, the sequences of the pRAS3 plasmid variants, pRAS3.1 and pRAS3.2 that were closely related to pTF-FC2 had been reported. However, what the authors of the pRAS3 plasmids report had missed during the annotation of the sequences is that the plasmids had a TA module completely unrelated to that of *pas* but in the same position between the *repB* and *repAC* genes. Once the newly discovered TA system had been shown to function as such (see Loftie-Eaton and Rawlings, 2009 below), it was clear to us that there must be a reason as to why the IncQ2 plasmids had acquired two unrelated TA systems in exactly the same position within the replicon. Our attempt to find out what that reason is, was the subject of the research reported this manuscript.

A previous observation that in the case of pTF-FC2, the *pasABC* genes could be deleted without affecting the plasmid copy number (PCN), although with a loss in plasmid stability (Smith and Rawlings, 1997) was confirmed in this study. This implied that having a TA system with its very strong promoter within the replicon did not affect the normal PCN and that this was controlled by something else. So the question we wished to address was what was the role of the *pas* with its strong autoregulated promoter? The expression of a β-galactosidase translation reporter gene fusion to the first few amino acids of the *repA* gene product gave approximately 480-520 Miller units irrespective of whether the *pas* was present or totally deleted. This suggested that expression had originated from the *oriT* region upstream of *mobB* and the tightly autoregulated *pas* did not add to this basal level of *repAC* expression. If the reporter gene translational fusion was made to the *pasA* gene product, then the level of reporter gene expression was approximately 7500 Miller units. This high level of expression was achieved because the PasA autorepressor was absent and therefore expression from the strong *pasABC* promoter was unregulated.

To demonstrate that the level of the *pas* autoregulator could affect *repAC* expression, an entire *pas* operon or only the *pas* promoter was cloned into a pGEM-T vector and placed *in trans* to the *repA*-translational reporter gene fusion. When the entire cloned *pas* was placed *in trans*, there was no difference in *repA*-reporter gene expression. This may be expected as the additional *pas* operon would itself have been autoregulated so that both the *pas* on pTF-FC2 and the additional *pas* on the co-resident plasmid were being expressed at basal levels due to trans-acting autoregulation. In contrast, when only the *pas* promoter was placed *in trans*, the promoter would be expected to bind to some of PasA repressor produced by the plasmid thereby reducing the amount of autoregulator able to bind to the *repA*-reporter construct. This is what happened, the level of *repA-lacZ* expression increased 2.7-fold. Interestingly, when the *pasABC* genes of the pTF-FC2 replicon were deleted, but the strong *pas* promoter was retained, the expression levels of *repA* and *repC* (as determined by qPCR) increased by 15- to 18-fold relative to a wild type replicon while level of *repB* expression was largely unchanged. Unexpectedly, the effect of this excess *repAC* expression was not to increase but rather to reduce the copy number of the pTF-FC2 replicon by approximately 2-fold. This implied that excess RepA and RepC was detrimental to plasmid replication. When the *repAC* genes were deleted from the pTF-FC2 replicon and instead expressed from an arabinose promoter on a co-resident plasmid, PCN increased as the amount of *repAC* expression increased. However, a point was rapidly reached whereby excess expression resulted in a decrease in copy number. This was probably the equivalent of what happens when the copy number of the plasmid within the cell becomes too high with the increased likelihood of the formation of RepC dimers that are ineffective at promoting plasmid replication.

We next tried to ascertain whether the presence of the *pas* with its strongly autoregulated promoter had a function in maintaining plasmid stability in addition to it being a post-segregational, cell-growth inhibiting, toxin-antitoxin system. To do this the *pas* was cloned into a low copy number plasmid that was placed into a cell and was expected to provide sufficient antitoxin to neutralize toxin-mediated post-segregational stability. The stability of a plasmid with a normal *pas*-containing replicon and a replicon from which the *pas* had been deleted were compared in the presence of a co-resident, low-copy number *pas*-encoding plasmid. After 260 generations the plasmid with the *pas*-containing pTF-FC2 replicon was still present in over 90% of cells, while the plasmid lacking the *pas* was present in a little over 40% of cells. It therefore appeared that the *pas* was contributing to plasmid stability independently of its ability to act as a post-segregation toxin-antitoxin stability mechanism.

Our conclusion was that one of the functions of the *pas* was to assist in preventing plasmid loss when the PCN in an individual cell fell to below a threshold. This situation could arise on cell division if one of the progeny cells received a much smaller number of plasmids than the other. A low copy number would also arise following plasmid mobilization when typically a single copy of plasmid is transferred during conjugation. In such circumstances, the quantity of PasA repressor in the cell would be low and a rapid transient expression of *pas* from its strong autoregulated promoter would occur. This increased expression would result in read-through into the *repAC* genes and the increase in *repAC* expression would result in a rapid increase in PCN. Once the PCN had reached its normal level, *pas* repression would again occur and expression of *repAC* would again be primarily from the promoter in the *oriT* that is under the regulatory control of RepB.

1.5.6 The pRAS3 plasmids isolated from potential *Aeromonas* fish pathogens

Loftie-Eaton, W. and Rawlings, D.E. 2009. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. J. Bacteriol. 191: 6436-6446. (Citations 2)

As introduced earlier, plasmids pRAS3.1 and pRAS3.2 are two natural variants of IncQ2 plasmids that were isolated from two different fish pathogens and sequenced by researchers in Norway. These workers were mainly interested in the tetracycline resistance genes that these plasmids carried and not in the plasmids themselves. The observation that the sequences of the plasmid backbones were identical with the exception of two features provided another opportunity for investigating the biology of plasmids that belong to the IncQ-family.

One of the two features that differed between the two plasmids was that plasmid pRAS3.1 had 4x22-bp iterons while pRAS3.2 had 3x22-bp iterons. The sequence of all iterons was identical. This suggested that we examine the effect of the number of iterons on PCN. The second feature was that pRAS3.1 had an additional 6-bp sequence inserted into the *mobB-mobA/repB* promoter region which is located in the *oriT*. By constructing plasmids that differed only in the number of iterons (identical *mobB* promoters) plus plasmids that differed only in the 6-bp *mobB* promoter insertion (identical numbers of iterons) we were able to isolate the effect of the additional iteron from the effect of the 6-bp promoter insertion.

In summary, the 6-bp insertion resulted in a 2-fold increase in expression in the direction of *mobB*, *mobA/repB* (and the rest of the replicon genes) while expression in the opposite direction (*mobCDE* genes) was unaltered. This 2-fold increase in expression in the direction of the replicon genes resulted in an equivalent ± 2-fold increase in PCN from an average of 23 to 41 copies and 31 to 59 copies in the case of a 5x22-bp iteron- or a 4x22-bp iteron-containing plasmid respectively. That this increase in copy number was due to additional *repB* transcription was confirmed by introducing a cloned *repB* gene under control of an arabinose promoter into the cell on a co-resident plasmid. Induction of *repB* with arabinose resulted in a 2.2- or 1.9-fold increase in PCN depending on whether the plasmid was a 6-bp promoter insertion variant or not. In contrast to *repB*, placement of *repC* under control of an arabinose promoter had no effect on copy number following arabinose induction, while when the same experiment was repeated with *repAC*, the copy number was reduced by about 30%. This inhibition of replication by excess RepAC was consistent with what we had found previously with pTF-FC2. A 6-bp insertion would be expected to have introduced an additional half-helix turn in the DNA of the promoter region. This affected the level of transcription possibly by a reduction in the ability of the RepB repressor to bind to the promoter or alternately by enhancing the efficiency of RNA polymerase to recognise the promoter.

The effect of an additional 22-bp iteron was to reduce the copy number of the plasmid from 59 to 41 in the case of a 6-bp *mobB* promoter insert variant and from 31 to 23 copies for the other variant. This was expected as it has previously been shown that an increase in the number of iterons resulted in a reduction in PCN (Lin and Meyer, 1986).

We speculated that pRAS3.2 with its 3x22-bp iterons was likely to have been the original plasmid (as 3x22-bp iterons is most common in IncQ plasmids) and that this plasmid had acquired a 6-bp insertion that resulted in an approximate doubling of the copy number. This was likely to have placed stress on the host with the result that there was selection for a plasmid variant where the copy number was reduced. The acquisition of an additional 22-bp iteron had achieved that copy number reduction. Of course, it may be that the acquisition of an additional iteron occurred first, with a reduction in copy number and subsequent selection of the 6-bp promoter insertion variant in which the copy number was increased. However, this is alternative is probably less likely.

In the same manuscript it was demonstrated that the mobilization frequencies of both pRAS3.1 and pRAS3.2 were approximately equal. Also that a construct containing the *oriT* region of the pRAS3 plasmids could be mobilized by RP4 when its own *mob* genes were provided by a co-resident plasmid but not by the *mob* genes of pTF-FC2 or pTC-F14. However, in the reverse experiments the *mob* genes of the pRAS3 plasmids were able to mobilize a plasmid containing the *oriT* regions of pTF-FC2 and pTC-F14. Based on these and previous mating experiments with pTF-FC2 and pTC-F14, we came to the conclusion that like R1162 (Becker and Meyer, 2003; Jandile and Meyer, 2006), the mobilization systems of the IncQ2 plasmids are relaxed and that the Mob proteins of the pRAS3 plasmids could mobilize *oriT* regions with considerable variation their sequences.

It was also demonstrated that the identical TA-systems of the pRAS3 plasmids were functional and that their presence increased plasmid stability. Sequence alignments suggested that the pRAS3 TAs were much more closely related to TA-systems that occurred on the chromosomes of various bacteria (e.g. species of *Bartonella*, *Xanthomonas, Aromatoleum*) than to systems that had previously been identified on plasmids.

We showed that the pRAS3 plasmids were compatible with members of the IncQ1α (RSF1010), IncQ1β (pIE1108) and IncQ1γ (pIE1130) incompatibility groups but were strongly incompatible with either the IncQ2α (pTF-FC2) or IncQ2β (pTC-F14) plasmids. However, this incompatibility was not associated with the replicons. A search for this strong incompatibility was made using a transposon mutagenesis system and by selecting for pTF-FC2 transposon mutants that could replicate in the presence of pRAS3.1. Using this approach we identified the source of the incompatibility as being an ORF (termed ORF3) immediately downstream of the *mobCDE* genes and that was transcribed together with those *mob* genes. The predicted product of this ORF had very high amino acid sequence identity (97 and 98%) to two ORFs of unknown function. Details of the search and the results obtained are in the manuscript. However, we felt that this incompatibility was taking us on a spurious side-track and we terminated this work. Once the cause of incompatibility had been identified we were able to determine by using the replicons only, that the pRAS3 plasmids belonged to a third group of the IncQ2 plasmids, that is, IncQ2γ.

Loftie-Eaton, W. and Rawlings, D.E. 2010. Evolutionary competitiveness of two natural variants of the IncQ-like plasmids, pRAS3.1 and pRAS3.2. J. Bacteriol. 192: 6182-6190. (Citations 1)

In the above manuscript we took the study of pRAS3.1 and pRAS3.2 further by addressing the question of which of the two plasmid variants had an evolutionary advantage over the other. We viewed evolutionary advantage as functioning at two levels, firstly within an individual cell and secondly at the level of a population of cells. Competitive advantage inside a cell could arise by one variant having increased stability, that is, a reduced chance of being lost on cell division or the ability to displace the other if both plasmids were co-resident in a cell. However, should a plasmid increase its competitiveness inside a cell and at the same time place too much of an additional demand on the host cell's overall metabolism, the plasmid with greater demand may fare worse at a population level because its host is outcompeted by a plasmid that placed less demand on the host.

During our analysis of the pRAS3 plasmid sequences a unique *Bst*EII restriction endonuclease site was detected within the iterons. This allowed an easy method of investigating the effect of the number of iterons on plasmid biology, beyond the 3x22-bp and 4x22-bp natural plasmid variants. Plasmid pRAS3.1 was digested with *Bst*EII and a concentrated ligation was carried out from which plasmids containing 3x, 4x, 5x and 7x22-bp iterons were isolated. We did not think it worthwhile searching for still more iteron variants and worked with these. Plasmids containing 1x or 2x22-bp iterons were not isolated and the assumption that such plasmids are likely to be non-viable is consistent with all natural IncQ-family plasmids having been found to contain a minimum of 3x22-bp iterons (or 3x20bp iterons with 2-bp spacers). A plasmid that contained the 6-bp insertion into the *mobB* promoter and 3x22-bp iterons had the highest PCN of 59. Increasing the number of iterons to 4x, 5x and 7x22-bp reduced the PCN to 41, 28 and 19 respectively. In displacement studies, a plasmid with 4x22-bp iterons displaced a plasmid with 3x22-bp iterons in all cases. This was irrespective of whether a 6-bp *mobB* promoter insertion was present or not and whether the 4x22-bp iteron-containing plasmid had a lower copy number than the competing 3x22-bp plasmid or not. The observation that the 6-bp insertion made no difference to plasmid displacement was not unexpected as we had shown that this insertion functions by increasing the quantity of RepB primase available. This increased intercellular primase would be available to all variants of the plasmid within the cell. When plasmids with 4x, 5x or 7x22-bp iterons were competed with a 3x22-bp iteron-containing plasmid, the latter plasmid was displaced by the plasmid with the larger number of iterons although displacement was strongest with 4x and progressively less with 5x and 7x22-bp iteron-containing plasmids. This was in spite of the 5x22-bp iteron-containing plasmid having half the copy number of the 3x22-bp iteron-containing plasmid. As the 22-bp iterons serve as a site for RepC-binding it could have been that there was insufficient RepC available to detect the full effect of having additional iterons. However expression of extra RepC under control of an arabinose promoter from a co-resident plasmid had no effect on the displacement.

It had been shown that cooperative binding occurs when π replication initiator protein of plasmid R6K binds to the 7x22-bp iterons of its replication origin (Bowers *et al*., 2007). We speculated that enhanced cooperative binding may occur in the pRAS3 variants with 4 or 5 iterons that gave those plasmids a selective advantage over plasmids with 3 iterons in spite of their copy number being lower. In the case of the 7x22-bp iteron plasmid, the advantage of cooperative binding might have been reduced by the much greater

difference in copy number and that this decrease in PCN reduced the advantage so that the 3 and 7 iteron-containing plasmids competed for replication approximately equally.

Up to this stage we had established that pRAS3.1 with its 6-bp iteron promoter insert and an additional 22-bp iteron had a higher copy number than pRAS3.2 and also displaced pRAS3.2 when the two plasmids were co-resident in the same *E. coli* host. The next topic that was addressed was whether either pRAS3.1 or pRAS3.2 placed an additional metabolic burden on the host sufficient to make the *E. coli* host cell that harboured either plasmid less competitive than a host containing the other plasmid. Before doing this experiment we considered it necessary to determine whether both plasmids were equally stable in the *E. coli* host in the absence of plasmid selection. The reason for this is that we did not wish to use antibiotic selection as that would itself place a metabolic burden on the host and we needed to compare each plasmid-containing host with a host that did not contain either plasmid. Furthermore, since the plasmids carried a TA system, if either plasmid was less stable than the other, loss of a plasmid would prevent further growth of the plasmidless cells and that could result in a slower growing population. Under these circumstances inhibition of growth due to plasmid loss would itself amount to being an additional apparent metabolic burden on the population although in a different sense to the metabolic burden due to a higher PCN with its increased gene expression and biosynthetic demands. In any event, both plasmids were equally stable after 100 generations in both Luria broth and M9 minimal medium.

As pRAS3 stability may have been due to the effectiveness of the TA system we decided to clone the TA system into a pACYC177 vector and place this *in trans* to pRAS3.1 and pRAS3.2 so as to neutralise the effect of the TA system. This cloned TA system was shown to be functional as it reduced the stability of a low copy number pOU82 (TA-containing) test plasmid when placed *in trans* to the pOU82-TA test plasmid. When pACYC177-TA was co-resident in the same cell as pRAS3.1 or pRAS3.2, both plasmids remained 100% stable even though their TA systems had been neutralized. The same applied to all of the pRAS3 derivatives with PCNs from ±59 to ±15. We found that all plasmids tested were 100% stable irrespective of whether their TA systems had been neutralized or not, in both LB and M9 medium as well as at 30 and 37^{o}C.

We were then in a position to carry out metabolic load experiments using *E. coli* cells containing pRAS3 derivatives in the absence of selection. The metabolic load of pRAS3.2 was calculated as being a 4.70% reduction in relative fitness compared to a plasmid-free cell while that of pRAS3.1 was higher at 7.48% with the difference of 2.78% being statistically significant. By testing other derivatives of pRAS3, the reduction in relative fitness was least for the lowest copy number plasmid, pRAS3.1.74 (PCN ±15) at 2.64% while the fitness reduction was highest for the plasmid with the highest copy number (PCN ±59) at 11.88%.

Therefore, although pRAS3.1 could displace a co-resident pRAS3.2 from a cell, it placed an additional 2.78% metabolic load on an *E. coli* host. Nevertheless, both plasmids clearly existed in natural environments and so the existence of either plasmid had not resulted in the elimination of the other. We would have liked to have carried out similar studies in the hosts from which the pRAS3 plasmids had been isolated. However, these had not been retained by the people who provided us with pRAS3.1 and pRAS3.2 and we were unable to transform a different strain of *Aeromonas salmonicida* that had been isolated from a local fish pathogen.

Loftie-Eaton, W and Rawlings D.E. 2012. Diversity, biology and evolution of IncQ-family plasmids. Plasmid 67: 15-34.

This is a review that I was asked to write by Dhruba Chattoraj who was editor of the journal, *Plasmid* at the time. Since Richard Meyer had recently written an excellent and comprehensive review on the replication and conjugation of IncQ plasmids, it was considered to be unnecessary to repeat that work. However, I was pleased to write a review as I felt that there were many other aspects concerning IncQ plasmids into which I had gained an insight while working with this plasmid family over a period of more than 25 years and that if I did not do this before retiring, those insights could be lost or recovered with considerable difficulty by the scientific community. I invited Wesley Loftie-Eaton to coauthor the review with me as he had recently finished a PhD in my laboratory and it could be helpful to his future career as a scientist. Furthermore, I hoped by that giving him increased exposure to the IncQ plasmids, he might wish to include work on IncQ plasmids in any future research he might do as after Richard Meyer and myself had retired, it seemed that there would be nobody left working on the biology of this plasmid group.

The review has been included in this volume of work and therefore the contents of the review will not be discussed in detail here. As the title suggests, the review focussed more on diversity and evolution than the molecular biology of replication and mobilization. By searching the database for IncQ plasmid replicons we had been able to identify four groups of IncQ-family plasmids. The main difference between them was that the primase gene of their replicons had been fused to the gene for the relaxase of four different lineages of mobilization genes. The IncQ1 group primase gene had been fused to a relaxase gene known as the MOB_{Q1} relaxase family (Garcillán-Barcia et al., 2009), while the IncQ2 and IncQ4 plasmids had been fused to MOB_{P14} and MOB_{P5} relaxase genes respectively. The IncQ3 plasmid primase genes had been fused to a relaxase gene that had not yet been assigned to a family. These four IncQ plasmid subgroups were supported by other features such as the phylogeny of their Rep proteins being more closely related within a subgroup than between subgroups and the nature of the genes (or absence thereof) that lie between the *repB* and *repAC* genes of their replicons.

We also presented strong evidence for a range of steps that had taken place during the evolution of IncQ plasmids. These evolutionary steps included incremental single base-pair changes such as found within the iterons and elsewhere, the acquisition of genes such as the different TA systems and other genes that lie between *repB* and *repAC* as well as gene swapping such as the *repC* gene of the pRAS3 plasmids (see review) and the *mobCDE* genes of pTC-F14. Finally, there was the fusion of the IncQ replicon to at least four different lineages of mobilization genes as described.

1.6 Additional references.

A comprehensive set of references to research on IncQ plasmids is given in the manuscripts that are included in this compilation. However, references to work described

in this introduction chapter that are in addition to those included in this compilation of manuscripts follow below.

Becker E.C. and Meyer R.J. 2003. Relaxed specificity of the R1162 nickase: a model for evolution of a system for conjugative plasmids. J. Bacteriol. 185: 3538-3546.

Bowers L.M., Krüger R. and Filutowicz M. 2007. Mechanism of origin activation by monomers of R6K-encoded π protein. J. Mol. Biol. **368**: 928-938.

Garcillán-Barcia M.P., Francia M.V., de la Cruz F. 2009. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol. Rev. 33: 657-687.

L'Abée-Lund T. and Sørum H. 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid **47**: 172-181.

Jandile S. and Meyer, R.J. 2006. Stringent and relaxed recognition of *oriT* by related systems for plasmid mobilization: implications for horizontal gene transfer. J. Bacteriol. 188: 499-506.

Lin L.S., Meyer R.J., 1986. Directly repeated, 20-bp sequence of plasmid R1162 DNA is required for replication, expression of incompatibility, and copy-number control. Plasmid **15**: 35-47.

Maisonneuve E., Shakespeare L.J., Jorgensen M.G. and Gerdes K. 2011. Bacterial persistence by RNA endonucleases. Proc. Natl. Acad. Sci. USA. **108**: 13206-13211.

Meyer, R. 2009. Replication and conjugative mobilization of broad host-range IncQ plasmids. Plasmid **62**: 57-70.

Rawlings, D.E., Gawith, C., Petersen, A. and Woods, D.R. 1983. Characterization of plasmids and potential genetic markers in *Thiobacillus ferrooxidans*. In: *Recent Progress in Biohydrometallurgy*. Eds. G. Rossi and A.E. Torma. pp 555-570. Associazione Mineraria. Sarda. Cagliari.

Sakai H., Komano T. 1996. DNA replication of IncQ broad-host-range plasmids in gram-negative bacteria. Biosc. Biotech. Biochem. **60**: 377-382.

Tanaka K., Kino K., Taguchi Y., Miao D.M., Honda Y., Sakai H., Komano, T. and Bagdasarian, M. 1994. Functional difference between the two oppositely orientated priming signals essential for the initiation of the broad host-range plasmid RSF1010 DNA replication. Nucl. Acids Res. **22**: 767-772.

Zhou H. and Meyer R.J. 1990. Deletion of sites for initiation of DNA synthesis in the origin of borad host-range R1162 J. Mol. Biol. **214**: 685-697.

Chapter 2. Manuscripts included in this dissertation.

The collection of twenty one manuscripts that have been included in this dissertation and discussed in chapter 1 are presented in chronological order. This is also the order in which they were discussed in chapter 1 except for manuscript 2.7 on transposon Tn*5467* found on pTF-FC2. This manuscript was described at the end of the discussion on pTF-FC2 .

JOURNAL OF BACTERIOLOGY, May 1984, p. 737–738
0021-9193/84/050737-02$02.00/0

Vol. 158, No. 2

Expression of a *Thiobacillus ferrooxidans* Origin of Replication in *Escherichia coli*

DOUGLAS E. RAWLINGS,* INGE PRETORIUS, AND DAVID R. WOODS

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 5 October 1983/Accepted 8 February 1984

A cryptic plasmid from an autotrophically grown arsenic-resistant strain of *Thiobacillus ferrooxidans* was isolated and cloned into pBR325. The origin of replication of pBR325 was deleted, and the recombinant plasmid was shown to replicate in *Escherichia coli*, using an origin of replication located on the *Thiobacillus* plasmid.

Thiobacillus ferrooxidans is an acidiphilic autotrophic bacterium which is used industrially to leach metals from mineral ores. Although genetic manipulation of *T. ferrooxidans* has the potential for the production of leaching bacteria with several desirable characteristics (2, 4), at present nothing is known about gene expression in this bacterium and no genetic system exists. Development of a genetic system for the organism and our understanding of gene expression in autotrophic bacteria would be greatly enhanced by the identification of genes which can be expressed in the heterotroph *Escherichia coli*. We describe the construction of a recombinant plasmid that is able to replicate in *E. coli*, using an origin of replication from the *T. ferrooxidans* plasmid.

Metal ion tolerance is a particularly attractive marker for genetic studies in *T. ferrooxidans*, as it is a useful laboratory marker and also has the potential of conferring an industrially significant characteristic to the organism (6a, 9). It has been shown previously that arsenic resistance can serve as a marker for *T. ferrooxidans* (Rawlings et al., in press). Since arsenic resistance has been shown to be plasmid borne in heterotrophic bacteria (3, 5), we investigated the plasmids in a *T. ferrooxidans* FC strain which was isolated from the acid leach liquor of a South African mine (Fairview Mine, General Mining Union Corporation Limited, South Africa) and which was very resistant to arsenic [2,048 mg of As(III) per liter]. Plasmids were isolated from the FC strain by the method of Mao et al. (8). This strain contained three plasmids (4.5, 12.4, and 27.6 kilobases [kb]). The 12.4-kb pTF-FC2 plasmid was the most prevalent and was chosen for further study. Restriction mapping (7) of pTF-FC2 showed that it had unique *Pst*I, *Xho*I, *Kpn*I, *Eco*RI, and *Apa*I restriction sites and two *Pvu*I restriction sites (Fig. 1). A recombinant plasmid, pDR401, was constructed by insertion of the *E. coli* plasmid pBR325 (1) into the *Pst*I site of pTF-FC2 (Fig. 1) (7). pBR325 contains the genes for ampicillin (Ap), chloramphenicol (Cm), and tetracycline (Tc) resistance. Cloning at the *Pst*I site insertionally inactivates the Ap^r gene, and *E. coli* transformants which were Cm^r (25 μg/ml) and Tc^r (20 μg/ml) but Ap^s (100 μg/ml) were selected on agar media. The recombinant plasmid pDR401 (17.8 kb) was isolated from the *E. coli* transformants by the method of Ish-Horowicz and Burke (6) and characterized by restriction mapping (7) and was able to retransform other *E. coli* recipients. *E. coli* transformants containing pDR401 did not show an increase in arsenic resistance, as they were inhibited by the same arsenic concentration in agar medium as the untransformed recipient strain [128 mg of As(III) per liter or 2,048 mg of As(V) per liter].

As digestion of DNA with the restriction enzymes *Sal*I and *Xho*I produces identical overlapping sticky ends, it is possible to construct a *Sal*I-*Xho*I deletion plasmid from pDR401 (Fig. 1). Removal of the *Sal*I-*Xho*I 3.1-kb fragment from pDR401 would result in a new plasmid which had lost the pBR325 origin of replication, approximately half of the Tc gene, and a 0.3-kb piece of the *T. ferrooxidans* pTF-FC2 DNA. The pDR401 plasmid was digested with *Sal*I and *Xho*I, ligated, and used in transformation experiments. *E. coli* transformants which were Cm^r, Tc^s, and Ap^s were selected and shown to contain the 14.7-kb deletion plasmid pDR412 (Fig. 1 and 2). Restriction enzyme analysis of pDR412 with *Pst*I indicated that a *Pst*I site had been deleted (Fig. 1 and 2). Digestion with *Pvu*I generated 3.5-, 5.0-, and 6.2-kb fragments and confirmed that a 3.1-kb fragment containing the pBR325 origin of replication gene had been removed (Fig. 1 and 2).

Experiments were carried out to localize sites essential for the replication in pDR412. Complete digestion of pDR412 with *Pvu*I produced two fragments of 3.5 and 6.2 kb which were composed entirely of *T. ferrooxidans* DNA (pTF-FC2) and a 5.0-kb fragment which contained the pBR325 Cm^r gene and ca. 2 kb of *T. ferrooxidans* plasmid DNA. These three fragments were religated and transformed into *E. coli*. Cm^r transformants were obtained at a very low frequency (20 transformants per μg of DNA). Unligated *Pvu*I control digests yielded no transformants. The isolation and restriction analysis of the plasmids from 32 *E. coli* transformants indicated that all three *Pvu*I restriction fragments were always present (Fig. 2). Smaller plasmids without a full complement of *Pvu*I sites were never obtained. The 5.0-kb fragment contains the Cm^r marker and will always be present since it is selected. The requirement for both the 3.5- and 6.2-kb fragments suggests that the *T. ferrooxidans* origin of replication is located at the *Pvu*I site which joins these two fragments. If this suggestion is correct, then the 3.5- and 6.2-kb fragments joined by the *Pvu*I site must always be present in the same orientation. However, the 5.0-kb fragment containing the Cm^r gene may be present in either orientation.

To confirm this suggestion, the pDR412 plasmids obtained by religation of the three *Pvu*I fragments were restricted with a *Pst*I-*Apa*I double digest. In all cases, the religated plasmids contained a 7.7-kb fragment which indicated that the 6.2-kb *Pvu*I fragment had been religated in the same orientation as in the uncut parent pDR412 plasmid. *Kpn*I-*Apa*I double digests of the religated pDR412 plasmids generated either

* Corresponding author.

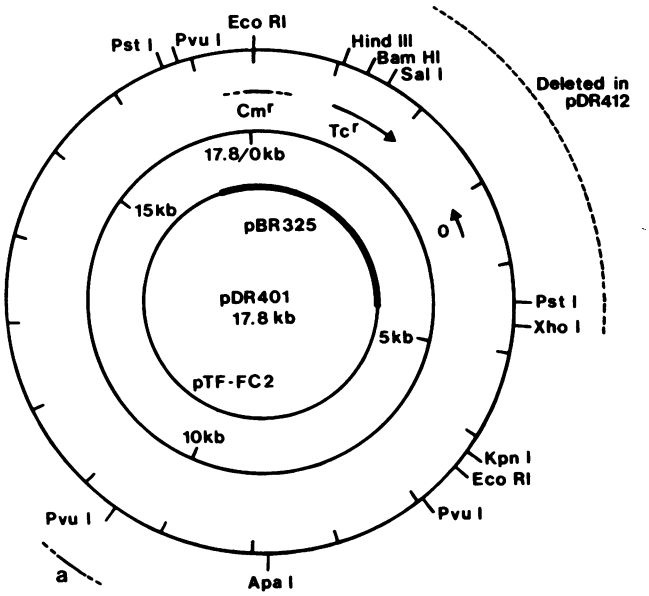


FIG. 1. Restriction endonuclease cleavage map of the plasmid pDR401 constructed by the cloning of *Pst*I-digested pBR325 into the single *Pst*I site of the *T. ferrooxidans* plasmid pTF-FC2. Deletion plasmid pDR412 was constructed by digestion of pDR401 with restriction endonucleases *Sal*I and /*Xho*I followed by religation. a, Site which is essential for replication of plasmid pDR412.

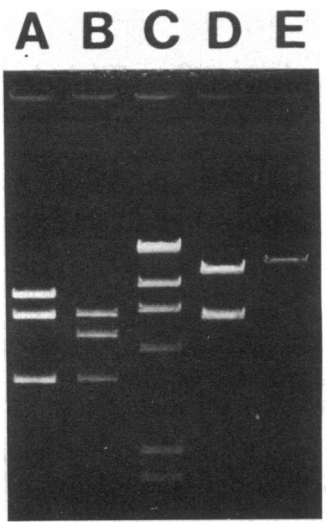


FIG. 2. Gel analysis of restriction endonuclease-cleaved pDR401 and pDR412 plasmids. Lanes A and D are pDR401 DNA digested with *Pvu*I and *Pst*I, respectively. Lanes B and E are pDR412 DNA digested with *Pvu*I and *Pst*I, respectively, showing the removal of the *Sal*I-*Xho*I 3.1-kb fragment from pDR401 with consequent removal of the pBR325 origin of replication and a *Pst*I cleavage site. Lane C is lambda DNA digested with *Hin*dIII. Electrophoresis was from top to bottom in 1% agarose in 89 mM Tris-borate (pH 8.3)–2.5 mM EDTA for 5 h at 50 mA.

2.5- and 12.2-kb fragments or 6.2- and 8.5-kb fragments which demonstrated that the 5-kb *Pvu*I fragment could be present in either orientation. These two orientations occurred in approximately equal proportions among the transformants. Since the *Apa*I site is approximately in the middle of the 3.5-kb *Pvu*I fragment, a *Pst*I-*Apa*I double digest would produce a 7.7-kb fragment for either orientation of this fragment. However, as the orientation of the 6.2-kb fragment was always fixed with respect to the 3.5-kb fragment, it is concluded that the site of an essential gene for replication incorporates the *Pvu*I site that joins them (Fig. 1), and it is likely that the 3.5-kb *Pvu*I fragment is also present in the reconstituted plasmids in the same orientation as the parent plasmid.

We have demonstrated that the recombinant plasmid pDR412, which contains a *T. ferrooxidans* origin of replication and the pBR325 Cm^r gene, is able to replicate in *E. coli*. Since we showed previously that chloramphenicol is a suitable marker for genetic studies in *T. ferrooxidans* (6a), pDR412 is a potentially useful selectable plasmid for future genetic manipulation experiments involving *T. ferrooxidans* and *E. coli*. The discovery that the origin of replication from an autotrophic bacterium is expressed in the heterotroph *E. coli* indicates that at least some signals for gene expression are similar in autotrophs and heterotrophs. This is encouraging and will facilitate future studies on the cloning and expression of other autotrophic genes in *E. coli*.

We acknowledge a research grant from General Mining Union Corporation Limited, South Africa.

LITERATURE CITED

1. **Bolivar, F.** 1978. Construction and charcterization of new cloning vehicles. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene **4**:121–136.
2. **Brierley, C. L.** 1982. Microbiological mining. Sci. Am. **247**:42–51.
3. **Brown, A. M. C., and N. S. Willets.** 1981. A physical and genetic map of the Inc N plasmid R46. Plasmid **5**:188–201.
4. **Curtin, M. E.** 1983. Microbial mining and metal recovery: corporations take the long and cautious path. Biotechnology **1**:229–238.
5. **Hedges, R. W., and S. Baumberg.** 1973. Resistance to arsenic compounds conferred by a plasmid transmissible between strains of *Escherichia coli*. J. Bacteriol. **115**:459–460.
6. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.

6a. **Livesey-Goldblatt, E., P. Norman, and D. Livesey-Goldblatt.** 1983. Gold recovery from arsenopyrite ore by bacterial leaching and cyanidation, p. 627–641. *In* G. Rossi and E. A. Torma (ed.), Recent progress in Biohydrometallurgy. Associazione Minereria Sarda, Cagliari.

7. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Mao, M. W. H., P. R. Dugan, P. A. W. Martin, and O. H. Tuovinen.** 1980. Plasmid DNA in chemoorganotrophic *Thiobacillus ferrooxidans* and *T. acidophilus*. FEMS Microbiol. Lett. **8**:121–125.
9. **Rawlings, D. E., A. Gawith, A. Peterson, and D. R. Woods.** 1983. Characterisation of plasmids and potential genetic markers in *Thiobacilus ferrooxidans*, p. 555–570. *In* G. Rossi and E. A. Torma (ed.), Recent progress in biohydrometallurgy. Associazione Minereria Sarda, Cagliari.

JOURNAL OF BACTERIOLOGY, May 1989, p. 2735–2739
0021-9193/89/052735-05$02.00/0

Vol. 171, No. 5

Identification and Sequence of the Basic Replication Region of a Broad-Host-Range Plasmid Isolated from *Thiobacillus ferrooxidans*

ROSEMARY A. DORRINGTON AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Rondebosch, 7700, South Africa

Received 27 September 1988/Accepted 1 February 1989

The minimum region required for replication of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2 in *Escherichia coli* was shown to be contained on a 2.05-kilobase fragment of DNA. A 184-base-pair fragment that was required in *cis* for plasmid replication was identified. This region was also involved in plasmid incompatibility. Nucleotide sequencing of this region revealed three perfectly conserved 22-base-pair tandemly repeated sequences. A comparison of this region with the equivalent region of the broad-host-range plasmid R1162 showed that the repeated sequences had 60% nucleotide homology. The 106-base-pair region immediately adjacent to the repeated sequences was 75% homologous. These plasmids were compatible.

Plasmid pTF-FC2 is a cryptic, 12.4-kilobase (kb) plasmid that was isolated from the acidophilic autotroph *Thiobacillus ferrooxidans*. The plasmid was cloned into the vector pBR325 and shown to be capable of replication in *Escherichia coli* from an origin of replication located on the *T. ferrooxidans* plasmid (17). The plasmid was mobilized between *E. coli* strains by the IncP plasmid RP4 (20), and the region responsible for mobilization was located (18). Although the extent of the host range of the *T. ferrooxidans* plasmid has not been widely investigated, it has been shown to replicate in *Pseudomonas aeruginosa* (18), *Klebsiella pneumoniae* (unpublished results), and *Thiobacillus novellus* (19).

The molecular mechanism and control of plasmid replication have become the subject of extensive research in recent years (22). Of particular interest is the ability of certain plasmids to replicate in a large number of bacterial species, whereas the host range of other plasmids is restricted to one or a few closely related species. Relatively few small broad-host-range plasmids have been identified. The most extensively studied of these are the plasmids of the IncQ incompatibility group. These include the similar or identical plasmids RSF1010 (5), R1162 (12), and R300B (2). An investigation into the molecular mechanism of replication of pTF-FC2 may help to identify features that are associated with the broad-host-range character of these plasmids. In addition, since *T. ferrooxidans* is used to leach a variety of metals from their ores (4), these studies can contribute to the construction of cloning vectors for this industrially important organism.

We report the isolation of the basic replication region of the broad-host-range *T. ferrooxidans* plasmid pTF-FC2 in *E. coli*. A region required for replication in *cis* when the intact replicon is supplied in *trans* was identified. This region was sequenced and compared with those of the IncQ plasmids.

MATERIALS AND METHODS

Bacterial strains and plasmids. Plasmids were maintained in *E. coli* K-12 strains JM105 (23) and LK111 (24). The *E. coli* DNA polymerase-deficient (*polA1*) strain GW125a (gift from G. Walker, Massachusetts Institute of Technology, Cambridge) was used to test for *polA*-independent plasmid replication. Plasmids used in this study are listed in Table 1.

Media and general recombinant DNA techniques. Bacteria were grown in Luria broth (LB) and on Luria agar (LA) plates at 37°C. Unless stated otherwise, 100 μg of ampicillin per ml and 25 μg of chloramphenicol per ml were used for plasmid selection. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke (7), and general DNA cloning techniques were as described in Maniatis et al. (11).

Determination of plasmid copy number. All plasmids tested for copy number were grown in the *polA E. coli* mutant GW125a. Two methods were used to determine plasmid copy number. In the first, total DNA was isolated from cells carrying the relevant plasmids. Precise quantities of total DNA were digested with *Pst*I restriction endonuclease, and the fragments were separated by electrophoresis in agarose gels. The gels were blotted and hybridized to nick-translated pDER412. The intensity of the hybridization signals was compared with that of signals obtained from known concentrations of plasmid. The quantity of plasmid relative to the *E. coli* chromosomal DNA was calculated by using the relative molecular sizes of the *E. coli* chromosome (4,000 kb) and pTV100 (8 kb). In this way it was possible to determine the number of plasmids per chromosome.

In the second method, plasmid copy number relative to pTV100 was determined by single-cell resistance to ampicillin by the method of Nördstrom et al. (14). Bacteria were grown overnight in LB containing concentrated ampicillin, and 10^5-, 10^6-, and 10^7-fold dilutions were spread onto LA plates containing various concentrations of ampicillin. Colonies were counted after incubation overnight, and the amount of ampicillin required to give 50% survival was determined.

Test for incompatibility. The ability of fragments of pTF-FC2 to displace pDER412 was used as the test for incompatibility. Plasmids to be tested were transformed into LK111 containing pDER412, and transformants were plated on LA containing ampicillin. Six colonies from each transformation were streaked onto separate plates containing ampicillin to give single colonies. From each plate, 10 colonies were picked and tested for resistance to chloramphenicol. Incompatibility with the IncQ plasmids was tested by substituting pKE462 for pDER412 and using tetracycline resistance for selection of pKE462.

Plasmid deletions and DNA sequencing. Deletions of

* Corresponding author.

TABLE 1. Plasmids used in this study

Plasmid	Antibiotic resistance	Description	Reference or source
pBR325	Tc^r Cm^r Apr^r		3
pUC19	Ap^r		23
Bluescript SK	Ap^r		Stratagene, San Diego, Calif.
pKE462	Tc^r As^r	7.5-kb *Eco*RI fragment from R46 carrying Tc^r and arsenic resistance cloned into R300B	Kim Ellis, Hammersmith, London
pDER401	Cm^r Tc^r	pTF-FC2 cloned into *Pst*I site of pBR325	16
pTT100	Ap	pTF-FC2 cloned into *Pst*I site of Bluescript SK	This study
pDER412	Cm	*Xho*I-*Sal*I deletion of pDER401 resulting in deletion of pBR325 origin of replication	16

pTV400 were made by the exonuclease III method of Henikoff (6). The DNA sequence was obtained from both strands by the chain-termination sequencing method with the Sequenase DNA sequencing kit of United States Biochemical Corp., Cleveland, Ohio.

RESULTS

***polA* independence of the pTF-FC2 origin of replication.** The *T. ferrooxidans* plasmid pTF-FC2 was previously cloned into the *Pst*I site of the vector pBR325, and the *oriV* of pBR325 was removed, to produce recombinant plasmids pDER401 and pDER412, respectively (17). We investigated whether the replication of the recombinant plasmids was independent of the *E. coli polA* gene. Plasmids pBR325, pDER401, and pDER412 were transformed into the *polA* mutant GW125a, and the transformants were tested for the presence of the plasmids by selection for growth on chloramphenicol. The presence of a plasmid was confirmed by restriction analysis of the plasmids isolated from transformants. Only pDER401 and pDER412, which contain pTF-FC2 plasmid DNA, were able to replicate in GW125a. Plasmids based on the pMB1/ColE origin such as pBR325 and related plasmids are dependent on *polA* for replication (9). Since the replication of the pTF-FC2 plasmid is *polA* independent, the ability to replicate in GW125a was used to identify fragments of pTF-FC2 involved in replication.

Determination of the minimal replicon of pTF-FC2 in *E. coli*. Plasmid pTF-FC2 was cloned into the Bluescript SK vector pTT100, and a number of subclones were constructed to identify the region of DNA involved in plasmid replication. A restriction map of pTT100 is shown in Fig. 1. The 5.0-kb *Cla*I-*Eco*RI fragment cloned into the Bluescript SK vector pTV100 was able to replicate in *E. coli* GW125a. Bluescript SK subclones pTV200 and pTV300, which contain the 2.6-kb *Cla*I-*Apa*I fragment and the 2.4-kb *Apa*I-*Eco*RI fragment, respectively, lost their *polA* independence. To identify the basic replicon further, a partial *Sau*3A library of pTV100 was constructed in the vector pUC19 and transformed into GW125a. A recombinant plasmid, pTV400, which contained a 3.2-kb pTF-FC fragment, was isolated. Exonuclease III shortening was used to produce a series of plasmids in which the 3.2-kb pTF-FC2 fragment had been progressively deleted from either end. Plasmids containing shortened pTV400 fragments were tested for the ability to replicate in GW125a, and the basic replication region was identified (Fig. 1). Plasmid pTV4101, which had a 600-base-pair (bp) deletion from the left-hand *Sau*3A site of pTV400, was able to replicate in GW125a, whereas pTV4111, which had an 850-bp deletion, had lost this ability. A 300-bp deletion from the right-hand *Sau*3A site of pTV400 (pTV4200) had no effect on replication, whereas a plasmid with a 650-bp deletion (pTV4220) was unable to replicate. *E. coli* GW125a cells containing pTV4210 with a 400-bp deletion produced colonies that were markedly smaller than those of cells carrying either pTV4200 or pTV400.

To identify the region of DNA required in *cis* for replication, deletions of pTV400 that were unable to replicate in *E. coli* GW125a were transformed into the same strain containing pDER412. A 185-bp DNA fragment common to both pTV4161 and pTV4210 was identified as the region of DNA required in *cis* for plasmid replication (Fig. 1).

The ability of different fragments of pTV100 to displace pDER412 was used to identify the region containing the plasmid incompatibility determinants. From the results in Fig. 1, it is clear that plasmid incompatibility is also located in the 184 bp of DNA common to pTV4161 and pTV4210. However, it is interesting that plasmid pTV4220, in which all three repeated sequences were deleted, retained partial incompatibility with respect to the pTF-FC2 replicon. Since pTV4220 retained that part of the replicon that encodes for the diffusable products required in *trans* for replication, it is most likely the presence or regulation of one of these products that is responsible for the partial incompatibility observed.

Plasmid copy number. Plasmid pTV100 in *E. coli* GW125a was estimated by the hybridization method to have a copy number of 12 to 15 plasmids per chromosome. All plasmids capable of *polA*-independent replication, with the exception of pTV4210, conferred an identical level of ampicillin resistance (800 μg/ml) to *E. coli* GW125a. The presence of pTV4210 resulted in a decrease in resistance to ampicillin (600 μg/ml).

Nucleotide sequence of DNA fragment required in *cis* for replication. The nucleotide sequence of the 329-bp DNA fragment corresponding to the region common to pTV4161 and pTV4200 was determined (Fig. 2). The most notable feature of this region is the presence of three perfectly conserved 22-bp tandemly repeated sequences (nucleotides 9 through 75). There are two sets of complimentary inverted repeat sequences (nucleotides 135 through 152 and 170 through 196) that are able to form potential stem-and-loop structures with ΔGs of −p5.4 and −14.7 kcal/mol, respectively (21). It is not known whether these inverted complimentary repeated sequences have any role in vivo. However, it is interesting that plasmid pTV4210, which appeared to be maintained at a lower copy number than pTV4200, lost one of the second set of complementary inverted repeat sequences.

Comparison of the DNA regions required in *cis* for replication of pTF-FC2 and the IncQ plasmids. The 329-bp DNA sequence of pTF-FC2 and the 370- and 210-bp *Hpa*II fragments of R1162 (12) required in *cis* for replication were

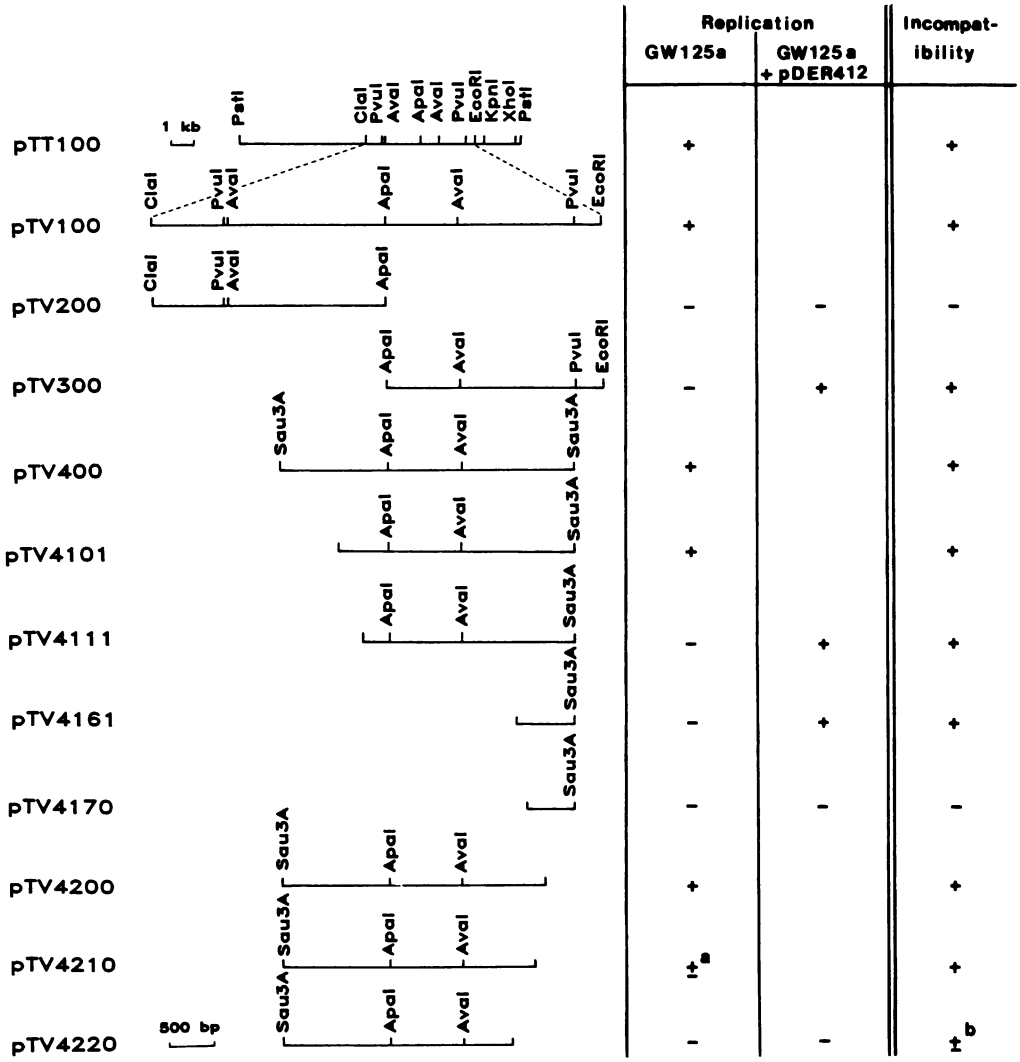


FIG. 1. Restriction endonuclease cleavage map of pTT100 and subclones, showing the identification of the minimal replicon, the region required in *cis* for replication, and the incompatibility determinants. Only pTF-FC2-derived DNA fragments are shown. Superscripts: a, colonies of reduced size; b, partial plasmid incompatibility (37 of 60 colonies were Cm^r).

aligned and compared. Substantial homology was found in the region shown in Fig. 3. The IncQ plasmids had a 60.1% homology (40 of 66 bp) with the pTF-FC2 plasmid in the region of the three repeated sequences. There was 75% homology between the DNA sequences immediately adjacent to the repeated sequences. Beyond this region the two sequences had only limited DNA homology. No further homology between the 329-bp pTF-FC2 DNA sequence and the remainder of the sequence of pTV4161 was found with the R1162 *Hpa*II fragments (results not shown).

Incompatibility between pTF-FC2 and the IncQ plasmid R300B. Since there was a considerable degree of homology between the repeated sequences and adjacent DNA regions of pTF-FC2 and the IncQ plasmid, we examined whether any incompatibility between pTV4161 and pKE462 (Table 1) could be detected. Plasmid pTV4161 carries a 650-bp fragment containing the three repeat sequences and 580 bp downstream. It was tested for its ability to displace pKE462. All 60 colonies tested were Tc^r, indicating that these two plasmids were compatible.

DISCUSSION

There are several similarities between the 12.4-kb cryptic *T. ferrooxidans* plasmid pTF-FC2 and the 8.9-kb IncQ plasmids R1162, RSF1010, and R300B. Both types of plasmid have a broad host range and are *polA* independent, and they have comparable copy numbers in *E. coli*. There are, however, some substantial differences. In RSF1010 the minimum replicon is spread over approximately 5.5 kb of DNA and is interspersed with regions that are not required for replication (1). The pTF-FC2 replicon is more compact and is confined to a 2.1-kb DNA fragment. Whether the broad-host-range properties of the parent plasmid are contained on this DNA fragment is under investigation.

A 184-bp region of plasmid DNA that is required in *cis* when the parent plasmid pDER412 is present in *trans* has been identified and sequenced. Nucleotide sequence comparisons between this fragment and the origin of replication of the IncQ plasmids R1162 and RSF1010 indicated that

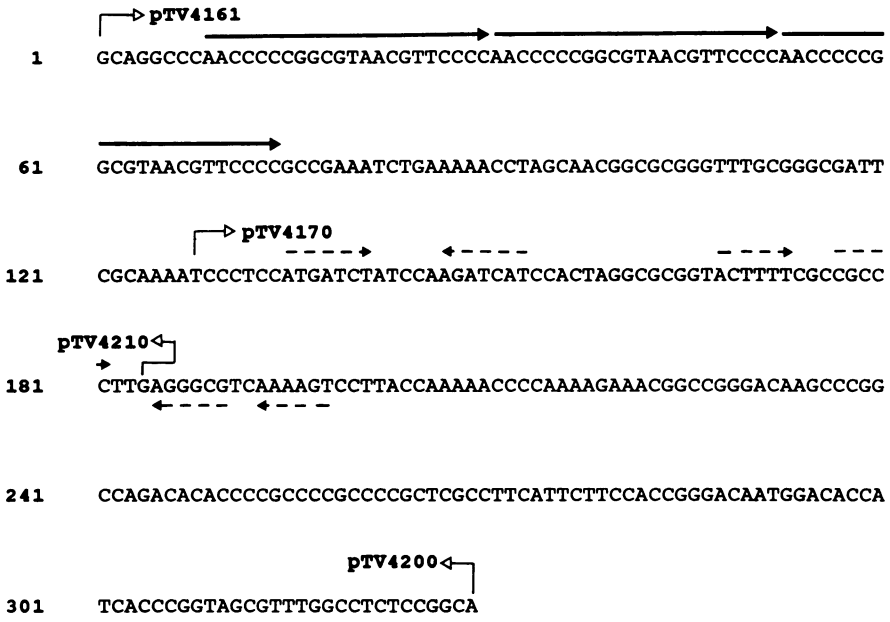


FIG. 2. Nucleotide sequence of the fragment of pTF-FC2 encompassing the region of DNA required in *cis* for replication. Solid arrows indicate the 22-bp repeated sequences, and broken arrows indicate complementary inverted repeated sequences.

pTF-FC2 has three consecutive 22-bp tandem perfectly conserved directly repeated sequences, whereas the IncQ plasmids have three and one-half perfectly conserved 20-bp repeated sequences (13, 15). If the two sets of repeated sequences including the two spacer nucleotides of the IncQ plasmids are aligned, there is 60% homology between them. From this comparison, it may be argued that the unit length of the repeated sequences of the IncQ plasmids is in fact 22 bp and that the first 2 bp are imperfectly conserved.

In R1162 two distinct domains, contained on adjacent 370- and 210-bp *Hpa*II fragments, are required in *cis* for plasmid replication (7). These domains are able to direct plasmid replication even when the distance between them is increased. The 184-bp sequence of pTF-FC2 has extensive homology with a sequence contained entirely within the 370-bp *Hpa*II fragment. Plasmid pTF-FC2 therefore does not appear to have a requirement for a domain equivalent to that on the 210-bp *Hpa*II fragment of the IncQ plasmids.

The 75% DNA homology between the 115 bp of DNA immediately adjacent to the repeated sequences of pTF-FC2 and the *oriV* of the IncQ plasmids is striking. The absence of homology outside this region may indicate structural conservation between *oriV* regions of the small broad-host-range plasmids.

The repeated sequences of the IncQ plasmids have been shown to be responsible for plasmid incompatibility and copy number control (10, 13). The 184-bp DNA sequence from pTF-FC2 containing the three repeats has also been shown to be involved in plasmid incompatibility. Although the repeated sequences of the two plasmids are 60% homol-

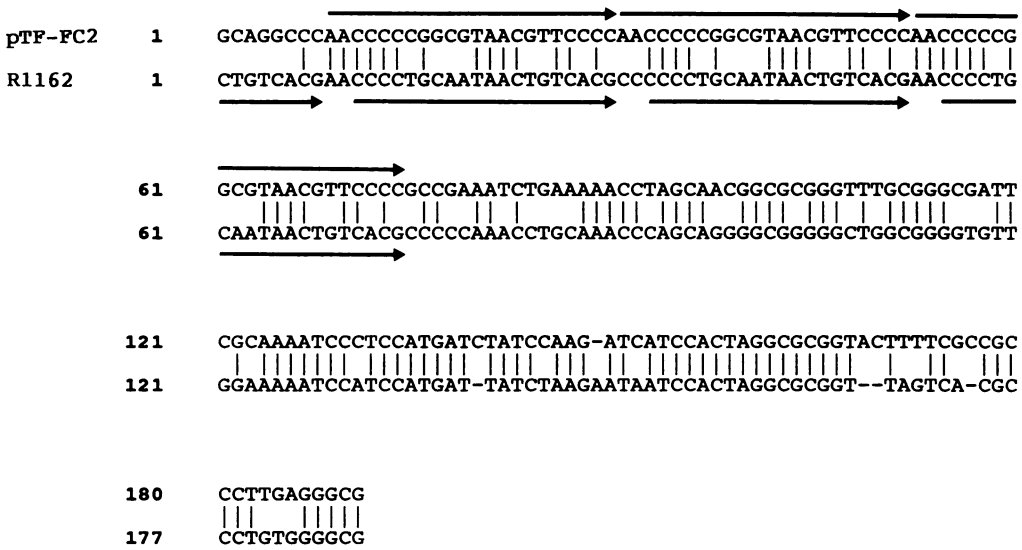


FIG. 3. Comparison of nucleotide sequence homology between the repeated sequences and adjacent regions of pTF-FC2 and R1162.

ogous, no incompatibility between the two sets of repeated sequences was detected.

The similarities between the nucleotide sequences of the pTF-FC2 origin of replication and those of the IncQ plasmids may reflect the features of a class of broad-host-range replicons to which both these plasmids belong. Whether the similarities extend to the diffusible products that are required in *trans* remains to be determined. Nucleotide sequencing of the rest of the basic replicon and *trans* complementation studies between pTF-FC2 and the IncQ plasmids are currently in progress.

LITERATURE CITED

1. **Bagdasarian, M. M., P. Scholz, J. Frey, and M. Bagdasarian.** 1986. Regulation of the *rep* operon expression in the broad host range plasmid RSF1010, p. 209–223. *In* S. B. Levy and R. P. Novick (ed.), Antibiotic resistance genes: ecology, transfer, and expression. 24 Banbury report. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
2. **Barth, P., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weight and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618–630.
3. **Bolivar, F.** 1978. Construction and characterisation of new cloning vectors. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene **4**:121–136.
4. **Brierley, C.** 1978. Microbiological mining. Sci. Am. **247**:42–51.
5. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
6. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**: 351–369.
7. **Ish-Horowicz, D., and F. Burke.** 1983. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
8. **Kim, Y.-J., L.-S. Lin, and R. Meyer.** 1987. Two domains at the origin are required for replication and maintenance of broad-host-range plasmid R1162. J. Bacteriol. **169**:5870–5872.
9. **Kingsbury, D. T., and D. Helinsky.** 1970. DNA polymerase as a requirement for the maintenance of the bacterial plasmid colicinogenic factor E. Biochem. Biophys. Res. Commun. **41**: 1538–1544.
10. **Lin, L.-S., Y.-J. Kim, and R. Meyer.** 1987. The 20 bp, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility in *vivo* and inhibits R1162 DNA replication in *vitro*. Mol. Gen. Genet. **208**:390–397.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **Meyer, R. J., M. Hinds, and M. Brasch.** 1982. Properties of R1162, a broad host range, high copy number plasmid. J. Bacteriol. **150**:552–562.
13. **Meyer, R. J., L.-S. Lin, K. Kim, and M. Brasch.** 1984. Broad host-range plasmid R1162: replication, incompatibility and copy-number control, p. 173–188. *In* D. R. Helinski, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
14. **Nördstrom, K., S. Molin, and H. Aagard-Hansen.** 1980. Partitioning of plasmid R1 in *Escherichia coli*. I. Kinetics of loss of plasmid derivatives deleted of the par region. Plasmid **4**:215–227.
15. **Persson, C., and K. Nördstrom.** 1986. Control of replication of the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated DNA sequences. Mol. Gen. Genet. **203**:189–192.
16. **Priefer, U. B., R. Simon, and A. Puhler.** 1985. Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. J. Bacteriol. **163**:324–330.
17. **Rawlings, D. E., I. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobaciluus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **158**:737–738.
18. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1986. Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the thiobacilli. Biotechnol. Bioeng. Symp. **29**:291–297.
19. **Rawlings, D. E., R. Sewcheran, and D. R. Woods.** 1986. Characterization of a broad-host-range mobilizable *Thiobacillus ferrooxidans* plasmid and the construction of *Thiobacillus* cloning vectors, p. 419–427. *In* R. W. Lawrence, R. M. R. Branion, and H. G. Ebnes (ed), Fundamental and applied biohydrometallurgy. Elsevier/North-Holland Publishing Co., Amsterdam.
20. **Rawlings, D. E., and D. R. Woods.** 1985. Mobilization of *Thibacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. **49**:1323–1324.
21. **Salser, W.** 1977. Globin mRNA sequences: analysis of base pairing and evolutionary implications. Cold Spring Harbor Symp. Quant. Biol. **42**:985–1002.
22. **Scott, J. R.** 1984. Regulation of plasmid replication. Microbiol. Rev. **48**:1–23.
23. **Yanisch-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
24. **Zabeau, M., and K. K. Stanley.** 1982. Enhanced expression of cro-beta-galactosidase fusion products under the control of the P_R promoter of bacteriophage lambda. EMBO. J. **1**:1217–1224.

JOURNAL OF BACTERIOLOGY, Oct. 1990, p. 5697–5705
0021-9193/90/105697-09$02.00/0

Vol. 172, No. 10

Characterization of the Minimum Replicon of the Broad-Host-Range Plasmid pTF-FC2 and Similarity between pTF-FC2 and the IncQ Plasmids

ROSEMARY A. DORRINGTON AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Private Bag, Rondebosch 7700, South Africa

Received 12 March 1990/Accepted 16 July 1990

The nucleotide sequence of a 3,202-base-pair fragment which contained the minimum region required for replication of the broad-host-range plasmid, pTF-FC2, has been determined. At least five open reading frames and a region that affected the host range were identified. Proteins corresponding in size and location to four of the five open reading frames were produced in an in vitro transcription-translation system. The predicted amino acid sequences of two of the proteins were aligned with those of the RepA and RepC proteins of the broad-host-range IncQ plasmid RSF1010 and found to be 43 and 60% homologous, respectively. Despite this similarity, neither the RepA nor the RepC protein of the IncQ plasmid was able to complement mutations in the pTF-FC2 *repA* and *repC* genes. Although there was a considerable amount of DNA homology between pTF-FC2 and RSF1010 in the *oriV* region and the region coding for the RepA and RepC proteins, no other homology between the two plasmids at either the DNA or protein level could be detected.

Plasmid pTF-FC2 is a 12.6-kilobase-pair, cryptic, broad-host-range plasmid that was originally isolated from the industrially important bacterium *Thiobacillus ferrooxidans*. The plasmid was cloned into the vector pBR325 and shown to replicate in *Escherichia coli* from an origin located on the pTF-FC2 portion of the recombinant plasmid (23). The host range of pTF-FC2 includes all the gram-negative bacteria that have been tested so far, namely, *Pseudomonas aeruginosa* (24), *Thiobacillus novellus* (25), *Klebsiella pneumoniae*, *Rhizobium meliloti*, *Agrobacterium tumefaciens*, and *Salmonella typhimurium* (D. E. Rawlings and R. A. Dorrington, unpublished results). A 329-base-pair (bp) region of the pTF-FC2 DNA that is required for replication in *cis* has been identified and sequenced (7).

Relatively few plasmids capable of replication in a large number of gram-negative bacteria have been identified. Of these, the most extensively studied are those belonging to the IncQ incompatibility group, which includes the similar or identical plasmids RSF1010 (8), R1162 (16), and R300B (3). The entire nucleotide sequence (8,685 bp) of the RSF1010 has recently been published (28).

Plasmid pTF-FC2 is similar to the IncQ plasmids in several respects. The *oriV* region of pTF-FC2 includes three 22-bp tandem, perfectly repeated DNA sequences (7), whereas the corresponding region of the IncQ plasmids has three-and-one-half 20-bp perfectly repeated DNA sequences separated by 2-bp spacer sequences (17, 18). The repeated sequences of pTF-FC2 have a 60% DNA homology with those of the IncQ plasmids, whereas the 115-bp sequence immediately adjacent to the repeat sequences is 75% homologous (7). Both plasmids are maintained in *E. coli* at a copy number of approximately 12 per chromosome (3, 7) and are mobilized at high frequency between gram-negative bacteria by IncP plasmids such as RP4 (1, 25, 26).

Despite these similarities, no incompatibility between pTF-FC2 and the IncQ plasmid R300B was detected (7). The structural similarity between the origins of pTF-FC2 and the IncQ plasmids may reflect general features of a class of broad-host-range plasmids to which these plasmids belong. A comparison of the molecular mechanisms of replication between plasmids such as pTF-FC2 and the IncQ plasmids could result in the identification of these features, which are associated with the phenomenon of broad host range.

We report the nucleotide sequence of the minimum replicon of pTF-FC2 in *E. coli* and *P. aeruginosa* and the identification of at least two proteins essential for replication in *E. coli* and a region which appears to be involved in host range. The origin of replication of pTF-FC2 has been compared with that of the IncQ plasmids, and the ability of the two plasmids to functionally complement each other has been investigated.

METHODS AND MATERIALS

Bacterial strains and media. *E. coli* strains were grown in Luria broth or on Luria agar plates. Antibiotics were added as required at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and tetracycline, 20 μg/ml, as required. A DNA polymerase I (*polA*) mutant of the *recA E. coli* AB1157 (15), called *E. coli* GW125a, was a gift from Graham Walker, Massachusetts Institute of Technology, Cambridge, and was used to test for PolA-independent plasmid replication. Plasmids were maintained in *E. coli* LK111 (31). The broad-host-range property of plasmids was tested by transformation of DNA into *P. aeruginosa* PAO1162 (*leu-38 rmo-11*), (2) which was grown on LNG (containing, per liter, 10 g of tryptone, 5 g of yeast extract, 5 g of NaCl, 0.4% KNO_3, and 0.4% glucose) agar plates containing 500 μg of carbenicillin per ml, 200 μg of tetracycline per ml, and/or 400 μg of chloramphenicol per ml, followed by reisolation of the plasmids. Plasmids used in this study were the vector pUC19 (30) and pKE462 (7), which has the 7.5-kilobase *Eco*RI fragment from R46 carrying the Tc^r and arsenic resistance markers cloned into R300B. Plasmid pDR412 (23) consists of the 12.3-kilobase *Pst*I-*Xho*I fragment of pTF-FC2 (Fig. 1) and a *Pst*I-*Sal*I fragment carrying the Cm^r gene, but not the origin of replication of pBR325 (4).

* Corresponding author.

General recombinant DNA techniques. Plasmid DNA was prepared from *E. coli* and *P. aeruginosa* strains by using the method of Ish-Horowicz and Burke (12), and analysis and cloning of DNA were as described by Maniatis et al. (15). *E. coli* strains were transformed by using the dimethyl sulfoxide method of Chung and Miller (5), and transformation of *P. aeruginosa* PAO1162 was performed as follows. An overnight culture was harvested, suspended in 0.4 volume of cold 0.15 M $MgCl_2$, and kept on ice for 5 min, after which the cells were pelleted and resuspended in 0.08 volume of 0.15 M $MgCl_2$. DNA was added to 200 μl of cells, and the mixture was incubated on ice for 60 min, at 37°C for 2 min, and again on ice for a further 5 min. After addition of 1 ml of LNG broth, the cells were incubated at 37°C for 1 to 2 h and plated out.

Sequential deletions of pTV400 were made by using exonuclease III (11), and the nucleotide sequence of these deletions was determined by the chain termination method with the Sequenase DNA-sequencing kit (United States Biochemical Corp., Cleveland, Ohio). The sequence was analyzed by using the Genepro (version 4.1) and University of Wisconsin Genetics Computer Group (UWGCG) (version 6.1) software (6) packages.

In vitro protein synthesis. Translation products were identified by using a procaryotic DNA-directed transcription-translation kit (code N.380; Amersham International plc, Amersham, England), and the products were analyzed by polyacrylamide gel electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence of pTV400 has been assigned the GenBank accession number M35249.

RESULTS

Functional analysis of the pTF-FC2 origin of replication. Recombinant plasmid pTV400 was constructed by cloning a 3,202-bp partial *Sau*3A fragment of pTF-FC2 into pUC19 (Fig. 1) and selecting for the ability to replicate in *E. coli* GW125a (7). Previously, we had shown that plasmids pTV4101, which had a 620-bp deletion from one side of the 3,202-bp fragment, and pTV4200, with a 334-bp deletion from the opposite end (Fig. 1), were the smallest plasmids capable of replication in *E. coli* GW125a with a normal copy number (7). Plasmid pTV4210 (Fig. 1), with a further 150 bp deleted from the distal end of pTV4200, was able to replicate at a reduced copy number (7). pTV400 and the deletion plasmids pTV4101, pTV4200, and pTV4210 were transformed into *P. aeruginosa* PAO1162 and examined for their ability to be maintained. Plasmids pTV400 and pTV4200 replicated normally, whereas pTV4210-containing colonies were smaller on carbenicillin plates than pTV4200-containing colonies were. No colonies containing either pTV4101 or pTV4100, which is 260 bp larger than pTV4101 (Fig. 1), were isolated.

Nucleotide sequence of pTV400. The complete nucleotide sequence of pTV400 (3,202 bp) is shown in Fig. 2. The sequence contains very few 6-bp recognition restriction enzyme digestion sites relative to its size. Previously mapped *Apa*I and *Ava*I restriction enzyme sites are located at nucleotides (nt) 1251 and 2067, respectively. Sequence analysis revealed unique sites for *Ava*II (nt 162), *Bal*I (nt 2419), *Dra*I (nt 2107), *Hin*cII (nt 1389), and *Xho*II (nt 1080). The 3,202-bp fragment has a G+C content of 59%, which corresponds closely to that calculated for the *T. ferrooxidans* chromosome (10) and to the average of 58.7% which we have calculated for the *T. ferrooxidans* genes previously cloned and sequenced in our laboratory (19–22).

Computer analysis of the 3,202-bp fragment by using the CODONPREFERENCE and TESTCODE routines of the UWGCG package indicated the presence of at least five potential coding regions, each preceded by a consensus ribosome-binding site (Fig. 3; Table 1). The amino terminus of open reading frame 2 (ORF2) overlaps with the carboxy terminus of ORF1 and has two possible ATG start codons at nt 1684 and 1714, each preceded by a consensus ribosome-binding site. A protein of 33,778 daltons (Da) would be produced if translation occurred from the first ATG, and one of 32,659 Da would be produced if translation commenced from the second. The carboxy terminus of ORF2 overlaps with the first two direct repeats of the *oriV*.

The *oriV* region of pTV400, which has previously been shown to consist of three sets of 22-bp direct repeated sequences and the 245-nt fragment immediately adjacent to the repeats (7), is situated between nt 2540 and 2869. Other features of the nucleotide sequence are a region between nt 655 and 677 containing a 14-bp match with the 22-bp repeated sequences in the *oriV* and two sets of inverted complementary repeats (nt 2673 to 2691 and 2703 to 2738) (Fig. 2). The biological function of these structural features is unclear, but deletion of half of the latter set of inverted repeats resulted in a reduced copy number (7).

Analysis of polypeptides encoded by the minimum replicon. Polypeptides from the pTF-FC2 minimum replicon were analyzed both in vivo and in vitro to ascertain whether polypeptides equivalent to those predicted from the nucleotide sequence could be detected. Polypeptides were expressed from cloned fragments and deletion plasmids by using an in vitro DNA-directed transcription-translation kit, and the products were analyzed by polyacrylamide gel electrophoresis. Since the predicted polypeptides varied in size, different concentrations of acrylamide were used to maximize resolution. Translation of the pUC19 vector resulted in the 30,500-Da β-lactamase protein (Fig. 4A, lane 5; Fig. 4B, lane 1), which was present in all the lanes. Plasmid pTV400, with the intact 3,202-bp fragment, produced additional polypeptides of molecular masses 33,500 Da, 32,000 Da (Fig. 4A, lane 1; Fig. 4B, lane 2), 10,000 Da, and 8,000 Da (Fig. 4B lane 2). These values correspond closely to the polypeptides predicted for ORF2, ORF1, ORF4, and either ORF3 or ORF5, respectively. Plasmid pTV4220 (nt 1 to 2353) has the *oriV* and 77 amino acid residues deleted from the carboxy terminus of ORF2. The 33,500 Da corresponding to ORF2 has disappeared, and a new 28,500-Da polypeptide, which corresponds closely to the predicted molecular mass of 25,679 Da (Fig. 4A, lane 2), has appeared, while the other polypeptides remained unchanged. Plasmid pTV4260 (nt 1 to 1739), which has the *oriV* and all except the first 17 amino acid residues of ORF2 deleted, showed a complete loss of the 33,500-Da protein while the other peptides were unchanged (Fig. 4A, lane 3; Fig. 4B, lane 4). Plasmids pTV4283 (nt 1 to 801) and pTV4282 (nt 1 to 870), which have both ORF1 and ORF2 deleted, no longer produced the 33,500- or 32,000-Da proteins (Fig. 4A, lane 4; Fig. 4B, lane 5) but still produced the 10,000- and 8,000-Da proteins (Fig. 4B, lane 5). From the codon preference data (Fig. 3) it was predicted that the ORF3 (8,000-Da) and ORF4 (10,000-Da) proteins are located within the first 571 bp of pTV400. This predicton correlates well with what was found in vitro. Plasmid pTV4290 (nt 1 to 660) produced proteins of 10,000 and 8,000 Da (Fig. 4B, lane 6), while pTV4101 (nt 615 to 3202) no longer produced these proteins (Fig. 4B, lane 3). Additional proteins of 30,000 and 28,000 Da (Fig. 4A, lanes 1 to 3) were observed. These proteins disappeared when

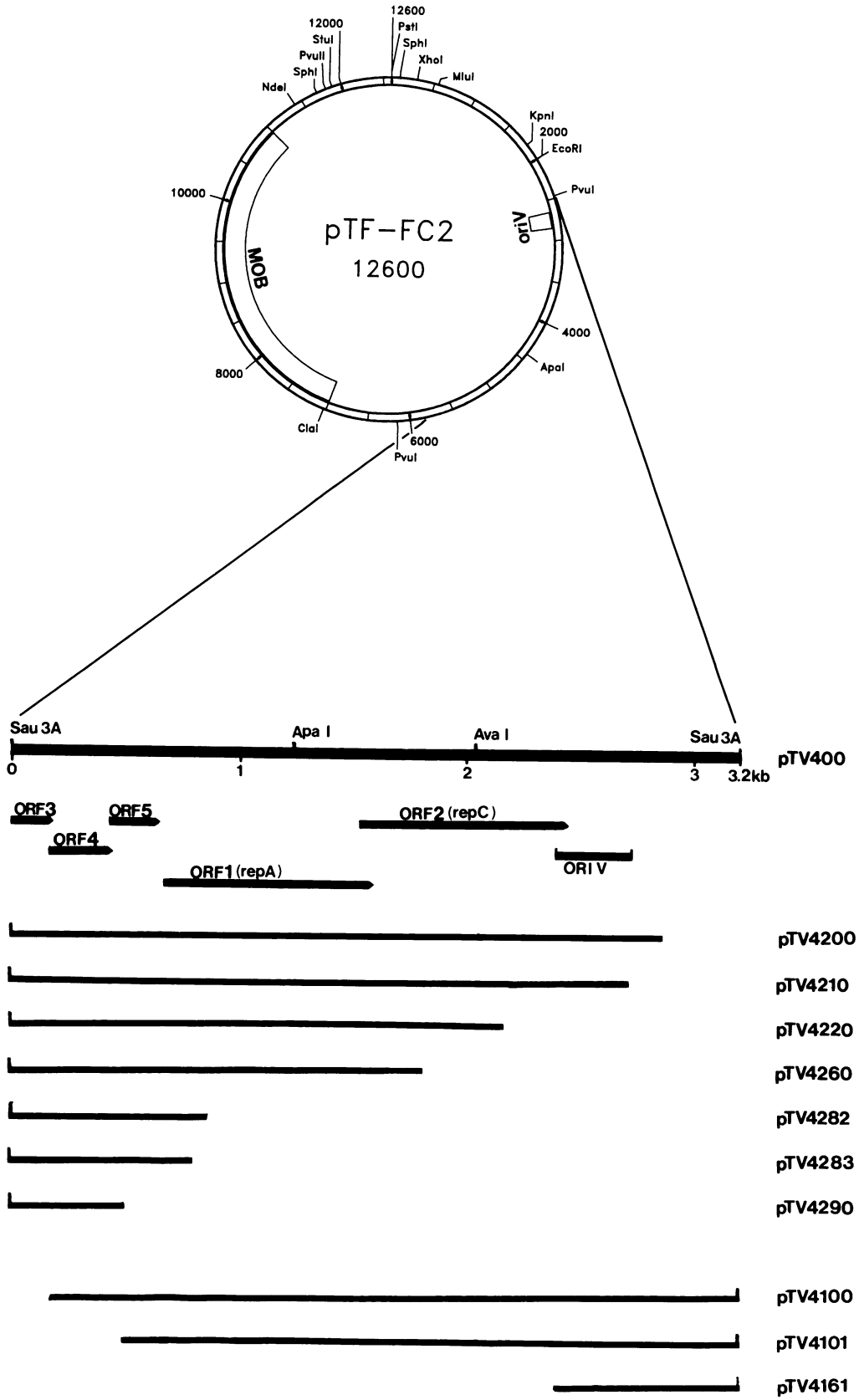


FIG. 1. Restriction endonuclease cleavage map showing the position and direction of potential ORFs and subclones used in this study. Only pTF-FC2-derived fragments are shown. The locations of the regions required for mobilization (MOB) and the vegetative origin of replication (oriV) are shown as open boxes.

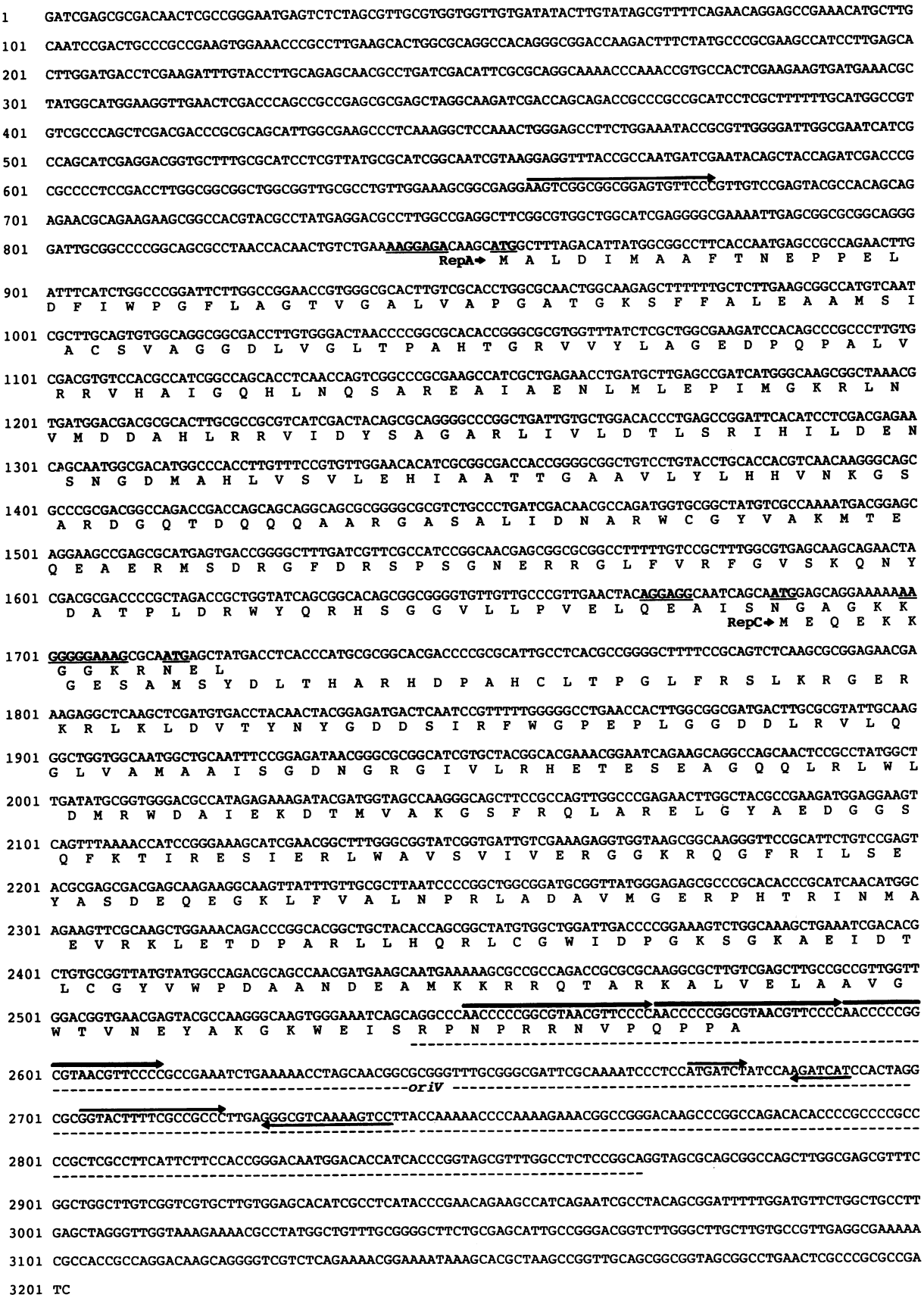


FIG. 2. Nucleotide sequence of the 3,202-bp *Sau*3A DNA fragment of pTF-FC2 contained in pTV400 and the predicted amino acid sequences of ORF1 (*repA*) and (*repC*). Solid lines below the nucleotides denote the possible ribosome-binding sites and start codons of each ORF. The extent of *oriV* is indicated by a dotted line below the sequence. Thick arrows above the sequence denote the three tandemly repeated 22-bp DNA sequences of *oriV* and the partially homologous sequence (14 of 22 bp) upstream of *repA*. Thin arrows above and below the nucleotide sequence show the two sets of inverted complementary repeats in *oriV*.

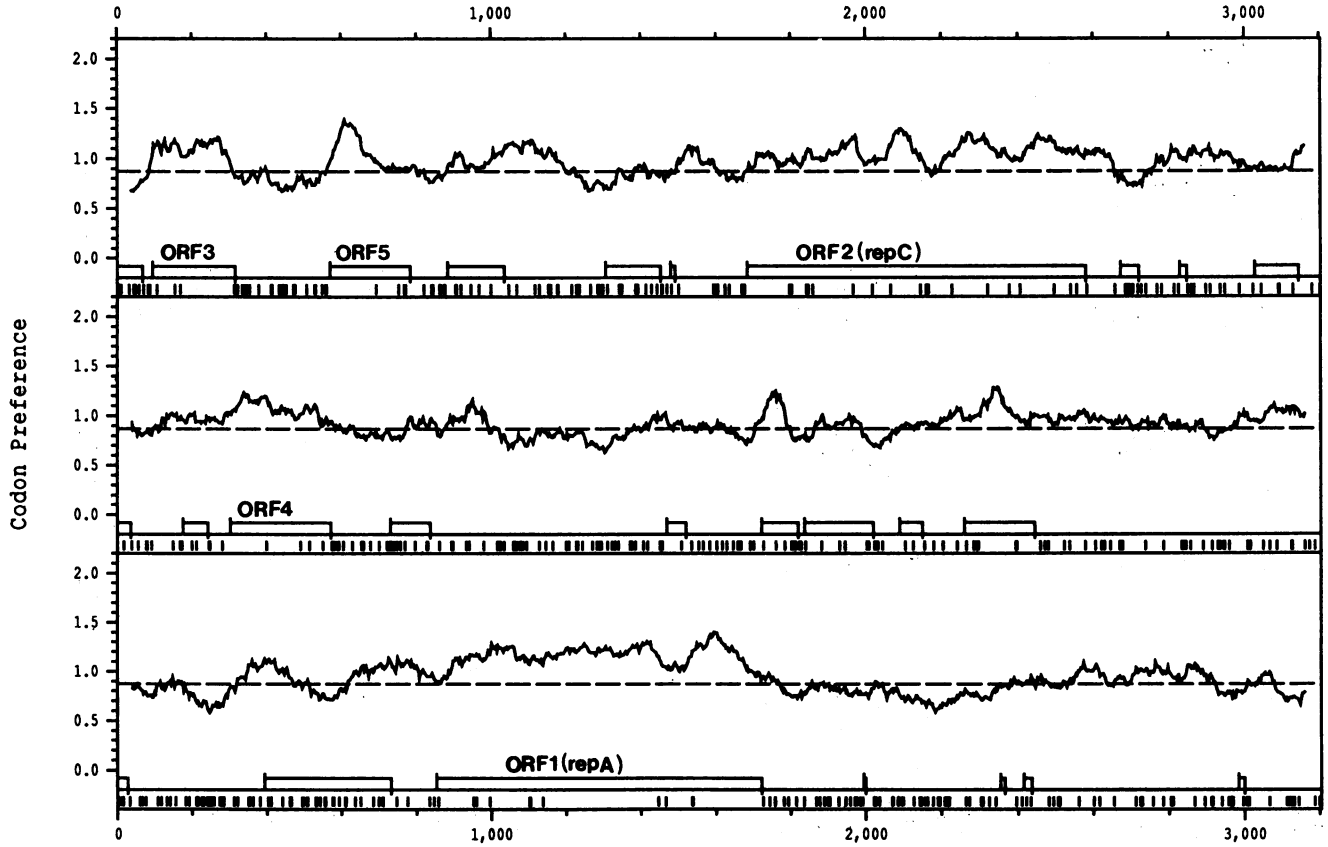


FIG. 3. Codon preference plot and rare codon usage for the predicted ORFs of the 3,202-bp fragment of pTV400. Rare codons are indicated by bars below open boxes, which represent the ORFs. ORFs were identified by using the UWGCG package codon preference subroutine. A codon preference table based on ORF1 and ORF2 was used to help identify ORF3, ORF4, and ORF5.

ORF1 was deleted (Fig. 4A, lane 4) and appeared to be degradation products of ORF1.

Comparison of ORF1 and ORF2 of pTF-FC2 with RepA and RepC of the IncQ plasmids. The predicted amino acid sequences of the ORF1 and ORF2 polypeptides were aligned and compared with the amino acid sequences of RepA and RepC of RSF1010 (28). The predicted ORF1 protein is 11 amino acids larger than RepA, and on alignment the two proteins have a similarity of 43% based on the perfectly conserved amino acid residues (Fig. 5). An alignment between the larger of the two possible ORF2 polypeptides and RepC of RSF1010 is also shown in Fig. 5, and the similarity was calculated at 60%. There is no homology between the remaining pTF-FC2 ORFs and any of the other RSF1010 proteins. Owing to the similarity between the ORF1 and ORF2 proteins of pTF-FC2 and RepA and RepC of RSF1010, the corresponding pTF-FC2 genes were named *repA* and *repC*, respectively.

Since there was a high degree of homology between the RepA and RepC proteins of pTF-FC2 and RSF1010, we wished to determine whether the IncQ proteins were able to complement mutations and deletions in the pTF-FC2 origin. Deletion plasmid pTV4161 contained only the region nt 2543 to 3202 (Fig. 1), which included the intact *oriV* but none of the ORFs, and was able to replicate in *E. coli* GW125a only when pDR412 was present (7). We substituted pDR412 with pKE462, a derivative of R300B, but were unable to detect replication of pTV4161. The R300B replicon was therefore unable to complement *oriV* of pTF-FC2.

The ability of the IncQ plasmid proteins to complement the pTF-FC2 RepA and RepC proteins individually was investigated by inactivating the proteins through the intro-

TABLE 1. Location of potential protein-coding regions on the origin of replication of pTF-FC2

ORF	Position of coding sequence (nt)	Translational initiation sites[a]	No. of amino acid residues	Mol mass (Da)
ORF1	852–1721	CUGUCUGAAAAGGAGACAAGCAUG	290	31,252
ORF2	1684–2580	AACUACAGGAGGCAAUCAGCAAUG	299	33,778
	1714–2580	GAAAAAAAGGGGGAAAGCGCAAUG	289	32,659
ORF3	94–315	UUUCAGAACAGGAGCCGAAACAUG	74	8,462
ORF4	302–571	UCGAAGAAGUGAUGAAACGCUAUG	90	10,318
ORF5	571–783	UCGUAAGGAGGUUUACCGCCAAUG	71	7,702

[a] The start codons are underlined, as are the nucleotides in the pre-start region that could pair with the 3′ end of the *E. coli* 16S rRNA (AUUCCUCCACU . . . 5′).

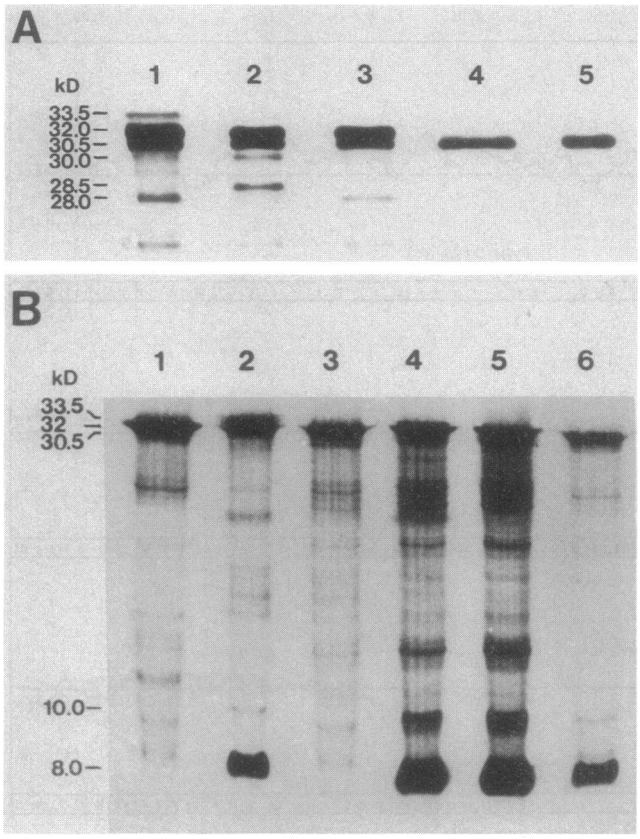


FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of polypeptides translated in vitro from pTV400 and deletions. (A) Lanes 1 to 5 show polypeptides from pTV400, pTV4220, pTV4260, pTV4283, and pUC19, respectively; proteins were separated on a 12% acrylamide gel. (B). Lanes 1 to 6 show polypeptides produced by pUC19, pTV400, pTV4101, pTV4260, pTV4282, and pTV4290, respectively; proteins were separated in a 20% acrylamide gel.

duction of frameshift mutations by using the *Apa*I and *Ava*I restriction enzyme sites which are located within the *repA* and *repC* genes, respectively. Mutations were introduced by digestion of pTV4100 with *Apa*I or *Ava*I and flushing the ends with the Klenow fragment of DNA polymerase I to produce plasmids pTV4102 (*repA*) and pTV4103 (*repC*). Neither mutant plasmid could be replicated in *E. coli* in the absence of pDR412. An in vitro DNA-directed transcription-translation system was used to test whether the frameshift mutations had inactivated the respective genes. Plasmid pTV4102, with a mutation in *repA*, produced only the 33,500-Da RepC protein, whereas pTV4103, with the mutation in *repC*, produced only the 32,000-Da RepA protein (data not shown).

We tested whether the individual RepA and RepC proteins of RSF1010 could complement mutations in their respective equivalents on the pTF-FC2 origin. Plasmids pTV4102 and pTV4103 were transformed into *E. coli* GW125a containing pDR412 or pKE462. Transformation with either plasmid resulted in more than 1,500 transformants per 100 ng of plasmid when pDR412 was present, but no transformants were detected in the presence of pKE462. We concluded that the IncQ replicon on pKE462 was unable to complement either the *repA* or *repC* mutant.

Alignment of the origin of replication of pTF-FC2 with the RSF1010 replicon. A nucleotide sequence alignment between the 3,202-bp fragment of pTF-FC2 and the completely sequenced IncQ plasmid, RSF1010, is shown in Fig. 6. There are two regions where DNA homology of more than 60% is apparent. One of these corresponds to the region that codes for the RepA and RepC proteins, and the other corresponds to the *oriV* region. There was no significant homology between the first 800 bp and the last 500 bp of the 3,202-bp fragment and the RSF1010 replicon.

DISCUSSION

There is a marked lack of 6-bp restriction endonuclease recognition sites in the nucleotide sequence of the 3,202-bp origin of replication of pTF-FC2. This feature has also been noted in other broad-host-range plasmids (29), and it has been suggested that this phenomonon may be essential for the survival of promiscuous plasmids.

Plasmid pTF-FC2 and the IncQ plasmids are similar in the structure and nucleotide sequence of their *oriV* regions (7), and from the nucleotide sequence reported here, it is clear that the similarity extends to the region encoding their respective RepA and RepC proteins. This high degree of homology is maintained at both the DNA and amino acid levels; this, along with the observation that the similarity is distributed evenly across both proteins, indicates that the two sets of proteins have similar functions in the replication of their respective origins. Despite these similarities, the IncQ RepA and RepC proteins were unable to complement mutations in their equivalent proteins on the pTF-FC2 origin.

The RepA protein of RSF1010 has been reported to be a DNA-dependent ATPase (27), and there is strong evidence that the RepC protein functions as an initiator of replication by binding to the 20-bp repeated DNA sequences in *oriV* of the IncQ plasmids (9, 14). Lin et al. (14) introduced point mutations in the 20-bp repeats of R1162 which resulted in a reduction in the degree of incompatibility between plasmids carrying these mutations and the wild-type R1162 origin as compared with plasmids carrying wild-type repeated sequences with the wild-type R1162 origin. They postulated that this was due to an altered binding efficiency of the RepC protein to the mutant repeated sequences. The absence of detectable incompatibility between the pTF-FC2 *oriV* and R300B (7), together with the finding that the two types of directly repeated sequences are only 60% homologous, suggests that the inability of the IncQ RepC protein to complement a pTF-FC2 *repC* mutant is due to its inability to bind to pTF-FC2 repeated sequences.

Besides the *repA* and *repC* gene products, the IncQ plasmids have an absolute requirement for the *repB* gene product for replication to occur in vitro in *E. coli* (27). The 35,900-Da protein produced by this gene is involved in primer synthesis at *oriV* during initiation of replication (28). The carboxy terminus of the *repB* gene is situated within 500 bp of the start of *repA*. No equivalent of this protein has been found in the 850 bp of sequence upstream of the pTF-FC2 *repA* start.

We have identified a 615-bp region which affects the ability of the pTF-FC2 replicon to be maintained in *P. aeruginosa*. This region codes for proteins of approximately 8,000 and 10,000 Da, which correspond closely to the peptides predicted for ORF3 or ORF5 and ORF4, respectively. In the IncQ plasmid, RSF1010, a small (10,000-Da) protein has been implicated in the determination of host range (28). There was no significant homology between this protein and the products of ORF3, ORF4, or ORF5.

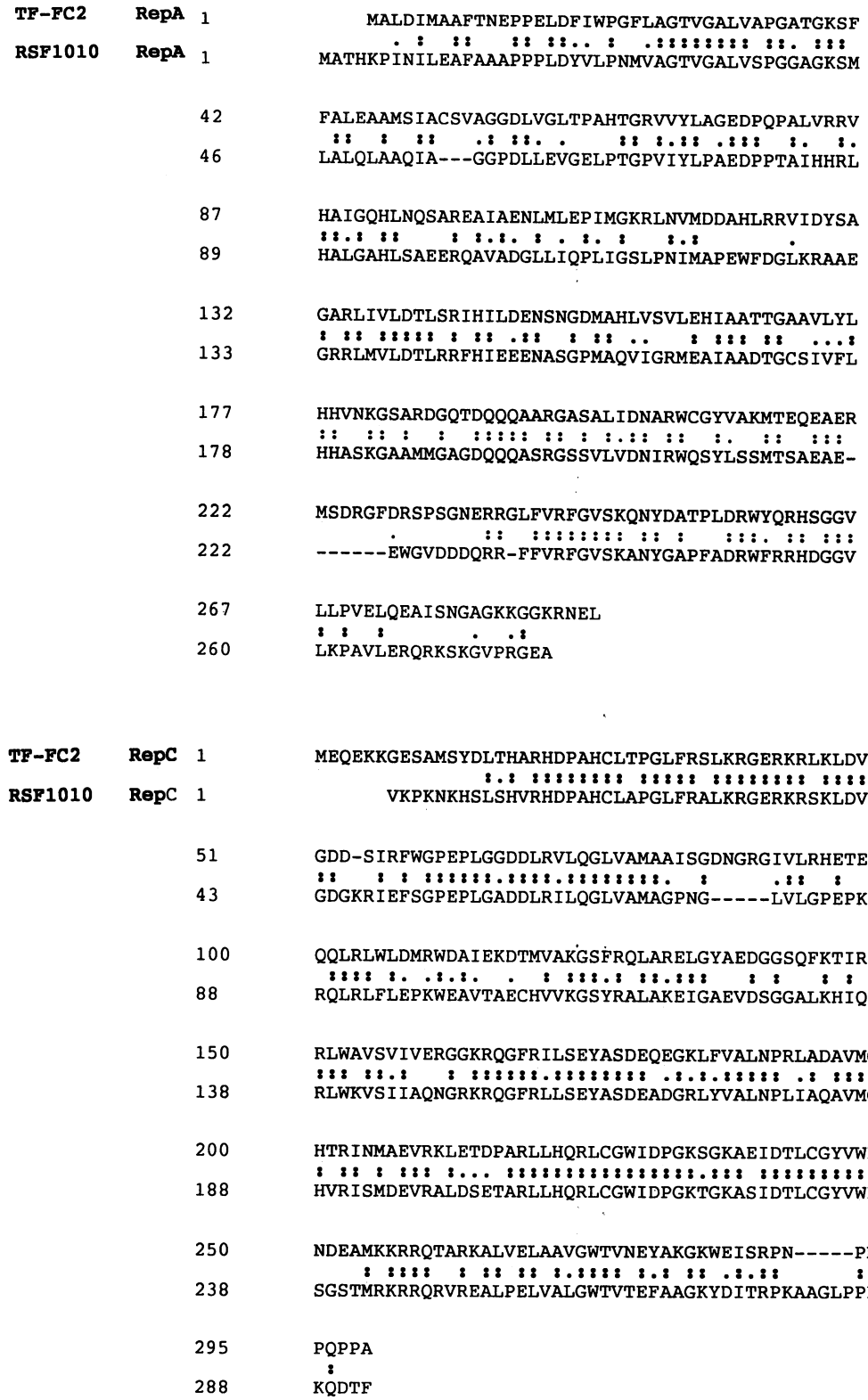


FIG. 5. Alignment of the amino sequences of the RepA and RepC proteins of pTF-FC2 and RSF1010 by using the Genepro computer sequence analysis package. Identical and conservative amino acid changes are indicated by double and single dots, respectively, between the sequences.

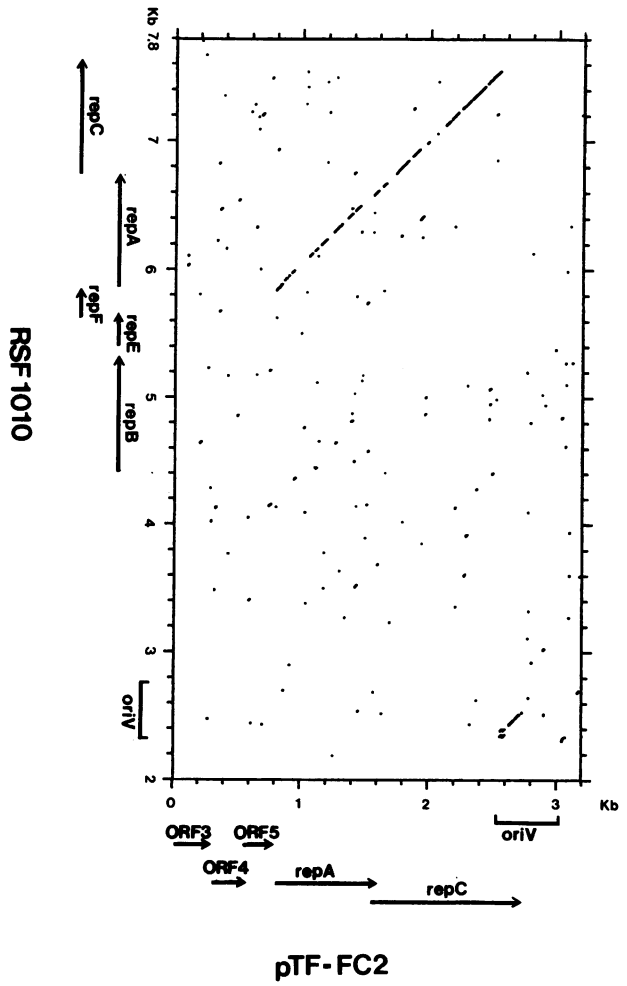


FIG. 6. Alignment of the nucleotide sequences of the 3,202-bp fragment of pTF-FC2 and nt 2000 to 7800 of RSF1010, which include all sequences involved in replication and mobilization but exclude the sulfonamide and streptomycin coding sequences (28).

The IncQ RepA and RepC proteins are expressed from a polycistronic mRNA (9), and their expression is controlled at the level of translation by an antisense RNA complementary to the ribosome-binding site and start of the *repA* gene (13). In addition, expression of IncQ *repC* appears to be translationally coupled to expression of *repA* (9). We were able to detect the pTF-FC2 RepC product when the front end of *repA* was deleted and replaced by a T7 promoter (Dorrington, unpublished results). In addition, the frameshift mutation in *repA* did not affect RepC production in vitro. Thus, unlike for its IncQ counterpart, the translation of RepC appears to take place even when uncoupled from that of RepA.

A comparison of the structure of the pTF-FC2 replicon with that of the IncQ plasmids is interesting. In both plasmids the *repA* genes, *repC* genes, and *oriV* regions have considerable nucleotide homology. In pTF-FC2, the two regions that show homology are contiguous, whereas in RSF1010, *oriV* is separated from *repA* and *repC* by a 2,484-bp fragment that includes the sulfonamide and streptomycin resistance genes. These genes have a different codon usage pattern from the rest of the genes found on RSF1010, and Scholz et al. (28) suggest that these genes may have been incorporated at a later stage in the development of the IncQ plasmids. The finding that the two homologous regions are contiguous in pTF-FC2 indicates that the two replicons may be derived from a common ancestor from which they have susequently diverged. In the IncQ plasmids, the *repA* and *repC* genes may have been separated from *oriV* by insertion of a transposable element such as Tn*4*, which carries sulfonamide and streptomycin resistance markers.

LITERATURE CITED

1. **Bagdasarian, M., M. M. Bagdasarian, R. Lutz, A. Nordheim, A. Frey, and K. N. Timmis.** 1982. Molecular and functional analysis of the broad-host-range plasmid RSF1010 and construction of vectors for gene cloning in Gram-negative bacteria, p. 183–197. *In* S. Mitsubishi (ed.) Bacterial Drug Resistance. Japan Scientific Societies Press, Tokyo.
2. **Bagdasarian, M., and K. N. Timmis.** 1982. Host-vector systems for gene cloning in *Pseudomonas*. Curr. Top. Microbiol. Immunol. **96**:47–67.
3. **Barth, P., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weight and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120**:618–630.
4. **Bolivar, F.** 1978. Construction and characterization of new cloning vectors. III. Derivatives of plasmid pBR322 carrying unique *Eco*RI sites for selection of *Eco*RI generated recombinant DNA molecules. Gene **4**:121–136.
5. **Chung, C. T., and R. H. Miller.** 1988. A rapid and convenient method for the preparation and storage of competent bacterial cells. Nucleic Acids Res. **16**:3580.
6. **Devereux, I., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence programs for the VAX. Nucleic Acids Res. **12**:387–395.
7. **Dorrington, R. A., and D. E. Rawlings.** 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171**:2735–2739.
8. **Guerry, P., J. van Emden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117**:619–630.
9. **Haring, V., P. Scholz, E. Scherzinger, J. Frey, K. Derbyshire, G. Hatfull, N. S. Willetts, and M. Bagdasarian.** 1985. Protein RepC involved in copy number control of the broad-host-range plasmid RSF1010. Proc. Natl. Acad. Sci. USA **82**:6090–6094.
10. **Harrison, A. P., Jr.** 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **38**:265–292.
11. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28**:351–369.
12. **Ish-Horowicz, D., and F. Burke.** 1983. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9**:2989–2998.
13. **Kim, K., and R. J. Meyer.** 1986. Copy number of broad-host-range plasmid R1162 is regulated by a small RNA. Nucleic Acids Res. **14**:8027–8046.
14. **Lin, L.-S., Y.-J. Kim, and R. J. Meyer.** 1987. The 20-base pair, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility *in vivo* and inhibits R1162 DNA replication *in vitro*. Mol. Gen. Genet. **208**:390–397.
15. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.
16. **Meyer, R., M. Hinds, and M. Brasch.** 1982. Properties of R1162, a broad-host-range, high-copy-number plasmid. J. Bacteriol. **150**:552–562.
17. **Meyer, R., L.-S. Lin, K. Kim, and M. Brasch.** 1984. Broad-host-range plasmid R1162: replication, incompatibility and copy-number control, p. 173–188. *In* D. R. Helinsky, S. N. Cohen, D. B. Clewell, D. A. Jackson, and A. Hollaender (ed.), Plasmids in bacteria. Plenum Publishing Corp., New York.
18. **Persson, C., and K. Nordström.** 1986. Control of replication of

the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated DNA sequences. Mol. Gen. Genet. **203**:189–192.

19. **Pretorius, I.-M., D. E. Rawlings, E. G. O'Neil, W. A. Jones, and D. R. Woods.** 1987. Nucleotide sequence of the gene encoding the nitrogenase iron protein of *Thiobacillus ferrooxidans*. J. Bacteriol. **169**:367–370.
20. **Ramesar, R. S., V. Abratt, D. R. Woods, and D. E. Rawlings.** 1989. Nucleotide sequence and expression of a cloned *Thiobacillus ferrooxidans recA* gene in *Escherichia coli*. Gene **78**:1–8.
21. **Rawlings, D. E.** 1988. Sequence and structural analysis of the α- and β-dinitrogenase subunits of *Thiobacillus ferrooxidans*. Gene **69**:337–343.
22. **Rawlings, D. E., W. A. Jones, E. G. O'Neil, and D. R. Woods.** 1987. Nucleotide sequence of the glutamine synthetase gene and its controlling region from the acidophilic autotroph *Thiobacillus ferrooxidans*. Gene **53**:211–217.
23. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **158**:737–738.
24. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1986. Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the thiobacilli. Biotechnol. Bioeng. Symp. **16**:281–287.
25. **Rawlings, D. E., R. Sewcheran, and D. R. Woods.** 1986. Characterization of a broad-host-range mobilizable *Thiobacillus ferrooxidans* plasmid and the construction of *Thiobacillus* cloning vectors, p. 419–427. *In* R. W. Lawrence, R. M. R. Branion, and H. G. Ebnes (ed.), Fundamental and applied biohydrometallurgy. Elsevier Biomedical Press, Amsterdam.
26. **Rawlings, D. E., and D. R. Woods.** 1985. Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. **49**:1323–1325.
27. **Scherzinger, E., M. M. Bagdasarian, P. Scholz, R. Lurz, B. Ruckert, and M. Bagdasarian.** 1984. Replication of the broad host range plasmid RSF1010: requirement for three plasmid encoded proteins. Proc. Natl. Acad. Sci. USA **81**:654–658.
28. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75**:271–288.
29. **Walker, G. C.** 1986. Plasmid biology of pKM101 of the *muc*AB genes. Banbury Rep. **24**:313–321.
30. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
31. **Zabeau, M., and K. K. Stanley.** 1982. Enhanced expression of cro-beta-galactosidase fusion products under the control of the PR promoter of bacteriophage lambda. EMBO J. **1**:1217–1224.

Gene, 108 (1991) 7–14

GENE 06141

The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*

(Recombinant DNA; plasmid replication; RepB primase; nucleotide sequence; *Thiobacillus ferrooxidans*)

Rosemary A. Dorrington, Soraya Bardien and Douglas E. Rawlings

Department of Microbiology, University of Cape Town, Rondebosch, 7700 (South Africa)

Received by M. Bagdasarian: 21 May 1991
Accepted: 9 August 1991
Received at publishers: 4 September 1991

SUMMARY

A 3202-bp fragment of plasmid pTF-FC2, cloned into pUC19, had previously been identified as the minimum region required for replication in either *Pseudomonas aeruginosa* or *Escherichia coli polA*$^-$ mutants. During the course of experiments to construct broad-host-range cloning vectors based on the pTF-FC2 replicon, it was found that the 3202-bp fragment had an absolute requirement for some function of the pUC19 vector. This requirement was eliminated in the presence of co-resident pTF-FC2 derivatives. An additional 1239-bp fragment from pTF-FC2, immediately adjacent to the 3202-bp fragment, was identified which restored the ability of the pTF-FC2 replicon to replicate autonomously. Sequence analysis of the region revealed a single open reading frame encoding a 40-kDa polypeptide, which was synthesised in an in vitro transcription/translation system. A comparison of the amino acid sequence of this protein with sequence data banks revealed limited homology with the RepB′ primase of the IncQ plasmid, RSF1010. An M13*Δlac*110 replication-deficient phage system was used to demonstrate that the 40-kDa protein did function as a primase with respect to replication at the origin of replication (vegetative) of pTF-FC2.

INTRODUCTION

Plasmid pTF-FC2 is a 12.4-kb, cryptic plasmid which was originally isolated from the Gram$^-$ acidophilic chemolithoautotroph, *Thiobacillus ferrooxidans* (Rawlings et al., 1984). The plasmid is able to replicate in a wide range of other Gram$^-$ bacteria such as *E. coli*, *P. aeruginosa* (Rawlings et al., 1986), *Thiobacillus novellus*, *Agrobacterium tumefaciens* and *Rhizobium meliloti* (Dorrington and Rawlings, 1989). In addition, the plasmid is mobilized at high frequency between strains of *E. coli* bacteria by plasmids belonging to the IncP incompatibility group (Rawlings et al., 1985). Unlike ColE1-based plasmids, derivatives of pTF-FC2 were found to be capable of replication in DNA polymerase I mutants (*polA*) of *E. coli* and were maintained at a copy number of twelve to 15 plasmids per chromosome (Dorrington and Rawlings, 1989).

In previous work a 3202-bp pTF-FC2 fragment was cloned into pUC19 and found to be able to support replication in *E. coli polA* mutants and *P. aeruginosa*. A 329-bp region that was required in *cis* for replication and contained the *oriV* was identified within this fragment. The *oriV* region had three perfectly conserved, tandem, 22-bp repeats as

Correspondence to: Dr. D. Rawlings, Department of Microbiology, University of Cape Town, Private Bag, Rondebosch (South Africa) Tel. (2721)650-3261; Fax (2721)685-3726.

Abbreviations: aa, amino acid(s); Ap, ampicillin; bp, base pair(s); Cm, chloramphenicol; kb, kilobase(s) or 1000 bp; Km, kanamycin; ORF, open reading frame; *oriV*, origin of plasmid replication (vegetative); *P.*, *Pseudomonas*; PA, polyacrylamide; PAGE, PA-gel electrophoresis; pfu, plaque-forming units; ^{R}, resistance/resistant; RBS, ribosome-binding site; SD, Shine–Dalgarno (sequence); SDS, sodium dodecyl sulfate; *ssi*, single-stranded-initiation (sites); Tc, tetracycline; UWGCG, University of Wisconsin Genetics Computer Group; ′ (prime), denotes a truncated gene or gene product; [ ], denotes plasmid-carrier state.

well as two sets of inverted complementary repeat sequences (Dorrington and Rawlings, 1989). The remainder of the 3202-bp fragment was shown to produce at least four proteins (Dorrington and Rawlings, 1990).

Comparisons between the nt sequences of pTF-FC2 and the broad-host-range IncQ (Scholz et al., 1989) plasmids revealed significant homology between the *oriV* regions of the plasmids. In addition, two pTF-FC2 proteins were found to have 43 and 60% aa sequence identity with the RepA and RepC proteins of RSF1010, respectively. In spite of the similarities between the proteins and *oriV* regions, the IncQ plasmids and pTF-FC2 were compatible and the IncQ plasmids were unable to complement pTF-FC2 *repA* and *repC* mutants (Dorrington and Rawlings, 1989; 1990). The RepA and RepC proteins are essential for the replication of RSF1010 (Scherzinger et al., 1984) and have been shown to have helicase and specific DNA-binding properties, respectively (Haring and Scherzinger, 1989). Besides RepA and RepC, a third protein, RepB, which has primase activity (Haring and Scherzinger, 1989), is essential for replication of RSF1010 (Scherzinger et al., 1984). No equivalent protein was identified on the pTF-FC2 minimum replicon which implied that pTF-FC2 used the host priming system (DnaG and RNA-Pol).

We describe experiments which show that the 3202-bp pTF-FC2 fragment, previously considered to contain the entire pTF-FC2 replicon (Rawlings and Dorrington, 1990), was unable to replicate autonomously in *E. coli* when the ColE1-type vector such as pUC19 or pBluescript was deleted from the hybrid plasmid. Furthermore, we show that a primase-like protein is encoded by a pTF-FC2 region upstream from the *repA* gene and that this protein enabled the replicon to function independently of the ColE1-type vectors.

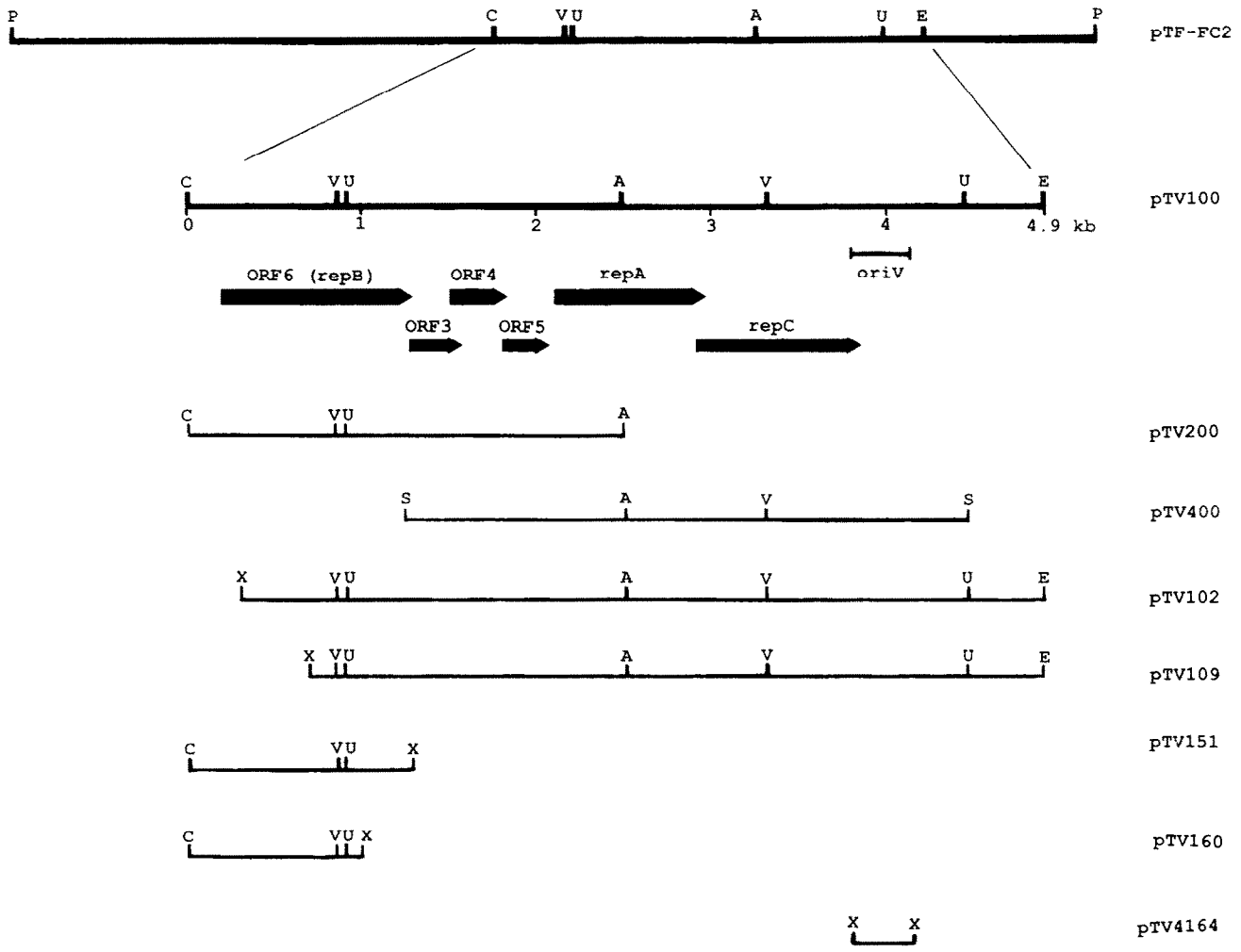


Fig. 1. Restriction map of the origin of replication of pTF-FC2 and of the subclones. Only pTF-FC2 derived fragments are shown. The construction of pTV400 has been described previously (Dorrington and Rawlings, 1989). Ordered deletions were generated from the *Cla*I and *Eco*RI ends using the exonuclease III method of Henikoff (1984). The position and direction of ORFs are indicated by solid arrows and the location of the 329-bp *oriV* region is shown. Restriction sites: A, *Apa*I; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sau*3A; U, *Pvu*I; V, *Ava*I; X, the end of a DNA fragment produced as a result of endonuclease III digestion.

RESULTS AND DISCUSSION

(a) Dependence of the previously identified replicon on pUC19

As the first step towards the construction of a broad-host-range vector, an attempt was made to link the previously identified minimal pTF-FC2 replicon to a selectable marker. Plasmid pTV400 (Fig. 1) was chosen for vector construction because it contained a 3202-bp partial *Sau*3A fragment of pTF-FC2 cloned into the *Bam*HI site of pUC19 and had retained the ability to replicate in *P. aeruginosa* and in the *E. coli polA*$^-$ strain, GW125a. Two experiments were carried out; in the first an attempt was made to delete pUC19 and replace the vector sequence with the Cm^R marker from pBR325 (Balbás et al., 1986). This experiment was unsuccessful. In the second experiment the Km^R marker from Tn*5* (De Bruin and Lupski, 1984) was cloned into pTV400, resulting in an Ap^R and Km^R plasmid, pTF100, which was capable of replication in the *polA*$^-$ *E. coli* GW125a strain (Fig. 2). Plasmid pTF100 was digested with *Eco*RI + *Hin*dIII so as to remove the pUC19 replicon, but retain the Km^R marker linked to the pTF-FC2 fragment. The *Eco*RI and *Hin*dIII sites were flushed using the *E. coli* DNA polymerase I Klenow fragment and the DNA was ligated and transformed into *E. coli* LK111. Km^R transformants were picked onto agar plates containing Ap, where sensitivity to Ap would indicate loss of the pUC19 replicon. Despite several repetitions of the experiment, no Ap^S colonies were obtained, all Km^R colonies remained Ap^R.

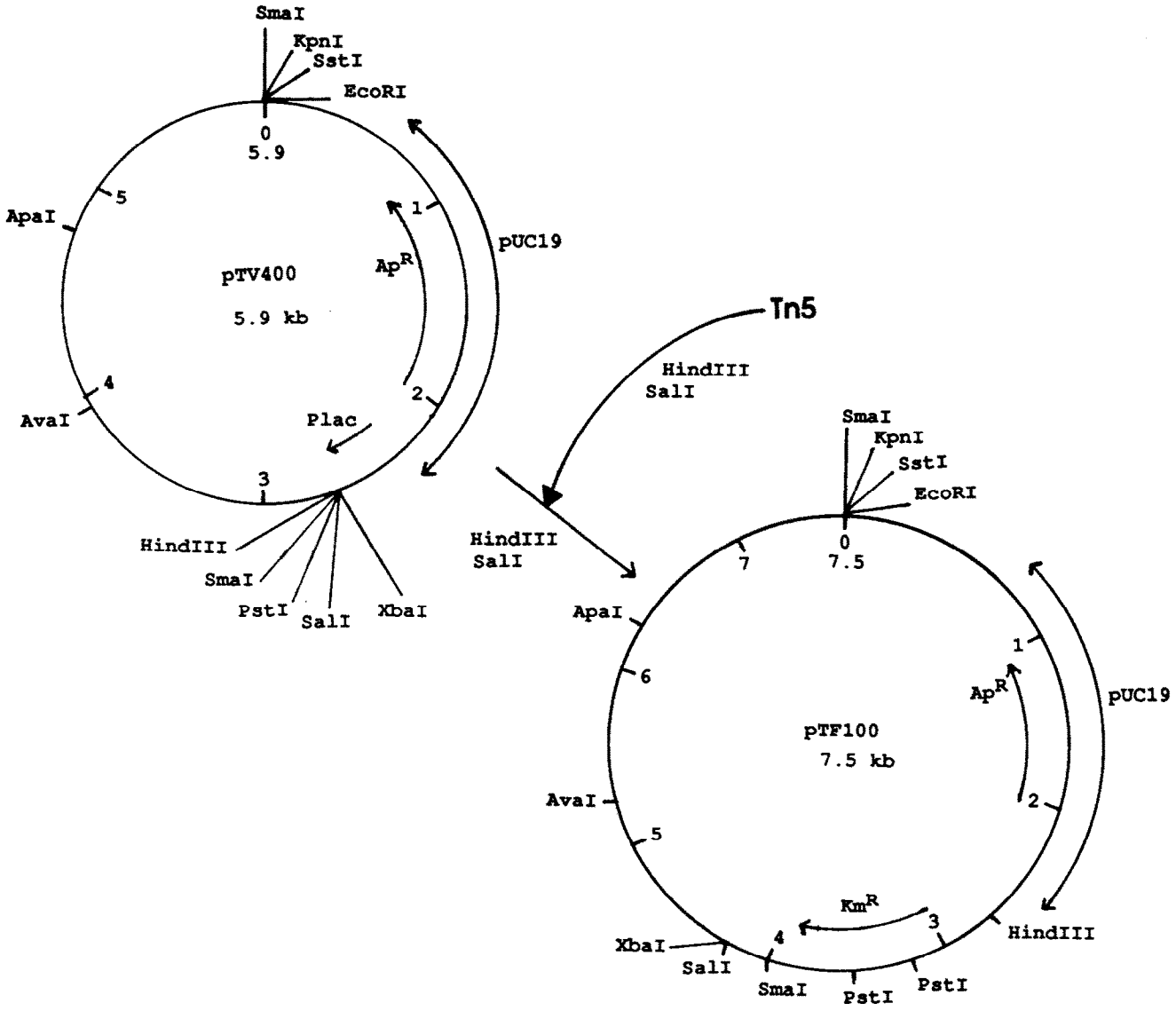


Fig. 2. Construction of pTF100. A 1.5-kb *Sal*I-*Hin*dIII fragment which carries the Km^R marker from Tn*5* was cloned into the *Sal*I + *Hin*dIII-digested pTV400. Recombinant plasmids were selected on plates containing Ap + Km. The construction was confirmed by restriction enzyme digestion and agarose gel electrophoresis.

To determine whether the requirement for pUC19 could be complemented by providing the pTF-FC2 derivative, pDER412 (Cm^R), in *trans*, ligations from the experiment described above were transformed into *E. coli* LK112 containing pDER412. Several $Cm^R Km^R Ap^S$ colonies were isolated. As LK112 was a *recA*$^-$ strain, it was unlikely that the $Km^R Cm^R$ phenotype was due to rescue of the Km^R marker by homologous recombination between pTF100 and pDER412. Restriction analysis of plasmids isolated from $Km^R Cm^R Ap^S$ colonies confirmed the presence of (in addition to pDER412) a plasmid corresponding to pTF100 from which the pUC19 replicon had been deleted. These results indicated that pDER412 was supplying a diffusible factor which enabled the 3202-bp fragment to replicate independently of the pUC19 replicon.

These experiments indicated that the use of *E. coli polA*$^-$ mutants to distinguish between replication from the *polA*$^-$ independent (pTF-FC2) and *polA*$^-$-dependent (ColE1-type vector) replicons was misleading. Although pUC19 was not capable of replication in either the *P. aeruginosa* or *E. coli polA*$^-$ strains, it nevertheless provided a function which enabled a pTF-FC2 fragment containing an incomplete replicon to be replicated in both organisms.

(b) Identification of an autonomously replicating fragment from pTF-FC2

A 5.0-kb *Cla*I-*Eco*RI fragment from pTF-FC2 was subcloned into the identical sites of the pBluescript SK vector (pTV100) and experiments were carried out to investigate whether this fragment was able to replicate autonomously in the absence of a ColE1-type origin. The Cm^R marker from pBR325 was cloned into pTV100 to produce an $Ap^R Cm^R$ plasmid. This plasmid, pTV101 (Table I), was digested to remove the pBluescript SK vector and religated. Restriction analysis of the plasmids from $Cm^R Ap^S$ colonies confirmed that pBluescript vector had been successfully deleted (data not shown).

A series of ordered deletions was generated from the *Cla*I site of pTV100 containing the Cm^R marker using the exonuclease III shortening method (Fig. 3). Two of these deletions, pTV102 and pTV109, were digested and ligated so as to remove the pBluescript SK vector but no $Ap^S Cm^R$

TABLE I

Strains and plasmids used in this study

	Relevant property	Source or Reference
Strains		
E. coli		
LK111	*thr*-1 *leu*-6 *thi*-1 *supE*44 *lacY*1 *tonA*21 *lacI*^{q} *lacZ*ΔM15	Zabeau and Stanley (1982)
LK112	a *recA*$^-$ derivative of LK111	Zabeau and Stanley (1982)
GW125a	a *polA*$^-$ derivative of AB1157	Dorrington and Rawlings (1989)
Plasmids		
pUC19	Ap^R	Yanisch–Perron et al. (1985)
pACYC184	Cm^R, Tc^R	Chang and Cohen (1978)
pBluescript SK	Ap^R	Stratagene, San Diego, CA
pDER401	Cm^R, Tc^R, 12.4-kb pTF-FC2 cloned into the *Pst*I site of pBR325	Rawlings et al. (1984)
pDER412	Cm^R, Δ*Xho*I-*Sal*I deletion of pDER401 resulting in deletion of the pBR325 origin of replication	Rawlings et al. (1984)
pTV100	Ap^R, 5.3-kb *Cla*I-*Eco*RI fragment of pDER401 cloned into the *Cla*I-*Eco*RI sites of pBluescript SK	This work
pTV101	Ap^R, Cm^R, the Cm^R gene of pBR325 cloned in the position of the *Eco*RI site of pTV100	This work
pTV155	Cm^R, the 1.3-kb pTF-FC2 fragment present on pTV151 (Fig. 1) cloned into the Tc^R gene of pACYC184	This work
Phage		
M13Δ*lac*110	partial 105-bp *ssi* deletion of phage M13	Honda et al. (1988)
M13Δ*lac*4164	M13Δ*lac*110 into which the 329-bp *oriV* region of pTF-FC2 (Fig. 1) has been cloned	This work

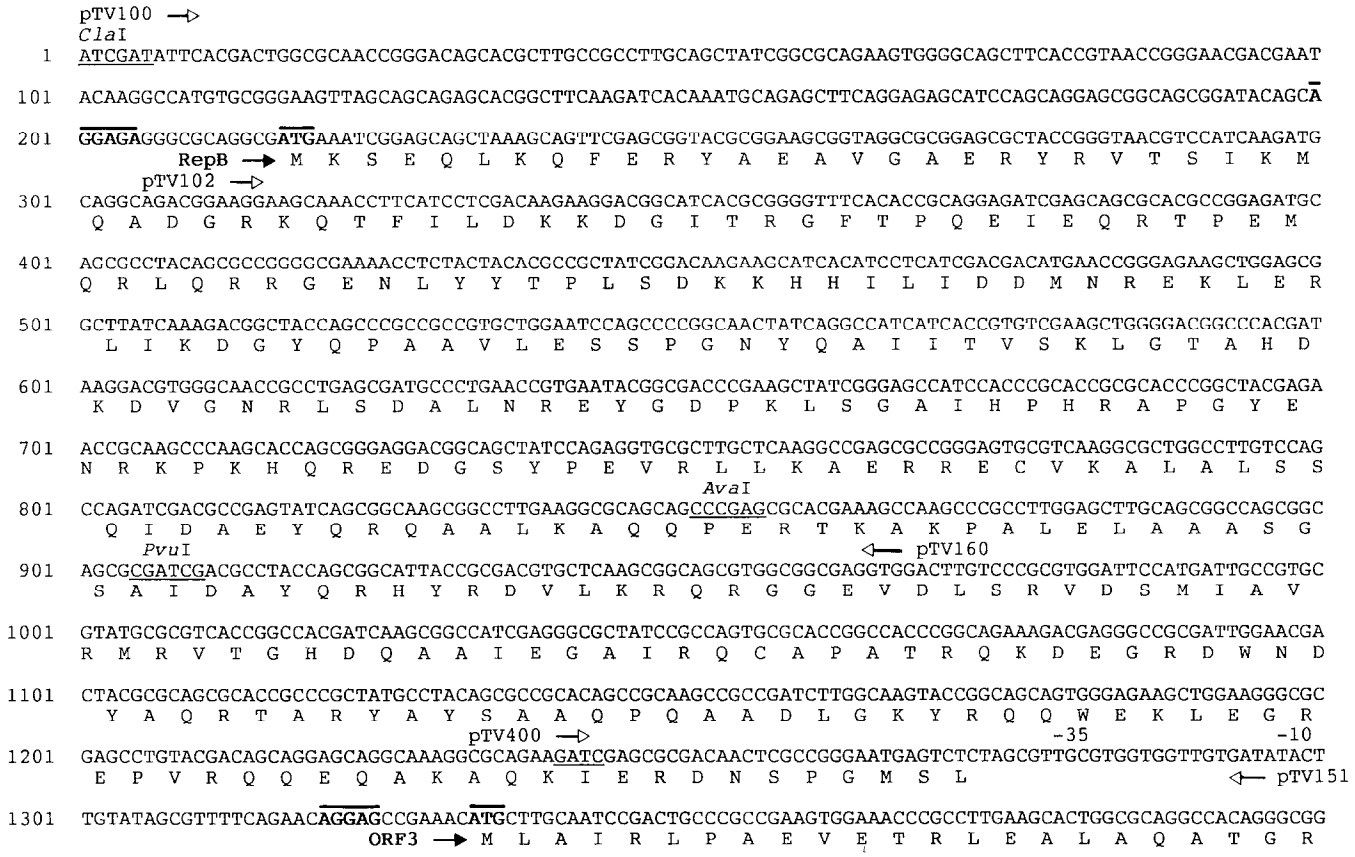


Fig. 3. Nucleotide sequence of 1400-bp of the pTF-FC2 replicon extending from the *Cla*I site of pTV100 and overlapping with the previously published pTV400 sequence (Dorrington and Rawlings, 1990). The predicted start codons of the RepB protein and as yet unidentified ORF3 are indicated by the blackened arrows. The starts of the numbered subclones (Fig. 1) are marked by open arrows. Restriction sites are underlined and indicated above the sequence. The underlined GATC sequence (nt 1239–1242) is a *Sau*3A site which marks the start of pTV400. Predicted RBS and ATG start codons are in bold type and overlined. Nucleotide sequence determination was by the dideoxy chain-termination method using a Sequenase DNA sequencing kit (version 2.0, U.S. Biochemical Corporation, Cleveland, OH). Overlapping sequence of the region between the *Cla*I site and the start of pTV400 was obtained in both directions. Sequence data were analysed using the UWGCG (version 6.2) software package. The sequence has been assigned the GenBank accession No. M64981.

transformants were obtained. Therefore, deletion of 300 bp from the *Cla*I site (pTV102), appeared to have resulted in the loss of ability to replicate autonomously.

(c) Analysis of the nt sequence of the additional region required for pTF-FC2 replication

The sequence of the region, extending for 1400 bp from the *Cla*I site towards and including the *Sau*3A site of the previously published pTV400 sequence (Dorrington and Rawlings, 1990), is shown in Fig. 3. Analysis of the sequence data revealed a 1056-nt ORF (ORF6) starting with an ATG codon at nt 217 (preceded by a 5′–AGGAGA RBS) and terminating with a TAG stop codon at nt 1273. The predicted protein encoded by ORF6 consisted of 352 aa with a calculated M_r of 40 155. A codon-preference plot of ORF6 using the codon-preference subroutine of the UWGCG package (Devereux et al., 1984) indicated that the codon usage of the potential ORF6 protein was similar to that of the other proteins previously identified on the 3202-bp fragment.

To determine whether ORF6 produced a translational product, pTV200, pTV151 and pTV160 (Figs. 1 and 4) were subjected to in vitro transcription/translation and the protein products analysed by SDS–PAGE. Plasmids pTV200 and pTV151 (*Cla*I site to nt 1296), both of which contained the whole of ORF6, each produced a translation product of 40 kDa which was not present in the pBluescript control (Fig. 4, lanes 2, 3 and 1, respectively). Plasmid pTV160 (nt 1–980) which had 98 aa residues deleted from the C terminus of ORF6 did not produce the 40-kDa protein, but instead produced a new 35-kDa polypeptide (Fig. 4, lane 4). This was larger than the calculated M_r of 29025. The sequencing data indicated that the most likely reason for the increase in apparent M_r was that a fortuitous fusion peptide had been generated with the new stop codon located within the pBluescript vector.

As the *lacZ* promoter of pBluescript SK was orientated

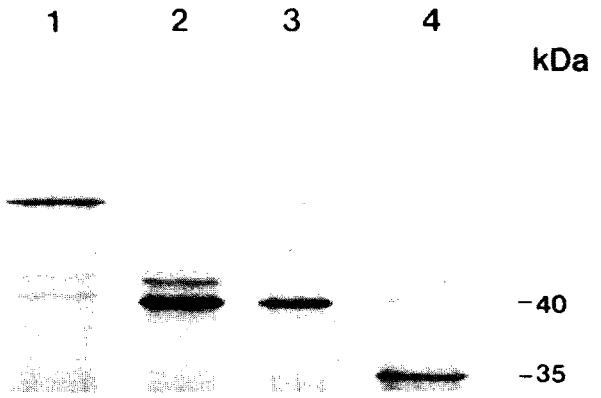


Fig. 4. SDS–PAGE analysis of polypeptides produced from the region containing ORF6. Lanes **1–4** represent polypeptides derived from pUC19, pTV200, pTV151 and pTV160 (Fig. 1), respectively. Translation was produced using a prokaryotic DNA-directed transcription/translation kit (code N.380, Amersham International plc, Amersham, U.K.), labelled with L-[^{35}S]methionine (specific activity 1500 Ci/mmol, Amersham) and separated using a 0.1% SDS–12% PA gel.

in the opposite direction to ORF6, it was likely that the protein was expressed from a promoter located upstream from the start of the ORF at nt 217. Sequence analysis of this region did not reveal any of the known *E. coli* consensus promoter sequences and confirmation of the presence of a *tsp* would require isolation of a transcript and primer extension experiments.

(d) Function of the ORF6 protein

The aa sequence of the ORF6 protein was aligned and compared with sequences in the GenBank/EMBL data bank (release 65) using the TFASTA subroutine of the UWGCG package. Only one sequence, that of the RepB′ protein of the IncQ plasmid RSF1010, showed limited, but significant, homology (23% identical aa residues) (Fig. 5).

```
pTF-FC2     1   MKSEQLKQFERYAEAVGAERYRVTSIKMQADGRKQTFILDKKDGITRGFTPQEIEQRTPE
                  :. .:  :.:      .     : :          :  . :. : ::
RSF1010     1       MKN-DRTLQAIGRQLKAMGCERFDIGVRDAT----TGQMMNREWSAAEVLQNTPW

           61   MQRLQRRGENLYYTPLSDKKH-HILIDDMNREKLERLIKDGYQPAAVLESSPGNYQAIIT
                  : .  :  .:  :    .:  .:.::     :.    .:  :: :.:.:: :::: .
           51   LKRMNAQGNDVYIRPAEQERHGLVLVDDLSEFDLDDMKAEGREPALVVETSPKNYQAWV-

          120   VSKLGTAHDKDVGNRLSDALNREYG-DPKLSGAIHPHRAPGYENRKPKHQREDGSYPEVR
                  :.. :   ..   .   :  :: ::      :  :  :. ::: ::    :  : :
          110   --KVADAAGGELRGQIARTLASEYDADPASADSRHYGRLAGFTNRKDKHTTRAGYQPWVL

          179   LLKAERRECVKALALSSQIDAEYQRQAALKAQQPERTKAK-PALELAAASGSAIDAYQRH
                :   .      . :: :                 :  :     .:.: :
          168   LRESKGKTATAGPALVQQAGQQIEQAQRQQEKARRLASLELPERQLSRHRRTALDEYRSE

          238   YRDVLKRQRGGEVDLSRVDSMIAVRMRVTGHDQAAIEGAIRQCAPATRQKDEGRDWNDYA
                  .:: :  ::: : :  .   :   :  :    .  :  ::
          228   MAGLVKR-FGD--DLSKCDFIAAQKLASRGRSAEEIGKAMAEASPALAERKPGHE-ADYI

          299   QRTARYAYSAAQPQAADLGKYRQQWEKLEGREPVRQQEQAKAQKIERDNSPGMSL
                ::          :  :    :    . : .
          284   ERTVSKVMGLPSVQLARAELARAPAPRQRGMDRGGPDFSM
```

Fig. 5. The aa alignment of the pTF-FC2 RepB (Fig. 3) and the RSF1010 RepB′ (Scholz et al., 1989) proteins. Identical and conservative aa changes are indicated by colons and single dots between the aa sequences, respectively.

There was a particularly well conserved region with 11/19 identical aa (aa 99–118 of ORF6 and aa 91–109 of RSF1010 RepB). On the strength of this evidence, the ORF6 was named *repB* and its 40-kDa protein RepB.

RepB′ of RSF1010 is a 35-kDa protein that is essential for replication (Scherzinger et al., 1984). It has been shown to have primase activity and is responsible for the de novo synthesis of primers at the *ssi* sites in the *oriV* (Haring and Scherzinger, 1989; Honda et al., 1989). The M13*Δlac*110 mutant, constructed and provided by Dr. N. Nomura, was used to test whether the pTF-FC2 RepB-like protein also had primase activity. This M13 mutant is defective in the region required for priming and produces small plaques with a reduced phage titer (Kim et al., 1981) unless a priming site and a primase which specifically recognizes the priming site is provided (Honda et al., 1989). The growth of phage M13*Δlac*110 was assayed in the presence of cells containing pACYC184 and pTV155 (the pTF-FC2 *repB*-like gene cloned into pACYC184). The phage with the defective priming site, produced small plaques and low phage titers when propagated in *E. coli* cells containing either pACYC184 or pTV155 (Fig. 6). The 329-bp *oriV* region of pTF-FC2 was cloned into M13*Δlac*110 to provide a priming site and the growth of this M13*Δlac*4164 phage construct compared in the presence and absence of pTV155. In the presence of the *repB*-like gene on coresident pTV155, large plaques were produced and the phage reached a titer three to four logs higher than when only the pACYC184 vector was present (Fig. 6).

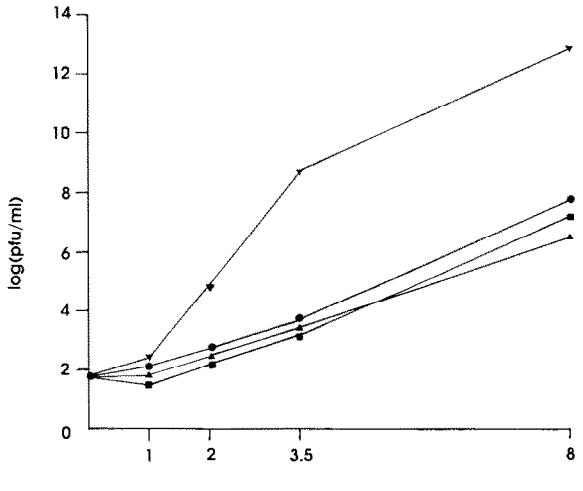


Fig. 6. Phage titers of M13*Δlac*110 recombinant phages. The 329-bp pTF-FC2 *oriV*-containing fragment was excised from pTV4164 and cloned into M13*Δlac*110 to produce M13*Δlac*4164. The 1239-bp fragment of pTF-FC2 present in pTV151 was subcloned into the vector pACYC184 to produce pTV155. Phage infection, growth and titers were determined as described by Honda et al. (1988). Growth of M13*Δlac*110 in *E. coli* LK111[pACYC184] (triangles) or LK111[pTV155] (squares), and M13*Δlac*4164 in LK111[pACYC184] (circles) or LK111[pTV155] (inverted triangles) was examined.

(e) Probable explanation for the ability of pUC19 to complement the primase-defective pTF-FC2 replicon

The role of pUC19 in the replication of the 3202-bp pTF-FC2 fragment in the absence of RepB is intriguing. Although pUC19 was unable to replicate in *E. coli* GW125a or in *P. aeruginosa*, the plasmid was able to complement a pTF-FC2 replicon from which the RepB had been deleted. The pUC19 vector was required in *cis*, although its orientation and distance relative to the pTF-FC2 *oriV* did not appear to affect replication (pTF100 has a 1.5-kb fragment inserted between the pTF-FC2 *oriV* and the pUC19 replicon). Replication also occurred if the pUC19 replicon of pTF100 was replaced with pACYC184 (data not shown). This implied that, like pUC19, pACYC184 could supply some function which enabled the 3202-bp fragment to replicate independently of the pTF-FC2-encoded primase.

A recent report on the priming of the IncQ plasmid RSF1010 has provided a possible explanation for these observations. Honda et al. (1991) showed that it was possible to delete the *ssi* sites which are essential for the priming of RSF1010 replication and replace them with primosome assembly sites from either pACYC184 or bacteriophage ϕX174. These constructs were no longer dependent on the RepB′ primase but required the *E. coli* primase, DnaG. Replication was, however, still dependent on RepA and RepC. It is likely that a similar situation occurred in the case of the primase deleted pTF-FC2 replicon. The primosome assembly sites of the cloning vectors pUC19 (Nomura et al., 1982; Abarzúa et al., 1984) or pACYC184 (Bahk et al., 1988) allowed replication of the 3202-bp pTF-FC2 replicon. Replication of the pUC19/3202-bp pTF-FC2 fragment in *E. coli polA* mutants was still dependent on the RepA and RepC proteins, however, as deletion or mutation of either protein prevented replication (Dorrington and Rawlings, 1990).

(f) Comparison of pTF-FC2 replicon with the IncQ plasmids

The pTF-FC2 and IncQ plasmids (Scholz et al., 1989) are therefore even more similar than was previously thought. The RepC, RepA and RepB proteins have 60%, 43% and 23% aa identity, respectively, with the % identity decreasing with increasing distance from the *oriV*. Although the *oriV* regions also shared homology, an average of 70% over a 190-bp region which included the repeated sequences, this similarity did not extend into the region of the RSF1010 *ssiA* and *ssiB* sites (Dorrington and Rawlings, 1989). The number, orientation and exact position of the pTF-FC2 priming sites is being investigated.

(g) Conclusions

(*1*) Deletion of the pUC19 replicon from pTV400 and pTF100 resulted in loss of the ability of the 3202-bp pTF-FC2 fragment to replicate in the absence of coresident pDER412. An additional 1239-bp region from pTF-FC2 was required to restore autonomous replication to the pTF-FC2 replicon.

(*2*) A 40-kDa protein, which had 23% aa identity with the RepB′ protein of the IncQ plasmid RSF1010 was found to be essential for pTF-FC2 replication.

(*3*) Using the M13*Δlac*110 system it was demonstrated that the 1239-bp region of the pTF-FC2 replicon includes a protein with primase activity that interacts specifically with sequences present in the 329-bp *oriV* fragment of pTF-FC2.

(*4*) This work illustrates the danger of making the assumption that since a plasmid like pUC19 was unable to replicate in *E. coli polA*$^-$ or *P. aeruginosa* strains, it is totally functionless in those hosts.

ACKNOWLEDGEMENTS

We thank Dr. N. Nomura for the gift of M13*Δlac*110 and Nikki Campbell for help in preparing the figures.

REFERENCES

Abarzúa, P., Soeller, W. and Marians, K.J.: Mutational analysis of primosome assembly sites. I. Distinct classes of mutants in the pBR322 *Escherichia coli* factor Y DNA effector sequences. J. Biol. Chem. 259 (1984) 14286–14292.

Bahk, J.-D., Sakai, H. and Komano, T.: Plasmid pACYC184 contains an *ssi* signal for initiation of single-strand phage DNA replication. Gene 65 (1988) 93–99.

Balbás, P., Soberón, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N. and Bolivar, F.: Plasmid vector pBR322 and its special-purpose derivatives – a review. Gene 50 (1986) 3–40.

Chang, A.C.Y. and Cohen, S.N.: Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. 134 (1978) 1141–1156.

De Bruin, F.J. and Lupski, J.R.: The use of transposon Tn*5* mutagenesis in the rapid generation of correlated physical and genetic maps of DNA segments cloned into multicopy plasmids – a review. Gene 27 (1984) 131–149.

Devereux, J., Haeberli, P. and Smithies, O.: A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12 (1984) 387–395.

Dorrington, R.A. and Rawlings, D.E.: Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. 171 (1989) 2735–2739.

Dorrington, R.A. and Rawlings, D.E.: Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. 172 (1990) 5697–5705.

Haring, V. and Scherzinger, E.: Replication proteins of the IncQ plasmid RSF1010. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram-negative Bacteria. Academic Press, London, 1989, pp. 95–124.

Henikoff, S.: Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28 (1984) 351–369.

Honda, Y., Sakai, H. and Komano, T.: Two single-strand DNA initiation

signals located in the *oriV* region of plasmid RSF1010. Gene 68 (1988) 221–228.

Honda, Y., Sakai, H., Komano, T. and Bagdasarian, M.: RepB′ is required in *trans* for the two single-strand DNA initiation signals in *oriV* of plasmid RSF1010. Gene 80 (1989) 155–159.

Honda, Y., Sakai, H., Hiasa, H., Tanaka, K., Komano, T. and Bagdasarian, M.: Functional division and reconstruction of a plasmid replication origin: molecular dissection of the *oriV* of the broad-host-range plasmid RSF1010. Proc. Natl. Acad. Sci. USA 88 (1991) 179–183.

Kim, M.H., Hines, J.C. and Ray, D.S.: Viable deletions of the M13 complementary strand origin. Proc. Natl. Acad. Sci. USA 78 (1981) 6784–6788.

Nomura, N., Low, R.L. and Ray, D.S.: Identification of ColE1 DNA sequences that direct single strand-to-double strand conversion by a ϕX174 type mechanism. Proc. Natl. Acad. Sci. USA 79 (1982) 3153–3157.

Rawlings, D.E. and Woods, D.R.: Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. 49 (1985) 1323–1325.

Rawlings, D.E., Pretorius, I. and Woods, D.R.: Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158 (1984) 737–738.

Rawlings, D.E., Sewcharan, R. and Woods, D.R.: Characterization of a broad-host-range mobilizable *Thiobacillus ferrooxidans* plasmid and the construction of *Thiobacillus* cloning vectors. In: Lawrence, R.W., Branion, R.M.R. and Ebnes, H.G. (Eds.), Fundamental and Applied Biohydrometallurgy. Elsevier, Amsterdam, 1986, pp. 419–427.

Scherzinger, E., Bagdasarian, M.M., Scholz, P., Lurz, R., Rückert, B. and Bagdasarian, M.: Replication of the broad host range plasmid RSF1010: requirement for three plasmid encoded proteins. Proc. Natl. Acad. Sci. USA 81 (1984) 654–658.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M. and Scherzinger, E.: Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 75 (1989) 271–288.

Yanisch-Perron, C., Vieira, J. and Messing, J.: Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene 33 (1985) 103–119.

Zabeau, M. and Stanley, K.K.: Enhanced expression of cro-β-galactosidase fusion proteins under the control of the P_R promoter of bacteriophage λ. EMBO J. 1 (1982) 1217–1224.

JOURNAL OF BACTERIOLOGY, Oct. 1992, p. 6230–6237
0021-9193/92/196230-08$02.00/0

Vol. 174, No. 19

Sequence Analysis and Characterization of the Mobilization Region of a Broad-Host-Range Plasmid, pTF-FC2, Isolated from *Thiobacillus ferrooxidans*

JÜRG ROHRER AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, P.B. Rondebosch 7700, South Africa

Received 15 June 1992/Accepted 1 August 1992

The nucleotide sequence of a 5,317-bp fragment which includes the region required for mobilization of broad-host-range plasmid pTF-FC2 was determined. A region of approximately 3.5 kb was required for plasmid mobilization, and *oriT* was localized on a 138-bp fragment. Polypeptides which corresponded in size and location to several of the open reading frames were detected in an in vitro transcription-translation system. Three open reading frames essential for plasmid mobilization and two which affect the mobilization frequency were identified. There was a distinct similarity in the sizes, amino acid sequences, and locations of the proteins from the mobilization region of pTF-FC2 and the Tra1 region of IncP plasmid RP4. Similarity in the structures and sequences of the *oriT* regions was also apparent. A sequence with 37-of-38-bp homology to the inverted repeated sequences of Tn*21* and an open reading frame with strong homology to the MerR regulatory protein was identified outside of the region required for mobilization.

Plasmid pTF-FC2 is a 12.4-kb broad-host-range plasmid that was isolated from the industrially important, acidophilic, chemolithoautotrophic bacterium *Thiobacillus ferrooxidans* (14). The plasmid has been shown to replicate in a wide range of gram-negative bacteria, and the minimum region required for replication has been isolated, sequenced, and characterized (1–3). Although the pTF-FC2 replicon was found to resemble most closely those of the IncQ plasmids (RSF1010, R300B, and R1162), the two plasmids were compatible and the replication proteins from the IncQ plasmids were unable to complement pTF-FC2 *rep* mutants. Plasmid pTF-FC2 was shown to be a nonconjugative plasmid that was capable of being mobilized at high frequency between *Escherichia coli* strains by IncP plasmid RP4 (15, 16). Since pTF-FC2 has great potential for the construction of broad-host-range, mobilizable vectors that are compatible with previously constructed broad-host-range vectors, we wished to characterize the mobilization region more fully.

The general model for mobilization entails several steps (13, 20). The strand to be transferred is nicked at a site (*nic* or *rlx*) which is located in the origin of transfer (*oriT*). The nicked strand is displaced via a rolling-circle type of replication mechanism (13) and is transferred, with the 5′ terminus leading, to the recipient cell. Conjugative DNA synthesis occurs in both donor and recipient cells, and recircularization in the recipient cell completes the cycle. Besides the presence of a coresident conjugative plasmid, essential features required by a mobilizable plasmid for conjugative transfer are an *oriT* region and mobilization proteins that specifically recognize this region (20). While the mobilization proteins may be provided in *trans*, the *oriT* region is required in *cis*.

In this study, the region of pTF-FC2 involved in plasmid mobilization was characterized by using the techniques of DNA sequencing, deletion, and site-directed mutagenesis analysis. We report here on the location of the region required for mobilization in *cis* and on the location and derived amino acid sequences of the mobilization proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Plasmids were maintained in *E. coli* K-12 strain JM105 (21). For mating experiments, *E. coli* S17-1 (Str^r) (18) containing the relevant plasmids was used as the donor. This strain has an RP4 plasmid derivative integrated into the chromosome which provides the conjugative functions needed for mobilization. *E. coli* CSH56 (Nal^r) was used as the recipient. All bacteria were grown in Luria broth or on Luria agar (LA) plates (10). Antibiotics were added to media as required at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 20 g/ml; nalidixic acid, 25 μg/ml; streptomycin, 15 μg/ml.

General DNA techniques. Plasmid DNA was prepared by the method of Ish-Horowicz and Burke (8). Standard methods were used for restriction digests, gel electrophoresis, and ligations (10). Ordered deletions for production of templates that would generate overlapping DNA sequence were constructed by using the exonuclease III method (7). Nucleotide sequence determination was done by the dideoxy-chain termination method using a Sequenase DNA-sequencing kit (version 2.0; United States Biochemical Corporation, Cleveland, Ohio). Polyacrylamide gels of 4, 5, and 6% were used for long, medium, and short runs, respectively. The DNA sequence of the entire 5,317-bp *Cla*I-*Pst*I fragment of pTF-FC2 was determined from both strands. DNA sequence data were analyzed by using the Genetics Computer Group Inc. software package (version 7.0).

Matings. Deletion plasmids were transformed into *E. coli* S17-1, and transformants were selected for growth on LA plates with ampicillin and streptomycin. Single colonies of donor and recipient bacteria were grown separately overnight at 37°C. Antibiotics were added to all donor cultures to maintain selection of the plasmids. Cells were washed once in LA and mixed at a 1:10 donor-to-recipient ratio. Samples (50 μl) were spotted onto an agar plate without antibiotics and incubated for 1 h at 37°C. Agar plugs were excised, and

* Corresponding author.

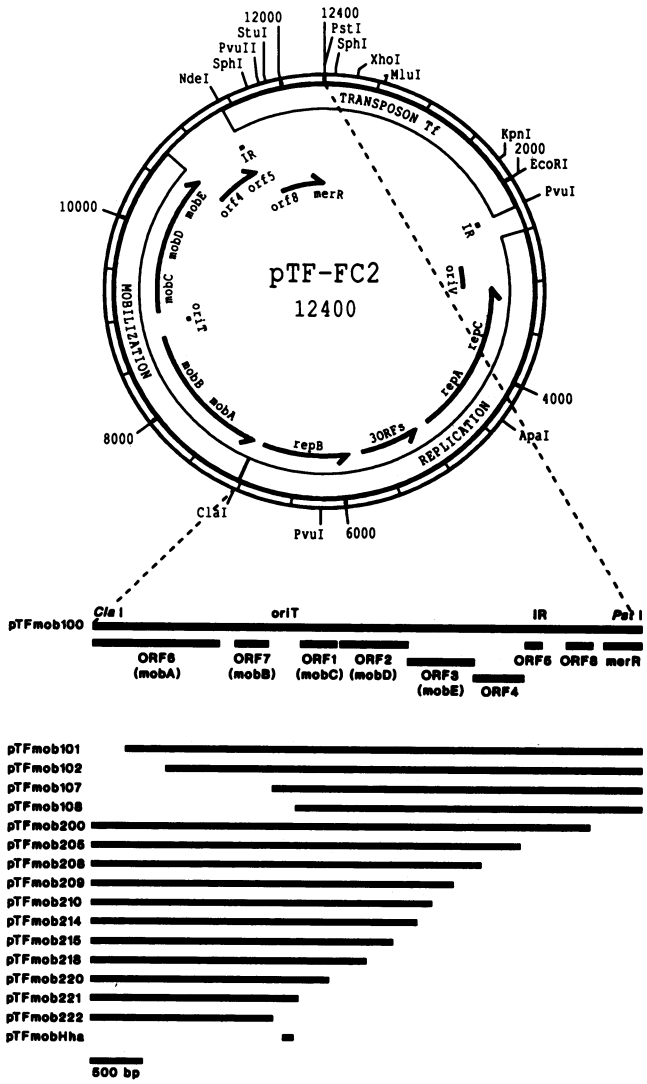


FIG. 1. Genetic and restriction endonuclease cleavage maps of pTF-FC2. The *Cla*I-*Pst*I mobilization region, showing the locations of potential ORFs, is depicted as a linear map, and the shortened subclones and *Hha*I fragment used in this study are shown below. The inverted repeats, which are homologous to those of transposon Tn*21*, are designated IR.

the cells were suspended in 5 ml of Luria broth and vortexed for 30 s. One milliliter was removed and washed three times with Luria broth to remove extracellular β-lactamase and prevent the growth of satellite colonies at low dilutions. Serial dilutions were plated onto LA ampicillin-streptomycin plates to count donors and onto LA ampicillin-nalidixic acid plates to count transconjugants. For experiments with pDER412 in *trans*, the shortened subclones were transformed into *E. coli* S17-1 cells already containing pDER412. Since pDER412 contains a chloramphenicol resistance marker (14), transformants were selected and donors were counted on LA ampicillin-chloramphenicol-streptomycin plates.

Protein analysis. Translation products were identified by using a prokaryotic DNA-directed transcription-translation kit (code L4500 *E. coli* S30 System; Promega Corporation, Madison, Wis.). Polypeptides were separated on sodium dodecyl sulfate (SDS)–15% polyacrylamide gels.

Site-directed mutagenesis. The protocol of Stanssens et al. (19) was used to introduce two nonpolar mutations into open reading frames (ORFs) 1 and 7. pTFmob100 was cloned into chloramphenicol-resistant mutagenesis vector pMc5-8 at the *Sal*I and *Xba*I sites. The resulting construct was named pTFmut100. Two primers corresponding to nucleotides 1662 to 1682 (5′-GGGGCCGGAACCaGCTGGACGC-3′ [ORF7]) and nucleotides 2182 to 2202 (5′-CGATGCTCAaAgCTTCA CCGC-3′ [ORF]) were used to introduce the mutations. The ORF7 primer inserted an extra A into the sequence, resulting in a *Pvu*II site and a simultaneous frame shift within the first 30 bp (see Fig. 2). The ORF1 primer substituted an A for a G and a G for a C, resulting in the introduction of a *Hin*dIII site (see Fig. 2). The *Hin*dIII site was filled in to give a frameshift mutation. All mutations were monitored by restriction digests and subsequent gel electrophoresis.

Nucleotide sequence accession number. The nucleotide sequence of the 5,317-bp *Cla*I-*Pst*I fragment of pTF-FC2 has been assigned GenBank accession number M57717.

RESULTS

Cloning of the pTF-FC2 mobilization region. Rawlings and Woods have shown that the mobilization region of pTF-FC2 is located on a 5.3-kbp *Cla*I-*Pst*I fragment (Fig. 1) (16). This region was subcloned into the *Cla*I and *Pst*I sites of the nonmobilizable BluescriptSK vector. The resultant plasmid, pTFmob100, was mobilizable at high frequency by plasmid RP4 or *E. coli* S17-1 and served as the source of DNA for this study.

Delineation of the region required for mobilization. Ordered sequential deletions from both ends of the *Cla*I-*Pst*I fragment were tested for the ability to be mobilized by the chromosomally located IncP plasmid of *E. coli* S17-1. Results of the mating experiments are shown in Table 1, column 1. Deletion pTFmob101 (319 bp) from the *Cla*I site was mobilized at a 9,000-fold-reduced frequency. With removal of a further 391 bp (pTFmob102), mobilization was longer detectable.

From the *Pst*I site, 1,564 bp (pTFmob208) could be deleted with no reduction in the mobilization frequency.

TABLE 1. Mobilization frequencies of pTFmob100 and subclones

Subclone	Mobilization frequency	
	Subclone only	Subclone with coresident pDER412
pTFmob100	9×10^{-1}	ND[a]
pTFmob101	1×10^{-4}	1
pTFmob102	$<3 \times 10^{-6}$	9×10^{-1}
pTFmob107	$<3 \times 10^{-6}$	9×10^{-1}
pTFmob108	$<3 \times 10^{-6}$	$<3 \times 10^{-4}$
pTFmob208	9×10^{-1}	ND
pTFmob209	6×10^{-1}	ND
pTFmob210	2×10^{-2}	ND
pTFmob214	6×10^{-3}	ND
pTFmob215	6×10^{-4}	ND
pTFmob218	$<3 \times 10^{-6}$	9×10^{-1}
pTFmob221	$<3 \times 10^{-6}$	9×10^{-1}
pTFmob222	$<3 \times 10^{-6}$	$<3 \times 10^{-6}$
pTFmobHha	$<3 \times 10^{-6}$	6×10^{-1}

[a] ND, frequency not determined.

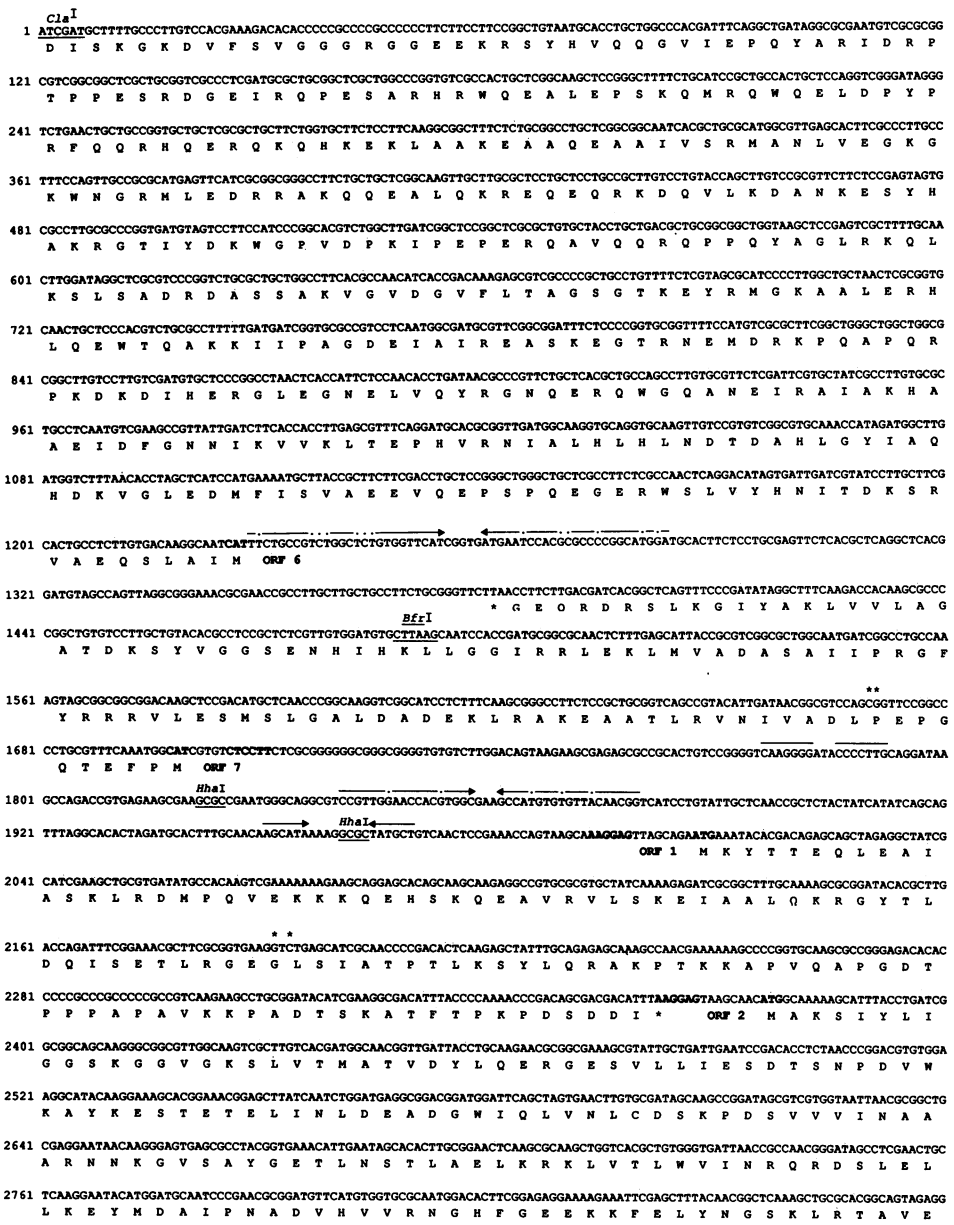


FIG. 2. Nucleotide sequence of the 5,317-bp *Cla*I-*Pst*I mobilization region. The amino acid sequences of the predicted ORFs are shown below the nucleotide sequence. Inverted repeats are indicated by converging arrows above the nucleotide sequence, and mismatches are denoted by dots above the relevant bases. Site-directed mutations are shown by asterisks above the sequence. In ORF 7, a T was inserted between C and G, and in ORF 1, the G and C were substituted by a C and a T, respectively. The inverted repeat showing homology to Tn*21* is underlined and in boldface. Potential ribosome-binding sites and start codons are in boldface. The 138-bp *Hha*I fragment containing *oriT* is indicated (bp 1824 to 1962).

When the 1,852-bp segment pTFmob209 was deleted, an approximately 1.5-fold reduction in mobilization frequency was detected. When a further 182-bp segment (pTFmob210) was deleted the frequency dropped 45-fold with respect to that of pTFmob208. Deletion of another 100 bp (pTFmob214) gave a 150-fold reduction, and a further 193-bp deletion (pTFmob215) gave a 1,500-fold reduction in frequency. Mobilization of subclone pTFmob218, having another 395 bp deleted, was no longer detectable. Therefore, pTFmob208 (3,755 bp) was the smallest subclone that was mobilizable at the frequency of pTFmob100.

The *oriT* region of pTF-FC2 was located by repeating the mating experiments by using *E. coli* S17-1 cells containing the subclone to be tested together with coresident pDER412. Plasmid pDER412 contains the 12.1-kbp *Pst*I-*Xho*I fragment of pTF-FC2 (Fig. 1) linked to a chloramphenicol-selectable marker, is compatible with the BluescriptSK cloning vector, and was used to provide the mobilization proteins in *trans*. The results of these mating experiments are shown in Table 1, column 2. Plasmid pTFmob107, which had a 1,743-bp deletion from the *Cla*I site, could be mobilized, while plasmid pTFmob108, with a 1,973-bp deletion, could not.

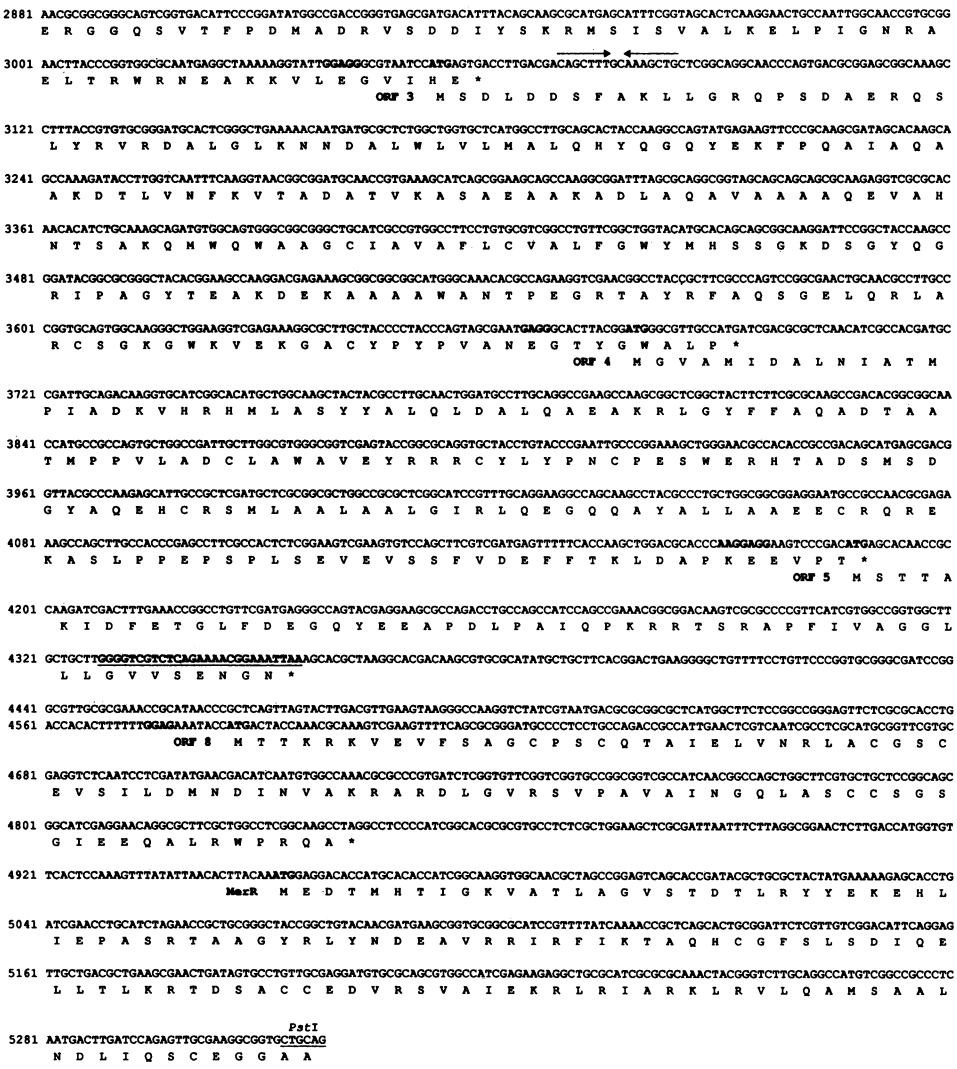


FIG. 2—*Continued.*

With respect to deletions from the *Pst*I side, pTFmob221, with a 3,311-bp deletion, was mobilized but pTFmob222, with a 3,555-bp deletion, was not. This indicated that the *oriT* region was located on the 263-bp fragment common to both pTFmob107 and pTFmob221. The location of the *oriT* region was confirmed by digestion of pTFmob107 with *Hha*I, flushing of the ends by filling in with the Klenow fragment of T4 DNA polymerase, and cloning of the fragments into BluescriptSK digested with *Hinc*II. A 138-bp sequence containing an *Hha*I fragment (pTFmobHha; Fig. 1 and 2) that was mobilized by *E. coli* S17-1(pDER412) was isolated, and the DNA sequence was determined. The 138-bp region was entirely contained within the 263-bp fragment common to pTFmob107 and pTFmob221.

Sequence analysis of the mobilization region. The nucleotide sequence of the complete 5,317-bp *Cla*I-*Pst*I fragment was determined (Fig. 2). The overall G+C content was found to be 58.7%, which is in close agreement with the 59% G+C content of the *T. ferrooxidans* chromosome (6). The Genetics Computer Group package was used to locate potential protein-coding regions. The CODONPREFERENCE subroutine, together with a codon preference table derived from the previously sequenced pTF-FC2 *repA* and *repC* genes, enabled several possible ORFs to be identified. Several of these (ORFs 1, 2, 3, 6, and 7) were shown to be translated by using an in vitro transcription-translation system (see below). The potential ORFs appeared to be translated divergently from the *oriT* region. All potential ORFs had an ATG start codon preceded by a clearly identifiable Shine-Dalgarno sequence, with the exception of ORF 6. There was also no termination codon for ORF 6 within the cloned pTF-FC2 fragment, and the sequencing data showed that ORF 6 terminates 42 bp within the BluescriptSK vector. Nevertheless, the truncated ORF 6 polypeptide was fully functional and essential for plasmid mobilization.

A 38-bp sequence with 37-of-38-nucleotide sequence homology to the inverted repeat of transposon Tn*21* was discovered close to the *Nde*I site and outside of the region required for mobilization. A second inverted repeat sequence with 38-of-38-nucleotide sequence homology to Tn*21* has been discovered elsewhere on the plasmid (Fig. 1 and unpublished data). This indicates that pTF-FC2 contains a transposon or the remnants of a transposon that seems to be related to Tn*21*.

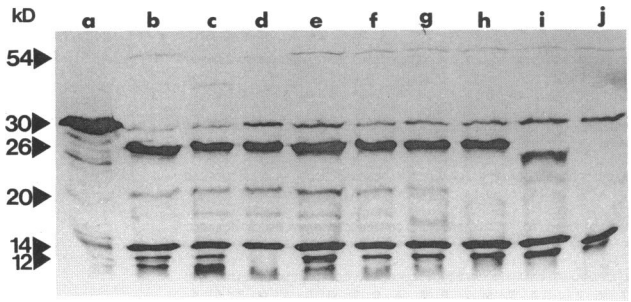


FIG. 3. SDS-PAGE analysis of polypeptides translated in vitro from pTFmob100 and deletions. Lanes: a, BluescriptSK control; b, pTFmob100 subclone; c to j, proteins produced from deletions pTFmob102 (c), pTFmob107 (d), pTFmob200 (e), pTFmob205 (f), pTFmob208 (g), pTFmob214 (h), pTFmob215 (i), and pTFmob220 (j). The polypeptide at 30.5 kDa corresponds to the β-lactamase complementing fragment from the BluescriptSK vector.

Identification of polypeptides transcribed from the ORFs. The cloned pTF-FC2 mobilization region was placed in an *E. coli*-derived in vitro transcription-translation system so as to detect which of the polypeptides predicted by sequence analysis were synthesized. The results are shown in Fig. 3. Five polypeptides in addition to those produced by the vector, were detected. The apparent molecular masses of the polypeptides were 54, 26, 20, 14, and 12 kDa (Fig. 3). The polypeptides synthesized from various deletions of the mobilization region were examined and compared with pTF mob100 so as to enable correlation of the polypeptides with an ORF. A series of deletions from the *Pst*I site, pTF mob200, pTFmob205, pTFmob208, pTFmob214, pTF mob215, and pTFmob220 (Fig. 1), were used to detect the synthesis of polypeptides corresponding to ORFs 8, 5, 4, 3, 2, and 1, respectively. No polypeptides corresponding to ORFs 8, 5, and 4 were found. The 20-kDa protein correlated to ORF 3, as it was no longer detectable in deletions pTFmob214, pTFmob215, and pTFmob220. The 26-kDa protein correlated with ORF 2, as deletion pTFmob215 resulted in a decrease in the apparent molecular mass of the 26-kDa polypeptide to 24 kDa and the protein was completely absent from pTFmob220. The 14-kDa protein correlated with ORF 1, as there was a decrease in the apparent molecular mass of the 14-kDa polypeptide to 13 kDa in deletion pTFmob220. The 54-kDa polypeptide was produced by pTFmob100 and all of the pTFmob200 deletions, but deletion pTFmob102 resulted in a decrease in the apparent molecular mass to 43 kDa and the polypeptide was no longer detectable in pTFmob107. The 12-kDa protein corresponds to ORF 7, as it is present in pTFmob100, pTFmob102, and all of the pTFmob200 deletions but not in pTFmob107. There were some differences between the apparent molecular masses of the Mob proteins, as determined by SDS-polyacrylamide gel electrophoresis (PAGE) (Fig. 3), compared with those derived from the sequencing data (Fig. 2). By SDS-PAGE, MobA, MobB, MobC, MobD, and MobE were 54, 12, 14, 26, and 20-kDa, respectively, whereas their sequence-derived molecular masses were 46.7, 11.6, 13.0, 25.3, and 23.1 kDa.

TABLE 2. Mobilization frequencies of pTFmut100 and mutant subclones

Subclone	Mobilization frequency	
	Mutant only	Mutant with coresident pDER412
pTFmut100	9×10^{-1}	ND[a]
pTFmut101	9×10^{-1}	ND
pTFmut102	$<3 \times 10^{-6}$	9×10^{-1}
pTFmut103	1×10^{-2}	9×10^{-1}
pTFmut104	2×10^{-4}	1×10^{-2}

[a] ND, frequency not determined.

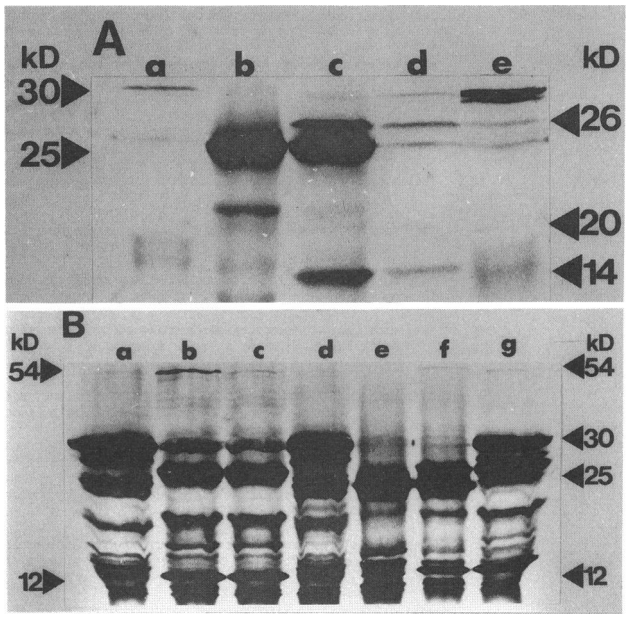
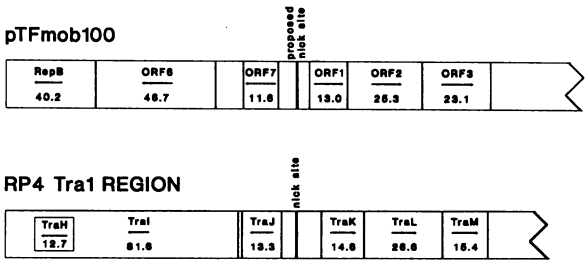


FIG. 4. SDS-PAGE analysis of polypeptides translated in vitro from site-directed mutations. (A) Lanes: a and b, mutagenesis vector controls pMa5-8 and pMc5-8; c, parental pTFmut100; d, pTFmut101; e, pTFmut102. (B) Lanes: a, BluescriptSK control; b, pTFmob100; c, pTFmut104; d, e, and f, pMa5-8, pMc5-8, and pTFmut100, respectively; g, mutant pTFmut103. The polypeptide at 30.5 kDa corresponds to the β-lactamase complementing fragment from the BluescriptSK and pMa58 vectors, and the 26-kDa polypeptide corresponds to the chloramphenicol acetyltransferase gene product from the pMc5-8 vector.

ORFs required for mobilization. The deletion studies described above showed that pTFmob208, containing ORFs 1, 2, 3, 6, and 7, was the smallest plasmid that could be mobilized at the same frequency as pTFmob100. From these results, it was concluded that ORF 3 affected the mobilization frequency whereas ORFs 2 and 6 were essential for mobilization. To establish whether the two inner ORFs, 1 and 7, were also required for mobilization, site-directed mutagenesis was used to introduce nonpolar mutations into these ORFs. A *Hin*dIII site was introduced into ORF 1 at position Gly-62 (Fig. 2), resulting in a silent mutation to Ala-62 (pTFmut101). Plasmid pTFmut101 was digested with *Hin*dIII, and the sticky ends were filled in to create a frameshift mutation, giving pTFmut102. Loss of the site was monitored by restriction digests and gel electrophoresis. Mating experiments showed that pTFmut101 was mobilizable at parental frequencies, while mobilization of pTFmut102 could not be detected (Table 2). In a similar manner, the unique *Bfr*I site internal to ORF 7 (Fig. 2) was used to introduce a frameshift mutation at position Leu-70. The resulting mutant, pTFmut104, was mobilizable at a frequency 4,500-fold lower than that of the parental plasmid

A.



B. Comparison of ORF1(MobC) of pTF-FC2 with TraK of RP4
Similarity: 51.8% Identity: 33.0% Gap: 5

```
  1 .MKYTTEQLEAIASKLRDMPQVEKKKQEHSKQEAVRVLSKEIAALQKRGY 49
    || |  | : |:        ||:       |   :  :: |    ||
  1 PKSYTDELAEWVESR......AAKKRRRDEAAVAFLAVRADVEAALASGY 44

 50 TLDQISETLRGEG.LSIATPTLKSYLQR...AKPTKKAPVQAPGDTPPPA 95
    |  | | :|: | : :   |::| .  |   |||   :: || :  |:||
 45 ALVTIWEHMRETGKVKFSYETFRSHARRHIKAKPADVPAPQAKAAEPAPA 94

 96 ...PAVKKPADTSKA............TFTPKPDSDDI. 118
       |  ::| : :||            || | ||  |:
 95 PKTPEPRRPKQGGKAEKPAPAAAPTGFTFNPTPDKKDLL 133
```

C. Comparison of ORF2(MobD) of pTF-FC2 with TraL of RP4
Similarity: 49.1% Identity: 24.8% Gap: 10

```
  1 MAKSIYLIGGSKGGVGKSLVTMATVDYLQERGESVLLIESDTSNPDVWKA 50
    ||| | ::  :|||||||| :     :|  ::|:  | |: |  |:  : :
  1 MAK.IHMVLQGKGGVGKSAIAAIIAQYKMDKGQTPLCIDTDPVNA.TFEG 48

 51 YKESTETELINLDEAD....GWIQLVNLCDSKPDSVVVINAAARNNKGVS 96
    ||       |  : : :     :  ||:|      | ||: |:|      :|
 49 YKALNVRRLNIMAGDEINSRNFDTLVELIAPTKDDVVIDNGAS.SFVPLS 97

 97 AY..GETLNSTLAELKRKLVTLWVINRQRDSLELLKEYMD...AIPNADV 141
      |  :: :   | |: : ||   |:      |: : :: :    :|   :
 98 HYLISNQVPALLQEMGHELVIHTVVTGGQALLDTVSGFAQLASQFPAEAL 147

142 HVVR.NGHFGE.EKKFELYNGSKLRTAVEERGGQSVTFPDMADRVSDDIY 189
     ||: |  :|  |      ::  |  ||   | :  : :| : :   :  :
148 FVVWLNPYWGPIEHEGKSFEQMKAYTANKARVSSIIQIPALKEETYGRDF 197

190 SKRMSISVALKE.LPIGNRAELTRWRNEAKKVLEGVIHE....... 227
    |  :   : : :|: :    :|| |   | |  |:: :
198 SDMLQERLTFDQALADESLTIMTRQR..LKIVRRGLFEQLDAAAVL 241
```

D. Comparison of ORF6(MobA) of pTF-FC2 with TraI of RP4
Similarity: 48.7% Identity: 26.2% Gap: 11

```
  1 ..MIALSQEAVRSK.DTINHYVLSWREGEQPSPEQVEEAVSIFMDELGVK 47
     ::|  ::  ||  |   | ::|:| || | :| :      :  :||
 51 AEVMATQHGNTRSEADKTYHLLVSFRAGEKPDAETLRAIEDRICAGLGFA 100

 48 DHQAIYGLHADTDNLHLHLAINRVHPETLKVVKINNGFDIEAAHKAIARI 97
    :|| : ::| |||||:|:|:|::||    :      ::   |      | :
101 EHQRVSAVHHDTDNLHIHIAINKIHPTRNTIHEPYRAY..RALADLCATL 148

 98 ENAQGWQREQNGRYQVLENGELGREHIDKDKPRQPAQPKRDMENRTGEKS 147
    |   |::|: ::                  : |       ||| : |  |
149 ERDYGLERDNHE.................TRQRVSENRANDMERHAGVES 181

148 AERIAIEDGAPIIKKAQTWEQLHRELAAKGMRYEKTGSGATLFVGD.VGV 196
          :  | :  || ||:||| |   |::    | |  :  ||   |
182 LVGWIKRECLPELQAAQSWEDLHRVLRENGLKLRERGNGFIFEAGDGTTV 231

197 KASSADRDASLSKLQKRLGAYQPPQRQQVAQREPEPIKPDVPGWKDYITG 246
    |||   || |  ||: |:||: |::  : : |     || :  : |  |:
232 KASTVSRDLSKPKLEARFGAFTPAEGGEAPRRREYRAKP.LKTRID.TTE 279

247 RKAHYSEKNADKLVQDKRQEQE....RKQLAEQQKARRDELMRGNWKGKG 292
      |:| : : : :   :: | :    |::   :   | : | |:  |  |
280 LYARY.QSERQEMGAVRKGELDTLRRRRDRLIEAAMRSNRLRRAAIKLLG 328

293 EVLNAMRSVIAAEQAAEKAALKE.....KHQKQREQHRQQFRPYPDLEQW 337
    |   | | : |  : | :| |       :: :|  | | | |:::|:
329 EGRIAKRLMYAQAHKALRADLDKINREYRQGRQAVQERTQRRAWADWLKA 378

338 QRMQKSPELAEQWRHRASEPQRIEGDRSEPPTPRDIRAYQPEIVGQQ..V 385
    : |          :| | :  :  :    :  ::  :|   : :    :
379 EAMKGDDKALAALRAREGRSDLKGNTIQGSGEAKPGHAAVTDNITKKGTI 428

386 HYSRKEEGGRGGGVSF.VDKGKSID..... 409
     |     : |::|  : | ::   |
429 IYRVGSSAVRDDGDRLQVSREATTDGLDAA 458
```

E. Comparison of ORF7(MobB) of pTF-FC2 with TraJ of RP4
Similarity: 44.3% Identity: 29.2% Gap: 2

```
  1 MPFETQGPEPLDAVINVRLTAAEKARLKEDADLAGLSMSELVRRRYFGRP 50
    |: ||        : | |   : |:  : | |  ||:|:|  :       |
  1 MADETKPTRKGSPPIKVYCLPDERRAIEEKAAAAGMSLSAYLLAVGQGYK 50

 51 IIASADAVMLKELRRI.......GGLLKHIHNESGGVYSKDTAGALVVLK 93
    | :  |   ::|| ||        |||||      :  : |  | :|
 51 ITGVVDYEHVRELARINGDLGRLGGLLKLWLTDDPRTARFGDATLLALLA 100

 94 AYIGK..........LSRDRQEG 106
       :|           : | | |
101 KIEEKQDELGKVMMGVVRPRAEP 123
```

FIG. 5. (A) Comparison of the genetic maps and mobilization proteins of pTF-FC2 and RP4. The relative positions of the ORFs are shown, and their sequence-derived molecular weights (10^3) are given below. A comparison of the amino acid sequences of pTF-FC2 (upper sequence) and RP4 (lower sequence) is shown in panels B, C, D, and E. The vertical lines indicate indentical amino acids. The double dots indicate conservative amino acid substitutions. The single dots indicate spaces in the sequence introduced to maximize alignment.

(Table 2). Since the *Bfr*I site is located near the carboxy terminus of ORF 7, it was possible that the protein had not been completely inactivated. To test this, site-directed mutagenesis was used to introduce a frame shift into ORF 7 at position Glu-9 (Fig. 2), resulting in pTFmut103. This mutation also resulted in a reduction in the mobilization frequency, but unexpectedly, the reduction was less than obtained with pTFmut104 (Table 2). To show that the mutations in ORFs 1 and 7 were nonpolar on ORFs 2, 3, and 6, PAGE was used to establish whether the mutants still produced protein products of a size corresponding to these ORFs (Fig. 4). Synthesis of the ORF 1, 2, and 3 polypeptides occurred in the case of the pTFmut101 point mutation, while the pTFmut102 frameshift mutation resulted in the loss of only the 14-kDa ORF 1 polypeptide (Fig. 4A, lanes d and e). Both of the frameshift mutations in ORF 7 resulted in the predicted loss of the 12-kDa polypeptide but not of the 54-kDa ORF 6 polypeptide (Fig. 4B, lanes c and g).

Comparison of the polypeptides involved in plasmid mobilization with those of plasmid RP4. The nucleotide sequence of the Tra1 region of IncP plasmid RP4 has recently been published (22). This region is involved in plasmid mobilization. There is a clear relatedness in the locations, sizes, and amino acid sequences of several of the proteins of the Tra1 region of RP4 and the mobilization region of pTF-FC2 (Fig. 5). A far lower degree of homology was detected between the predicted amino acid sequence of ORF 3 and the TraM protein of RP4 (38.1% similarity and 15.6% identity; data not shown).

Similarity among the *oriT* regions of pTF-FC2, RP4, and R64. The *oriT* regions of RP4 (13), R64 (9), and pTF-FC2 were aligned and compared (Fig. 6). All of the *oriT* regions contained a 17- to 19-bp complementary inverted repeat sequence capable of forming a stem-loop structure. The Genetics Computer Group package SQUIGGLES subroutine produced *G* values of −16.7, −17.1, and −18.4 kcal (1

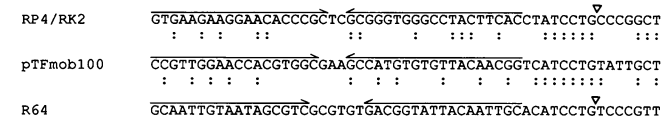


FIG. 6. Comparison of the *oriT* regions of RP4/RK2, pTF-Fmob100, and R64. The inverted repeats are marked with arrows, and the nick sites of RP4 and R64 are indicated by wedges. The double dots indicate identical nucleotide matches.

cal = 4.184 J)/mol for RP4, R64, and pTF-FC2, respectively. The complementary inverted repeats were followed by an 8-bp nucleotide sequence containing an ATCCTG nucleotide hexamer which was perfectly conserved in all three plasmids. It has been shown that the nick sites for RP4 (13) and R64 (5) are located immediately after this conserved hexamer.

Amino acid homology with other polypeptides. The predicted amino acid sequences of ORFs not similar to the proteins of the IncP Tral region were screened for homology against the GenBank-EMBL DNA sequence data bases (release 65) by using the TFASTA subroutine of the Genetics Computer Group sequence analysis package. A high degree of sequence similarity to previously reported genes was found for only one of the additional predicted polypeptides. MerR had a high level of similarity to the *merR* gene products of gram-negative bacteria (Fig. 7). No amino acid homology was detected between the mobilization proteins of pTF-FC2 and proteins from the mobilization regions of other plasmids.

DISCUSSION

A 5,317-bp *Cla*I-*Pst*I fragment of pTF-FC2 was sequenced, and a 3,755-bp region sufficient for mobilization of pTF-FC2 was identified. This mobilization region contained five ORFs and *oriT*. Two of the ORFs were located on one side of *oriT*, and three were located on the other (Fig. 5A). Three of these, ORFs 1, 2, and 6, were essential for mobilization, while ORFs 3 and 7, although not essential, affected the mobilization frequency. The ORFs are situated such that they appear to be translated divergently from *oriT*. Polypeptides corresponding to all five ORFs were detected. Since all five ORFs are involved in mobilization, the genes for ORFs 6, 7, 1, 2, and 3 have been named *mobA*, *mobB*, *mobC*, *mobD*, and *mobE*, respectively (Fig. 1).

The locations and sizes of the genes surrounding the *oriT* region of pTF-FC2 have a strong resemblance to the equivalent Tral region of plasmid RP4 (22). In RP4, *oriT* is flanked on one side by the *traK* and *traL* genes, which together are referred to as the leader operon (12). The *traJ* and *traI* genes

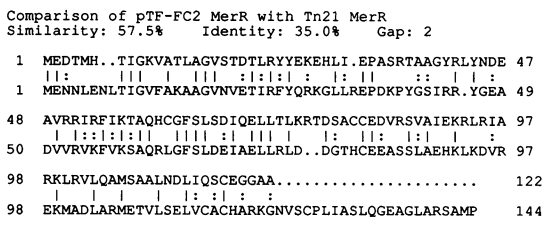


FIG. 7. Comparison of the amino acid sequences of the MerR polypeptides from pTF-FC2 (upper sequence) and Tn*21* (11) (lower sequence). The vertical lines indicate identical amino acids. The double dots indicate conservative amino acid substitutions.

are located on the opposite side of *oriT* and are considered to form a relaxase operon (12). In pTF-FC2, the *oriT* region is flanked by *mobA* and *mobB* on the left and *mobC*, *mobD*, and *mobE* on the right (Fig. 5). Similarity in size and amino acid sequence between TraK-MobC, TraL-MobD, and TraJ-MobB and the partial sequence of TraI with MobA suggests that the two regions are related. The *oriT* regions of the two plasmids also have clear structural and sequence similarities (Fig. 6). The TraH protein of RP4 is translated from an ORF within the TraI-coding region and has been shown to bind and stabilize the TraI-TraJ-*oriT* complex (13). Although the sequences of the RP4 TraI protein and the truncated pTF-FC2 MobA polypeptide are similar, the region of similarity does not extend to the region that encodes the TraH protein of RP4. The truncated MobA protein is nevertheless sufficient to enable pTF-FC2 mobilization. Either a TraH equivalent is not essential for mobilization of pTF-FC2 or the TraH protein of RP4 is nonspecific and able to act in *trans* to mobilize pTF-FC2.

The replicon of pTF-FC2 is of the same type as the IncQ plasmids; however, there was no detectable similarity between the mobilization regions of pTF-FC2 and IncQ plasmid RSF1010 (17). There was also no apparent similarity with other mobilizable plasmids, including a recently published mobilization region of a 6.7-kb plasmid isolated from *T. ferrooxidans* ATCC 33020 (4). Plasmid pTF-FC2 appears to be a natural hybrid with an origin of replication which is related to the IncQ plasmids and mobilization genes and an *oriT* region that resembles that of the IncP plasmids. It is interesting that the region where pTF-FC2 changes from IncP like to IncQ like lies within the large continuous MobA-RepB reading frame. The *Cla*I site cuts the large ORF into two polypeptides, the N-terminal MobA portion of which is required for mobilization and the C-terminal RepB portion of which has been shown to be a primase essential for replication (3). A similar situation exists for IncQ plasmid RSF1010, in which a large 77.9-kDa MobA-RepB polypeptide gives rise to a C-terminal 35.9-kDa RepB′ polypeptide which also functions as a primase (17).

The discovery of what appears to be a Tn*21*-related transposon containing an ORF with homology to *merR* genes is also interesting. Plasmid pTF-FC2 has been cryptic with respect to a phenotypically selectable marker, and this discovery is the first indication of a possible plasmid phenotype. The size of the DNA fragment between the two Tn*21*-like inverted repeat sequences is, however, only 3.4 kbp, and the unsequenced 2.3-kbp portion is too small to contain both a *merA* (mercury reductase) gene, which is typically 1.65 kbp, and a *tnpA* (transposase) gene, which in the case of Tn*21* is about 3.0 kbp. The 2.3-kbp *Pst*I-*Pvu*I fragment is being sequenced to determine exactly which genes are present on the pTF-FC2 transposonlike element.

REFERENCES

1. **Dorrington, R. A., and D. E. Rawlings.** 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171**:2735–2739.
2. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172**:5697–5705.
3. **Dorrington, R. A., and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene **108**:7–14.
4. **Drolet, M., P. Zanga, and P. C. K. Lau.** 1990. The mobilization and origin of transfer regions of a *Thiobacillus ferrooxidans*

plasmid: relatedness to plasmids RSF1010 and pSC101. Mol. Microbiol. **4:**1381–1391.
5. **Furuya, N., and T. Komano.** 1991. Determination of the nick site at *oriT* of IncI1 plasmid R64: global similarity of *oriT* structures of IncI1 and IncP plasmids. J. Bacteriol. **173:**6612–6617.
6. **Harrison, A. P.** 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. Annu. Rev. Microbiol. **40:**265–292.
7. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
8. **Ish-Horowicz, D., and F. Burke.** 1983. Rapid and efficient cosmid cloning. Nucleic Acids Res. **9:**2989–2998.
9. **Komano, T., A. Toyoshima, K. Morita, and T. Nisioka.** 1988. Cloning and nucleotide sequence of the *oriT* region of the IncI1 plasmid R64. J. Bacteriol. **170:**4385–4387.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Nucifora, G., S. Silver, and T. K. Misra.** 1989. Down regulation of the mercury resistance operon by the promoter-distal gene *mer*D. Mol. Gen. Genet. **220:**69–72.
12. **Pansegrau, W., D. Balzer, V. Kruft, R. Lurz, and E. Lanka.** 1990. *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. Proc. Natl. Acad. Sci. USA **87:**6555–6559.
13. **Pansegrau, W., G. Ziegelin, and E. Lanka.** 1988. The origin of conjugative IncP plasmid transfer: interaction with plasmid-encoded products and the nucleotide sequence at the relaxation site. Biochim. Biophys. Acta **951:**365–374.
14. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **158:**737–738.
15. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1986. Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the thiobacilli. Biotechnol. Bioeng. Symp. **29:**291–297.
16. **Rawlings, D. E., and D. R. Woods.** 1985. Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. **49:**1323–1325.
17. **Scholz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271–288.
18. **Simon, R., U. Priefer, and A. Pühler.** 1983. A broad-host-range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Biotechnology **1:**784–794.
19. **Stanssens, P., C. Opsomer, Y. M. McKeown, W. Kramer, M. Zabeau, and H. J. Fritz.** 1989. Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. Nucleic Acids Res. **17:**4441–4453.
20. **Willetts, N., and B. Wilkins.** 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. **48:**24–41.
21. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
22. **Zielegin, G., W. Pansegrau, B. Strack, D. Balzer, M. Kröger, V. Kruft, and E. Lanka.** 1991. Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. DNA Sequence **1:**303–327.

Molecular Microbiology (1993) 9(5), 1051–1059

Regulation of mobilization of the broad-host-range plasmid pTF-FC2

Jurg Rohrer and Douglas E. Rawlings*
Department of Microbiology, University of Cape Town, P B Rondebosch, 7700, South Africa.

Summary

The mobilization region of the broad-host-range plasmid pTF-FC2 consists of an *oriT* and five genes arranged in two operons that are divergently transcribed from the *oriT*. The transcriptional starts of both operons were identified and the quantity of transcript from the *mobC–mobE* promoter (P1) was at least 10-fold greater than that from the *mobA–mobB* promoter (P2). A translational fusion between the first protein of each operon and a *lacZ* reporter gene was constructed and used to demonstrate that *mob* gene expression was autoregulated. Analysis of the *oriT* resulted in the detection of a putative integration host factor (IHF)-binding site and an intrinsically bent region. In the absence of IHF, the mobilization frequency and expression from P1 were reduced. The presence of *ssi* sites on both strands within the *oriT* region was demonstrated by using an M13 phage mutant, defective in its mechanism for priming DNA replication. Initiation of DNA synthesis at the *oriT* did not require a plasmid-encoded primase.

Introduction

Plasmid pTF-FC2 is a 12.2 kb, multicopy, broad-host-range plasmid that was isolated from the biomining bacterium *Thiobacillus ferrooxidans* (Rawlings *et al.*, 1984). The plasmid was found to have a replicon that clearly resembled that of the broad-host-range IncQ plasmids. However, the IncQ plasmid R300B was unable to complement pTF-FC2 *repA* or *repC* mutants and the two plasmids were compatible (Dorrington and Rawlings, 1990; Dorrington *et al.*, 1991). Although pTF-FC2 is a non-conjugative plasmid, it is mobilized at a high frequency between *Escherichia coli* strains by a co-resident IncP plasmid, RP4 (Rawlings and Woods, 1985). The ability to be mobilized together with its broad-host-range replicon enables pTF-FC2 to be used in the construction of vectors that can be used in a large variety of Gram-negative bacteria (Rawlings *et al.*, 1986).

Two features are required for the mobilization of a non-conjugative plasmid by a co-resident conjugative plasmid. These are the origin of transfer (*oriT*, required in *cis*) and *trans*-acting mobilization proteins that specifically recognize the *oriT* region. The *oriT* includes binding sites necessary for the nicking and initiation of DNA transfer, as well as single strand initiation (*ssi*) sites that are required for the triggering of complementary DNA synthesis in both donor and recipient cells (Willetts and Wilkins, 1984; Pansegrau *et al.*, 1988). The functions of plasmid encoded mobilization proteins, although not fully understood, include proteins that are required to form the relaxation complex and subsequent translocation of the ssDNA molecule to the recipient (Pansegrau *et al.*, 1990a). In addition, some plasmids such as R64 and RP4 encode for a specific primase that is required for the initiation of conjugation-associated DNA synthesis at the *ssi* sites and for which the host primase is unable to substitute (Rees *et al.*, 1987; Lanka and Barth, 1981).

The minimum region required for the mobilization of pTF-FC2 was shown to be located on a 3755 bp fragment, which consisted of an *oriT* site flanked by five genes arranged such that two were on one side and three on the opposite side of the *oriT* (Fig. 1). Of the five polypeptides, three (MobA, MobC, and MobD) were essential for mobilization while two (MobB and MobE) affected mobilization frequency (Rohrer and Rawlings, 1992). In RP4, the proteins of the Tra1 region are arranged into three operons, a leader operon on one side of the *oriT*, and the relaxase and primase operons on the other (Ziegelin *et al.*, 1991). There was a distinct similarity in the organization, size and amino acid sequences of four of the pTF-FC2 Mob polypeptides with the proteins of the leader and relaxase operons of the Tra1 region of RP4.

The direction of translation of the mobilization proteins of pTF-FC2 suggested the presence of two divergent promoters in the *oriT* region. In this paper we report on the location of two divergent promoters within the *oriT* region of pTF-FC2 and investigate the effect of plasmid-encoded proteins on a reporter gene that was regulated by these two promoters. In addition we tested the effect of integration host factor on mobilization and whether the *oriT*

Received 11 January, 1993; revised 12 April, 1993; accepted 16 May, 1993. *For correspondence. Tel. (21) 650 3261; Fax (21) 650 4023.

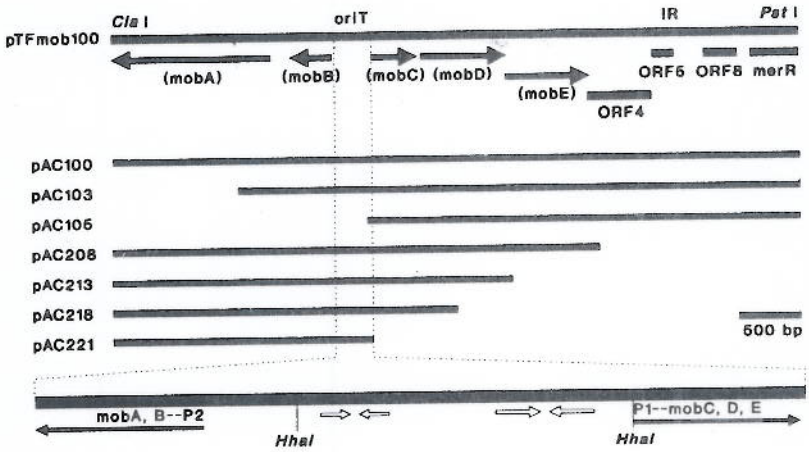


Fig. 1. The *ClaI–PstI* fragment of pTF-FC2 showing the five mobilization genes and other ORFs. The deletions subcloned into pACYC184 and referred to in the text are indicated below the linear representation. Below the deletions is an expanded view of the *oriT* region showing the location of promoters P1 and P2, the *Hha*I restriction sites used in the M13 subcloning and two sets of inverted repeats (non-shaded arrows). Solid arrows represent the direction of transcription.

```
1557 CCAAAGTAGCGGCGGCGGACAAGCTCCGACATGCTCAACCCGGCAAGGTCGGCATCCTCT
     G  F  Y  R  R  R  V  L  E  S  M  S  L  G  A  L  D  A  D  E

                                                    5'-GCGTC CAGCGGTTC
1617 TTCAAGCGGGCCTTCTCCGCTGCGGTCAGCCGTACATTGATAACGGCGTC CAGCGGTTC
     K  L  R  A  K  E  A  A  T  L  R  V  N  I  V  A  D   L  P  E

     Sau96I
     CGGCCCC-->                                          !
1676 CGGCCCCTGCGTTTCAAATGGCATCGTGTCTCCTTCTCGCGGGGGGCGGGCGGGGTGTGT
     P  G  Q  T  E  F  P  M  <--MobB

                          <--pTFmob222
                    HhaI
1736 CTTGGACAGTAAGAAGCGAGAGCGCCGCACTGTCCGGGGTCAAGGGGATACCCCTTGCAG

                                   BglI
1796 GATAAGCCAGACCGTGAGAAGCGAAGCGCCGAATGGGCAGGCGTCCGTTGGAACCACGTG

                                                        ****
1856 GCGAAGCCATGTGTGTTACAACGGTCATCCTGTATTGCTCAACCGCTCTACTATCATATC

                          -35                    HhaI        -10
     *********
1916 AGCAGTTTAGGCACACTAGATGCAC TTTGCA ACAAGCATAAAAGG CGC TATGCT G

                                                    <--GCTGTCTCGTCGAT
     HincII  !
1971 TCAACTCCGAAACCAGTAAGCAAAGGAGTTAGCAGAATGAAATACACGACAGAGCAGCTA
                               MobC-->      M  K  Y  T  T  E  Q  L

     CTC-5'
2031 GAGGCTATCGCATCGAAGCTGCGTGATATGCCACAAGTCGAAAAAAAGAAGCAGGAGCAC
     E  A  I  A  S  K  L  R  D  M  P  Q  V  E  K  K  K  Q  E  H

                                   Sau3A
2091 AGCAAGCAAGAGGCCGTGCGCGTGCTATCAAAAGAGATCGCGGCTTTGCAAAAGCGCGGA
     S  K  Q  E  A  V  R  V  L  S  K  E  I  A  A  L  Q  K  R  G

                          XmnI
2151 TACACGCTTGACCAGATTTCGGAAAC GCTTCGCGGTGAAGGTCTGAGCATCGCAACCCC
     Y  T  L  D  Q  I  S  E  T   L  R  G  E  G  L  S  I  A  T  P
```

Fig. 2. Nucleotide sequence of the *oriT* region. The two oligonucleotides used in the primer extension studies are shown above the sequence together with the direction of primer extension. Transcription starts are indicated by the exclamation marks. The P1 σ^{70}-type promoter sequence is boxed and in bold. The extent of pTFmob222 is shown, the arrow refers to the DNA still remaining on this fragment. The direction of *mobB* and *mobC* transcription are indicated by the arrows. Ribosome-binding sites and ATG start codons are in bold. The 509 bp fragment cloned into the β-galactosidase fusion vector pSKS107 is indicated by the breaks in the sequence at position 1667 bp and at the *Xmn*I site. Inverted repeats are indicated by converging arrows, restriction sites used in the bending studies are shown and the stars above the DNA sequence denote the consensus IHF-binding site.

region was able to support initiation of DNA synthesis utilizing either a host or plasmid-encoded primase.

Results

Identification of the transcription initiation sites within the oriT *region*

To determine the transcriptional start sites, primer extension experiments were carried out from within *mobB* and *mobC* towards the *oriT* (Fig. 2). Total RNA was isolated from *E. coli* cells harbouring the plasmid pTFmob100 and an identical amount was used to identify the start of both transcripts. The primer extension product for P1 (Fig. 1) gave a strong signal which lined up with base pair 1977 (Fig. 3A). Analysis of the sequence suggested that this promoter was of the *E. coli* σ^{70}-RNA polymerase type (Harley and Reynolds, 1987). A –10 recognition sequence (TATGCT), which corresponded closely to the consensus –10 region (TATAAT), was located 7 bp upstream from the start. The –35 region (TTTGCA) corresponded well to the consensus (TTGACA) sequence and there was a 17 bp spacing between the –35 and –10 regions (Fig. 2).

The second *oriT* promoter, P2 (Fig. 1), gave a much weaker primer extension signal compared to P1. The transcription start lined up with base pair 1721 (Fig. 3B) and according to the guidelines of Harley and Reynolds (1987) there were no correctly spaced sequences resembling the –10 and –35 σ^{70} promoter regions. Since no clear promoter could be identified in the region of the P2 transcriptional start and as there were possible promoters elsewhere within the *oriT* region, additional primer extension experiments were carried out to look for other transcriptional starts. None were found. Construct pTFmob222 (Fig. 2) was a fortuitous exonuclease III-derived deletion plasmid that had been isolated during the pTfmob100 sequencing exercise (Rohrer and Rawlings, 1992) and which extended exactly 40 bp beyond the P2 transcription start (Fig. 2). We wished to determine whether the MobA and MobB polypeptides were still produced from pTFmob222 in an *E. coli*-derived *in vitro* transcription–translation system. In pTFmob222, the *lacZ* promoter from the pBluescript-SK cloning vector was in the same direction as P2 and synthesis of MobA (54 kDa) and MobB (12 kDa) could have been the result of transcription from either promoter. The pTFmob222 insert was therefore recloned into the pBluescript-KS vectors which placed the *lacZ* promoter in the opposite orientation to the *mobA* and *mobB* genes. MobA and MobB proteins were still produced from this construct (data not shown). This provided additional evidence that the non-σ^{70}-consensus-type promoter was located within 40 bp of the transcription start.

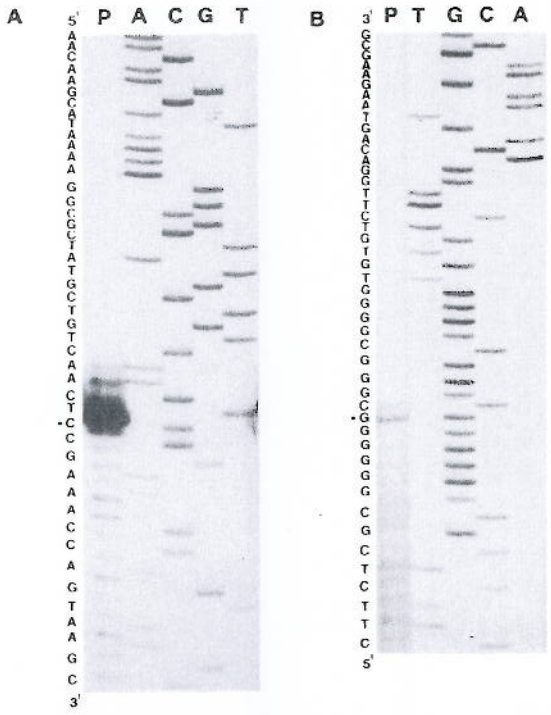


Fig. 3. Primer extension analysis of promoters P1 and P2. Sequencing ladders were generated from plasmid pTFmob100 (Fig. 1) using the two oligonucleotide primers shown in Fig. 2.
A. The primer extension product for P1 with the most prominent signal lining up with the C at base pair 1977.
B. The primer extension from P2 with the signal lining up with the G at base pair 1721. Identical amounts of RNA and primer were used for both experiments.

Regulation of expression from the oriT *promoters by Mob proteins*

In order to investigate the regulation of expression from the promoters of the *oriT* region, fusions with a β-galactosidase reporter gene were constructed. A 509 bp fragment encompassing the *oriT* region and the starts of the *mobB* and *mobC* genes was used (Fig. 2). A P1–*mobC*–*lacZ* translational fusion, pSKSP1, was made by cloning the 509 bp fragment into the vector pSKS107 (Casadaban *et al.*, 1983) in a two-step procedure such that amino acid E-55 of MobC was fused to 13 codons of pUCBM21 and this construct was fused to codon 4 of the *lacZ* gene. A P2–*mobB*–*lacZ* reporter construct, pSKSP2, was made by a similar procedure in which amino acid L-11 of MobB was fused to 16 codons of the pUCBM21 vector, which was fused to codon 4 of the *lacZ* gene of pSKS107. Successful recombinants gave blue colonies when plated on Luria agar with X-gal. The P1 fusion (pSKSP1) was not detected when incubated at 37°C, but when grown at room temperature (22°C) small intensely blue colonies appeared which contained the desired construct. The P2 reporter subclone (pSKSP2, Fig. 2) grew readily at 37°C.

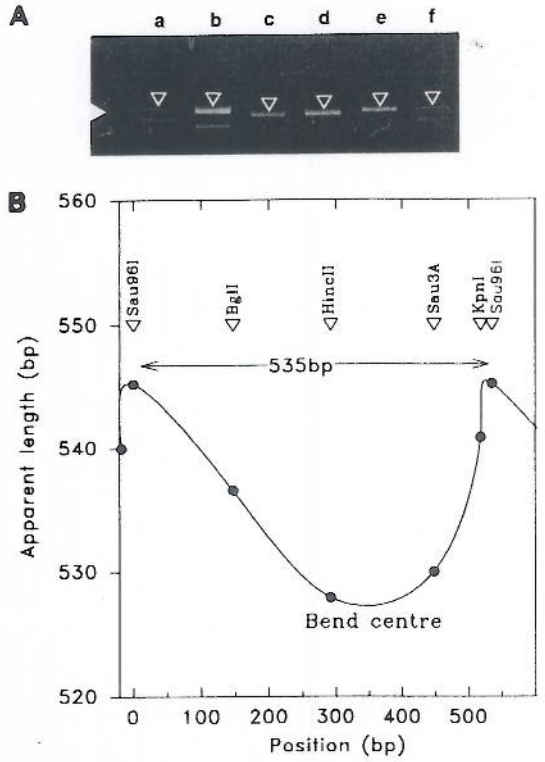


Fig. 4. A. Polyacrylamide gel to detect intrinsic bending at the *oriT*. Two copies of the 509 bp fragment (Fig. 2) concompassing the oriT were cloned in tandem and digested with a restriction enzyme that cuts once within each copy to produce fragments of identical length. The exact positions of the restriction sites are indicated in Fig. 2. DNA digested with: lanes: a and f *Sau*961; lane b *Bgl*I; lane c *Hinc*II; lane d *Sau*3A and lane e *Kpn*I. The *Kpn*I site was introduced at position 1667-bp (Fig. 2) during dimer construction.
B. Graphic representation of the polyacrylamide gel showing the relative position of the restriction sites.

Expression from P1 in *E. coli* LK112 was high (23 793 units), while expression from P2 was very low (79 units) (Table 1). To establish whether any plasmid-encoded proteins affected promoter activity, selected deletions (Fig. 1) were subcloned into the compatible vector pACYC184 (Chang and Cohen, 1978). The deletions were selected so as to sequentially remove MobE (pAC213), MobD (pAC218) and MobC (pAC221) from the right-hand side, and MobA (pAC103) and MobB (pAC105) from the left-hand side of *oriT* (Fig. 1). Clone pAC100 was the original pTFmob100 subclone cloned into pACYC184, while pAC208 had previously been shown to be the smallest subclone mobilizable at parental frequencies, and contained all the elements necessary for mobilization (Rohrer and Rawlings, 1992). The β-galactosidase assays clearly showed that pAC100 and pAC208 affected expression from either promoter (Table 1). Therefore all the factors involved in P1 and P2 regulation appeared to be located within the region of pTF-FC2 required for mobilization.

When a complete set of MobA, B, C, D and E proteins was present on a co-resident plasmid (pAC100 and pAC208), the high level of constitutive expression of the P1–β-galactosidase fusion protein was reduced by approximately half. Removal of MobA had no significant effect on pSKSP1 expression, while removal of MobB resulted in a further drop in expression. From the other side, removal of MobE resulted in increased expression levels, removal of MobD had no noticeable effect, while removal of MobC resulted in high expression levels similar to pSKSP1 on its own.

Low-level expression from the P2–β-galactosidase fusion increased from 79 to 114 units when the complete set of mobilization proteins was present. Removal of MobA resulted in increased expression levels of 247 units, while removing MobB reduced levels to 151 units. Deletions removing MobC to MobE seemed to have no effect on pSKSP2 expression levels when compared to values obtained when a complete set of mobilization proteins was present.

Isolation of plasmids from a given amount of cells followed by ethidium bromide staining indicated that the copy number did not vary noticeably within a set of experiments. This indicated that alterations in expression level were not due to changes in copy number of either the fusion or complementing plasmids.

Intrinsic bending of DNA in the oriT *region*

DNA sequences which are intrinsically bent have been reported to be associated with certain strong promoters (Plaskon and Wartell, 1987), origins of replication of phage (Zahn and Blattner, 1987), yeast autonomously replicating sequences (Snyder *et al.*, 1986) and chloroplast DNA (Hsieh *et al.*, 1991). Statically bent DNA has also been reported to occur in the *oriT* region of plasmid RP4. A visual search for phased poly-A tracts within the *oriT* region of pTF-FC2 revealed a region extending from

Table 1. Results of the β-galactosidase fusion studies for promoters P1 and P2. Values are given in Miller units.

Plasmid co-resident with P1 or P2	Genes present[a]	Promoter Subclone	
		promoter 1*	promoter 2
None	NA	23 793 ± 3514	79 ± 8
pAC100	*A,B,C,D,E*	10 214 ± 1447	114 ± 9
pAC103	*B,C,D,E*	9381 ± 1281	247 ± 34
pAC105	*C,D,E*	6744 ± 689	151 ± 19
pAC208	*A,B,C,D,E*	11 540 ± 922	130 ± 15
pAC213	*A,B,C,D*	14 495 ± 948	134 ± 15
pAC218	*A,B,C*	13 621 ± 1498	129 ± 20
pAC221	*A,B*	25 044 ± 6620	139 ± 17

a. Refers to the mobilization genes present on the pACYC184 subclones. NA, not applicable.

1921 bp to 2180 bp that contained a high proportion of poly-A sequences (usually associated with static bending; Wu and Crothers, 1984). This 260 bp region overlapped the P1 promoter region and part of the *mobC* gene (Fig. 2). We therefore wished to investigate whether the DNA within this region was intrinsically bent. The standard test for static bending (Wu and Crothers, 1984) involved the cloning of the 509 bp *oriT* fragment as a dimer in a head to tail fashion such that upon digestion with a restriction enzyme (that cut only once in each copy), fragments of DNA were obtained that had identical molecular masses (Fig. 2). The different mobilities of the restriction digests in a polyacrylamide gel indicated the presence of curved DNA (Fig. 4A). The apparent length of each fragment was plotted against the position of the corresponding restriction endonuclease. A curve linking the data points indicated that bending took place over a fairly large segment centred in the vicinity of the *Hin*cII site (Fig. 4B).

Effect of integration host factor on mobilization and promoter activity

Also located within the *oriT* region was a putative IHF-binding site with a 1 bp mismatch to the conserved nucleotides of the consensus sequence (5'-pyrimidine-AA-N_4-TTGAT-A/T-3'; Leong *et al.*, 1985). The IHF site was located just upstream of promoter P1 (Fig. 2). To establish the effect of IHF on mobilization, mating studies were done using IHF mutants, while the effect of IHF on promoter activity was established using the previously constructed reporter gene subclones (pSKSP1 and pSKSP2) in the same IHF mutants.

The IHF mutant strain *E. coli* NCM796 and its parent strain RK4353 were used as donors in the mating experiments as outlined in the *Experimental procedures*. The mating frequency of pTFmob100 from *E. coli* RK4353 to LKIII was 0.004 (±0.001) while the frequency from the IHF mutant was 0.0002 (±0.0002), indicating that while not essential, IHF did affect mobilization. The mating frequency of the conjugative plasmid R751 was decreased by less than twofold from 0.023 (±0.008) in the parental strain to 0.016 (±0.006) in the IHF-mutant strain. This indicated that the decrease in the frequency of pTF-FC2 mobilization from the IHF mutant was due to the effect of the IHF mutation on pTF-FC2 mobilization rather than due to a non-specific effect of the IHF mutation on the conjugation process or on the overall physiology of the cell.

Expression from promoter P1 was also affected by IHF (Table 2). In the parental strain the P1–*lacZ* construct on its own resulted in a β-galactosidase expression level of 15 736 units. A co-resident plasmid pDER405 (Rawlings and Woods, 1985) containing a tetracycline marker was used to supply the mobilization proteins. Use of pDER405 was necessary as the pACYC184 subclones all contained a chloramphenicol-resistance marker, making selection of these subclones in the mutant strain impossible as the transposon used to inactivate the *hip* gene carried a chloramphenicol-resistance marker. When the mobilization proteins were provided *in trans* the levels dropped 10-fold. In both cases, the observed values were lower than those obtained in similar experiments using a different *E. coli* strain M8820 (described above). However, when the IHF mutant was used, the expression level of pSKSP1 on its own dropped to 12 337 units (not significantly different to the parental RK4353 value), but with the mobilization proteins *in trans* the value dropped to about half of the parental value. In regard to expression from P2 (Table 2), pSKSP2 on its own showed no significant change in expression levels between strains RK4353 and NCM796 (132 and 128 units respectively) while the expression levels with the mobilization proteins provided *in trans* dropped to zero. This unexpected result was due to instability of the pSKSP2 construct in the presence of pDER405 which resulted in its rapid loss from the population and hence the observed expression levels of 0 to 2 units.

Table 2. Results of the β-galactosidase expression studies in *E. coli* strain RK4353 and the IHF-mutant strain NCM796.

	RK4353		NCM796	
Plasmid	own	pDER405	own	pDER405
pSKSP1	15 736 ± 1886	1553 ± 450	12 337 ±2228	748 ± 248
pSKSP2	132 ± 21	ND	128 ± 20	ND

ND, not determined due to instability of the pSKSP2 fusion in strains containing co-resident pDER405.

Mobilization-associated single-stranded DNA priming takes place within the minimum 206 bp oriT *region isolated from pTF-FC2 and does not require a plasmid-encoded primase*

In a previous paper we showed that the mobilization region of pTF-FC2 had a striking similarity to the Tra1 region of the IncP plasmid RP4 (Rohrer and Rawlings, 1992). Initiation of DNA synthesis in the *oriT* region of the plasmid RK2 (identical to RP4) requires a plasmid-encoded primase (Yakobson *et al.*, 1990) whose gene is situated within the Tra1 region (Ziegelin *et al.*, 1991). We wanted to test whether the primase (*repB* gene) on pTF-FC2, which is required to initiate DNA synthesis at the *oriV* (Dorrington *et al.*, 1991), was also able to initiate DNA synthesis at the *oriT*. To test this the M13Δ*lac110*

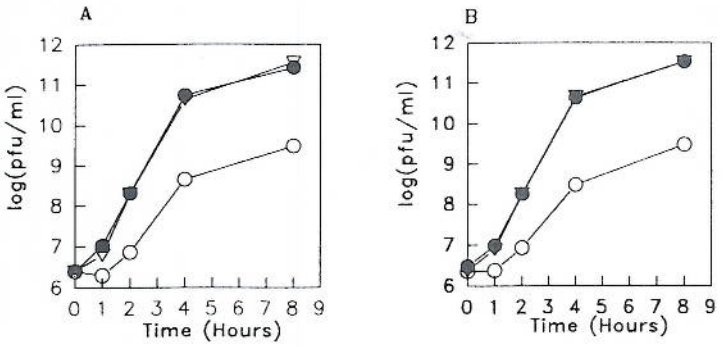


Fig. 5. Growth curve of mutant M13 phage constructs with and without the *HhaI oriT*-containing fragment. Symbols: solid circles, M13Hha1; triangles, M13Hha2 and hollow circles, M13 mutant (negative control).
A. Without co-resident pDER412.
B. With co-resident pDER412.

phage mutant which is defective in the region required for the priming of DNA synthesis was used. This M13 mutant produces small plaques with a reduced phage titre (Kim *et al.*, 1981) unless a priming site that is recognized by a host- or plasmid-encoded primase is provided (Honda *et al.*, 1989).

The 206 bp *Hha*I *oriT*-containing fragment (Fig. 2), thought to contain *ssi* sites, was subcloned into the *Pst*I site of M13Δ*lac110* in both orientations (M13Hha1 and M13Hha2). When growth of the M13Hha1 or M13Hha2 phages was compared to M13Δ*lac110* over an 8 h period, it was found that the *oriT*-containing M13 phages were able to replicate at an increased efficiency and reached a titre approximately 100-fold higher than the M13Δ*lac110* control (Fig. 5A). To test whether pTF-FC2 encoded for an *oriT* specific primase, the mutant M13 and M13 *oriT*-containing clones were propagated in strains containing co-resident pDER412. The presence of pDER412 did not result in an additional increase in the rate of phage growth or affect the final titre of M13 obtained (Fig. 5B). This indicated that the 206 bp *oriT* fragment had at least one *ssi* site on either strand which was recognized by an *E. coli* host-specified primase.

Discussion

Divergent promoters are a common feature of plasmid *oriT* regions and have been reported for several plasmids including RP4/RK2 (Ziegelin *et al.*, 1991), RSF1010/R1162 (Derbyshire *et al.*, 1987) and pTF1 (Drolet and Lau, 1992). Divergent transcription is likely to generate an area of negative supercoiling between two RNA polymerases that are moving apart (Liu and Wang, 1987; Liley and Higgins, 1991). Drolet and Lau (1992) suggested that this reduced supercoiling may stimulate unwinding of the DNA and allow the formation of DNA secondary structures such as the 10 bp inverted repeat adjacent to the nick site of pTF1. These workers had demonstrated that the MobL protein bound to a single-stranded DNA *oriT* sequence but not to a double-stranded *oriT* fragment and the unwinding model was suggested as a possible mechanism that could result in the exposure of single-stranded DNA to MobL. In contrast, the two proteins (TraI and TraJ) that react with the *oriT* of RP4, interact only with supercoiled DNA (Pansegrau *et al.*, 1990b). As the transcription start sites of the *oriT* region of pTF-FC2 have been located, it is clear that the 206 bp *Hha*I *oriT* fragment is missing the entire P2 promoter and the −10 element of P1. The 206 bp fragment when cloned into pUC19 should therefore no longer be subject to unwinding as a result of divergent transcription. Nevertheless this pUC19–*oriT* construct was mobilized at high frequency between *E. coli* strains (Rohrer and Rawlings, 1992). Promoter-driven unwinding as a result of divergent transcription in the *oriT* region would therefore not appear to be an essential prerequisite for efficient DNA transfer in pTF-FC2.

The β-galactosidase fusion experiments indicated that transcription from both promoters P1 and P2 was autoregulated by one or more proteins in their respective operons. Transcription from P1 was clearly inhibited by MobC and weakly inhibited by MobE, while transcription from P2 was inhibited by MobA. It is possible that regulation by these proteins could be the direct result of a repressor-type mechanism or as an indirect result of the assembly of a relaxation complex at *oriT*. Direct evidence for the mechanism of regulation of transcription would require the overproduction and partial purification of the mobilization proteins followed by DNA-binding studies.

As an identical amount of RNA was used in both primer extension experiments, the amount of RNA transcribed from the σ^{70}-like P1 promoter was clearly much greater than that from promoter P2. The level of β-galactosidase expression from P1 was also greater than from P2 (approximately 300-fold). However, it should be noted that although it is reasonable to compare β-galactosidase activity of a given promoter within a set of expression experiments, comparisons between promoters are not valid. This is because cells containing the P1 promoter fusion were grown at 22°C but those with the P2 fusion at

37°C. This temperature change was necessary to overcome the inability of the *E. coli* host cells containing the P1 fusion to grow at 37°C. Presumably, expression from the unregulated promoter was so high that it titrated components needed for *E. coli* chromosomal σ^{70} promoters, or the large amounts of β-galactosidase, permease and acetylase (*lacY* and *lacA* were also present on the fusion constructs) interfered with cell growth.

IHF-enhanced expression from the pSKSP1 construct when the mobilization proteins were provided *in trans*, but not on its own. This suggests that one or more of the mobilization proteins interact with IHF to affect expression from P1. The affect of IHF on P2 in the presence of the mobilization proteins was not clear. Plasmid isolation from colonies containing pSKSP2 and co-resident pDER405 indicated rapid loss of pSKSP2. This explained why β-galactosidase expression was barely detectable in these assays. Plasmid instability has previously been reported for subclones containing the F plasmid *oriT*. If the nick site was positioned on the lagging strand with respect to plasmid replication, the constructs were found to be unstable in the presence of the mobilization proteins (Everett and Willetts, 1982). In the case of pSKSP2, the nick site (Pansegrau and Lanka, 1991) would be on the lagging strand and could explain the loss of pSKSP2 in the presence of co-resident pDER405, which contains a full set of mobilization genes.

The extent of intrinsic bending detected in the DNA over a fairly large region, which included the transcriptional start and *N*-terminal portion of the *mobC* gene, was small (approximately 5% variation in relative mobility) but clearly discernable. The centre of DNA curvature was situated between the σ^{70}-like P1 promoter and the translational start of the MobC protein. The degree of curvature was not as marked as for the RP4 *oriT* (approximately 13% variation in relative mobility) and most of the curvature occurred outside of the *Hha*I fragment, which has been shown to contain a fully functional pTF-FC2 *oriT*. If static DNA curvature does play a role in the region it is most likely to affect the expression of promoter P1. A near consensus IHF-binding site (Leong *et al.*, 1985) was located at positions –51 to –63 bp from the transcriptional start of promoter P1. Binding of IHF to this site is likely to have a far greater affect on the amount of DNA curvature than the intrinsic bend (Friedman, 1988). As demonstrated, the absence of IHF decreased both expression of the *mobC–mobE* operon and the frequency of mobilization. From these studies it is unclear whether the decrease in mobilization frequency from the *E. coli* IHF-mutant donor was solely a result of reduced transcription from P1 or whether the lack of IHF affected bending in the *oriT* region thereby reducing rate of activity at the *oriT*. It is likely that IHF plays a dual role.

The Tra1 region of plasmid RK2/RP4 includes a gene (*traC*) that encodes for an *oriT* specific primase (Yakobson *et al*, 1990; Miele *et al.*, 1992). Immediately downstream of the *mobA* gene of pTF-FC2 is the *repB* gene, which has been shown to function as a primase that is required for replication. This primase was however, not essential for the initiation of DNA synthesis at *oriT* (Fig. 5). Both strands of the *oriT* region contained putative *ssi* recognition sites for an *E. coli* priming mechanism (Fig. 2).

Studies on the promoters of broad-host-range plasmids are of interest not only as a means of understanding the mobilization process, but because they can provide an insight into the question of what enables a promoter to be recognized by bacteria that belong to several different genera. In addition to *E. coli*, plasmid pTF-FC2 has been shown to replicate in and be mobilized by bacteria as diverse as *Thiobacillus novellus* (Rawlings *et al.*, 1986), *Rhizobium meliloti*, *Agrobacterium tumefaciens* and *Pseudomonas aeruginosa* (B. Samuels and D. E. Rawlings, unpublished data). Studies on whether the transcript starts of the pTF-FC2 promoters in different bacteria are identical are in progress.

Experimental procedures

Bacterial strains, plasmids and media

E. coli strain JM105 (Yanisch-Perron *et al.*, 1985) was used for the maintenance of plasmid constructs and prepapration of pTFmob100 RNA. Strain M8820 (Castihlo *et al.*, 1984), which has the *lac* operon deleted and is RecA$^-$, was used in the β-galactosidase gene fusion assays. For the IHF studies, strains RK4353 (Stewart and MacGregor, 1982) and NCM796 (*hip*::Cm; a gift from S. Kustu) were used. RK4353 is the parent of the IHF mutant NCM796, which has a transposon inserted into the *hip* gene. Bacteria were grown in Luria broth (LB) or on Luria agar (LA) plates (Maniatis *et al.*, 1982). For the phage experiments JM105 cells infected with the phage were grown on H-agar plates. H-agar consisted of 10 g l^{-1} tryptone, 8 g l^{-1} of NaCl and 15 g l^{-1} agar while the sloppy covering contained 7 g l^{-1} agar. Antibiotics were added to the media at the following concentrations: ampicillin, 100 μg ml^{-1}; chloramphenicol, 20 μg ml^{-1}; trimethoprim, 200 μg ml^{-1}; rifampicin, 50 μg ml^{-1} and tetracycline 15 μg ml^{-1}.

General DNA techniques

Plasmid DNA was prepared according to the method of Ish-Horowicz and Burke (1983). Standard methods, as suggested by suppliers, were used for all restriction digests, ligations and gel electrophoresis. A DNA sequencing kit (Sequenase, Version 2.0, United States Biochemical Corporation) was used for primer extensions and to check the correctness of the *lacZ* fusion constructs. Sequencing reactions were run on 6% polyacrylamide gels.

Beta-galactosidase assays

Fusion constructs pSKSP1 and pSKSP2 were transformed into *E. coli* M8820 and cells containing pSKSP1 were selected on

LA plates containing ampicillin and X-gal at room temperature, while cells containing pSKSP2 were selected on similar plates but at 37°C. Fresh colonies were picked off the plates and grown up at their respective temperatures till an optical density at 600 nm (OD_{600}) of 0.4 was reached. Beta-galactosidase assays were carried out in a microfuge tube according to Miller (1983). To ensure accuracy, 12 colonies of each type (pSKSP1 or pSKSP2 on their own and with co-resident pACYC184 constructs) were assayed with four values for each colony being obtained. This process was repeated four times.

RNA isolation

E. coli JM105 cells harbouring the plasmid pTFmob100 (*ClaI–PstI* fragment containing the mobilization region cloned into the pBluescript-SK vector; Rohrer and Rawlings, 1992), were grown up overnight at 37°C in the presence of ampicillin. A 250 ml sample of this overnight culture was added to 25 ml of fresh LB, grown to an OD_{650} of 0.5 and RNA extracted as described by Aiba *et al.* (1981).

Primer extensions

Primers were labelled according to the following protocol. Five microlitres (1 mg) of primer was added to 2 µl of 1 M Tris pH 8, 1 µl 100 mM DTT, 2 µl 100 mM $MgCl_2$, 8 µl [γ-^{32}P]-ATP and 1 µl polynucleotide kinase. The reaction was incubated at 37°C for 30 min. The labelled primer was kept at –20°C for a maximum of two weeks.

To 20 µl (100 mg) of RNA, 2 µl of labelled primer, 2 µl of 3 M sodium acetate and 50 µl of 99% ethanol were added. The mixture was incubated at –20°C for 30 min and microfuged at 4°C for 20 min. The supernatant was removed and the pellet dried at room temperature. To the dried pellet 30 µl of RNA-hybridization buffer (supplied by Ambion) was added. After 5 min incubation at 85°C the samples were placed at their respective annealing temperatures for no less than 16 h. Primer 1 was annealed at 60°C while primer 2 was annealed at 76°C. After annealing, samples were ethanol precipitated and dried. Reverse transcriptase buffer (20 µl; 50 M Tris-Cl pH 8, 8 mM $MgCl_2$ and 2 mM DTT), 1 µl RNAse inhibitor (ex-human placenta, Boehringer Mannheim), 2 µl 10 mM dNTPs and 50 U reverse transcriptase were added to the pellet. The reaction was allowed to proceed for 60 min at 42°C and precipitated with ethanol. The pellet was resuspended in 10 µl of 'sequenase' loading buffer and after denaturation was loaded onto a sequencing gel.

Primase site detection

Essentialy the method of Honda *et al.* (1988) was used to detect the presence of single-strand initiation sites (*ssi*) and a mobilization specific primase. The bacterial strain used for the phage studies was JM105. Initial selection for M13Δ*lac110* recombinants was on H-Agar plates using a blue/white X-gal assay. Intact phages were isolated and titred according to Maniatis (1982).

Growth curve experiments were performed as follows. A colony of JM105 was picked from a minimal medium plate, inoculated into 5 ml of LB and incubated for 2 h at 37°C. 1.2×10^8 p.f.u. were added to 400 ml of this culture and incubated for 10 min at room temperature. This suspension was added to 20 ml of fresh LB and immediately 1 ml removed (time 0) and the phage titred. One millilitre samples were also titred at 1, 2, 3.5 and 8 h.

IHF matings

The two *E. coli* strains RK4353 and NCM796 were initially transformed with the conjugative IncPβ plasmid R751 (Tri^R). Plasmid pTFmob100 was transformed into both cell lines. Overnight samples of each donor type were mixed with the recipient (LKIII Rif^R) in a ratio of 1:10 and incubated on LA at 37°C for 60 min. Mating pairs were disrupted by vortexing the agar plug in a 0.85% saline solution for 60 s. A dilution series was plated onto LA-ampicillin-trimethoprim-streptomycin plates to count donors and ampicillin-rifampicin plates to count transconjugants. Mating frequencies were calculated as number of transconjugants per donor.

References

Aiba, H., Adhya, S., and de Crombrugghe, B. (1981) Evidence for two functional *gal* promoters in intact *Escherichia coli* cells. *J Biol Chem* **256:** 11905–11910.

Casadaban, M.J., Martinez-Arias, A., Shapira, S.K., and Chou, J. (1983) β-Galatosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. *Methods in Enzymology* **100:** 293–308.

Castihlo, A.B., Olfson, P., and Casadaban, M. J. (1984) Plasmid insertion mutagenesis and *lac* gene fusion with mini-mu bacteriophage transposons. *J Bacteriol* **158:** 488–495.

Chang, A.C.Y., and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. *J Bacteriol* **134:** 1141–1156.

Derbyshire, K.M., Hatfull, G., and Willetts, N. (1987) Mobilization of the non-conjugative plasmid RSF1010: a genetic and DNA sequence analysis of the mobilization region. *Mol Gen Genet* **206:** 161–168.

Dorrington, R.A., and Rawlings, D.E. (1990) Charactezization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. *J Bacteriol* **172:** 5697–5705.

Dorrington, R.A., Bardien, S., and Rawlings, D.E. (1991) The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. *Gene* **108:** 7–14.

Drolet, M., and Lau, P.C.K. (1992) Mobilization protein–DNA binding and divergent transcription at the transfer origin of the *Thiobacillus ferrooxidans* pTF1 plasmid. *Mol Microbiol* **6:** 1061–1071.

Everett, R., and Willetts, N. (1982) Cloning and mutation and location of the F origin of conjugal transfer. *EMBO J* **1:** 747–753.

Friedman, D.I. (1988) Integration host factor: a protein for all reasons. *Cell* **55:** 545–554.

Harley, C.B., and Reynolds, R.P. (1987) Analysis of *E. coli* promoter sequences. *Nucl Acids Res* **15:** 2343–2361.

Honda, Y., Sakai, H., Komano, T., and Bagdasarian, M. (1989) RepB' is required *in trans* for the two single-strand DNA initiation signals in *oriV* of plasmid RSF1010. *Gene* **80:** 155–159.

Hsieh, C-H., Madeline, W., and Yang, J. (1991) The sequence-directed bent DNA detected in the replication origin of *Chlamydomonas reinhardtii* chloroplast DNA is important for the replication function. *Mol Gen Genet* **225:** 25–32.

Ish-Horowicz, D., and Burke F. (1983) Rapid and efficient cosmid cloning. *Nucl Acids Res* 9:2989–2998.

Kim, M.H., Hines, J.C., and Ray, D.S. (1981) Viable deletions of the M13 complementary strand origin. *Proc Natl Acad Sci USA* **88:** 179–183.

Lanka, E., and Barth, P.T. (1981) Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. *J Bacteriol* **148:** 769–781.

Leong, J.M., Nunes-Düby, S., Lesser, C.F., Youderian, P., Susskind, M.M., and Landy, A. (1985) The ϕ80 and P22 attachment sites. *J Biol Chem* **260:** 4468–4477.

Lilley, D.M.J., and Higgins, C.F. (1991) Local DNA topology and gene expression: the case of the *leu-500* promoter. *Mol Microbiol* **5:** 779–783.

Liu, L.F., and Wang, J.C. (1987) Supercoiling of the DNA template during transcription. *Proc Natl Acad Sci USA* **84:** 7024–7027.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Miele, L., Strack, B., Kruft, V., and Lanka, E. (1992) Gene organization and nucleotide sequence of the primase region of IncP plasmids RP4 and R751. *DNA Sequence* **2:** 145–162.

Miller, J.H. .(1983) *Experiments In Molecular Genetics.* Cold Spring Harbor, New York: Cold Spring Harbor, Laboratory Press.

Pansegrau, W., and Lanka, E. (1991) Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. *Nucl Acids Res* **19:** 3455.

Pansegrau, W., Ziegelin, G., and Lanka, E. (1988) The origin of conjugative IncP plasmid transfer: interaction with plasmid-encoded products and the nucleotide sequence at the relaxation site. *Biochim Biophys Acta* **951:** 365–374.

Pansegrau, W., Balzer D., Kruft V., Lurz R., and Lanka E. (1990a) *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. *Proc Natl Acad Sci USA* **87:** 6555–6559.

Pansegrau, W., Ziegelin, G., and Lanka, E. (1990b) Covalent association of the *traI* gene product of plasmid RP4 with the 5'-terminal nucleotide at the relaxation nick site. *J Biol Chem* **265:** 10 637–10 644.

Plaskon, R.R., and Wartell, R.M. (1987) Sequence distributions associated with DNA curvature are found upstream of strong *E. coli* promoters. *Nucl Acids Res* **15:** 785–796.

Rawlings, D.E., and Woods, D.R. (1985) Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. *Appl Environ Microbiol* **49:** 1323–1325.

Rawlings, D.E., Pretorius, I.-M., and Woods, D.R. (1984) Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli. J Bacteriol* **158:** 737–738.

Rawlings, D.E., Pretorius, I-M., and Woods, D.R. (1986) Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the thiobacilli. *Biotechnol Bioeng Symp* **29:** 291-297.

Rees, C.E., Bradley, D.E., and Wilkins B.M. (1987) Organization and regulation of the conjugation genes of IncI1 plasmid ColIb-P9. *Plasmid* **18:** 223–236.

Rohrer, J., and Rawlings, D.E. (1992) Sequence analysis and chracterization of the mobilization region of the broad- host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans. J Bacteriol* **174:** 6230–6237.

Snyder, M., Buckman, A.R., and Davis, R.W., (1986) Bent DNA at a yeast autonomously replicating sequence. *Nature* **324:** 87–89.

Stewart, V., and MacGregor, C.H. (1982) Nitrate reductase in *Escherischia coli* K-12: involvement of *chlC*, *chlE* and *chlG* loci. *J Bacteriol* **151:** 788–799.

Willetts, N., and Wilkins, B. (1984). Processing of plasmid DNA during bacterial conjugation. *Microbiol Rev* **48:** 24–41.

Wu, H-M., and Crothers, D.M. (1984) The locus of sequence-directed and protein-induced DNA bending. *Nature* **308:** 509–513.

Yakobson, E., Deiss, C., Hirata, K., and Guiney, D.G. (1990) Initiation of DNA synthesis in the transfer origin region of RK2 by the plasmid-encoded primase: detection using defective M13 phage. *Plasmid* **23:** 80–84.

Yanisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.

Zahn, K., and Blattner, R.F. (1987) Direct evidence for DNA bending at the lambda replication origin. *Science* **236:** 416–422.

Ziegelin, G., Pansegrau, W., Strack, B., Balzer, D. Kröger, M., Kruft, V., and Lanka, E. (1991) Nucleotide sequence and organization of genes flanking the transfer origin of promiscuous plasmid RP4. *DNA Sequence* **1:** 303–327.

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1995, p. 4223–4229 Vol. 61, No. 12
0099-2240/95/$04.00+0

Structure and Function of Tn*5467*, a Tn*21*-Like Transposon Located on the *Thiobacillus ferrooxidans* Broad-Host-Range Plasmid pTF-FC2

ANNE-MARIE CLENNEL, BRUCE JOHNSTON, AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 6 February 1995/Accepted 25 September 1995

A 3.5-kb region of plasmid pTF-FC2, which contains a transposon-like element designated Tn*5467*, has been sequenced, and its biological activity has been investigated. The transposon is bordered by two 38-bp inverted repeat sequences which have sequence identity in 37 of 38 and in 38 of 39 bp to the *tnpA* distal and *tnpA* proximal inverted repeats of Tn*21*, respectively. Within these borders, open reading frames with amino acid similarity to a glutaredoxin-like protein, a MerR regulatory protein, and a multidrug-resistant-membrane transport-like protein were found. The gene for the glutaredoxin-like protein was expressed in *Escherichia coli* and enabled growth of a glutathione-requiring *E. coli trxA gshA* mutant on minimal medium and the reduction of methionine sulfoxide to methionine. In addition, there were two regions which, when translated, had homology to 85% of the N-terminal region of the Tn*21* resolvase (*tnpR*) and to 15% of the C terminus of the Tn*21* transposase (*tnpA*). A region containing *res*-like sites was located immediately upstream of the partial *tnpR* gene. Neither the partial transposase nor the resolvase genes of Tn*5467* were biologically active, but Tn*5467* was transposed and resolved when the Tn*21* transposase and resolvase were provided in *trans*. Tn*5467* appears to be a defective transposon which belongs to the Tn*21* subgroup of the Tn*3* family.

Plasmid pTF-FC2 is a 12.2-kb, broad-host-range, mobilizable plasmid that was originally isolated from an industrial strain of the biomining bacterium *Thiobacillus ferrooxidans* (15). The plasmid has a replicon which clearly resembles that of the identical or nearly identical IncQ plasmids RSF1010, R300B, and R1162. Plasmid pTF-FC2 is, however, compatible with the IncQ plasmids, and the *repA* or *repC* gene products of the IncQ plasmids are unable to complement *repA* or *repC* mutants of pTF-FC2 (5). Interestingly, the mobilization region of pTF-FC2 is similar in structure to that of the IncPα plasmids RP4 or RK2 and the IncPβ plasmid R751 (17). It consists of five Mob proteins and an origin of transfer (*oriT*) arranged in two operons in a manner similar to that of the Tra1 region of IncP plasmid RP4. In addition, four of the Mob proteins have low but clear sequence identity to Tra proteins of the IncP plasmids. Plasmid pTF-FC2, therefore, contains an IncQ-like replicon and an IncP-like mobilization region and appears to be a natural hybrid which is related to these two well-studied groups of plasmids.

The Tn*3* family of transposons consists of a number of subgroups of which the Tn*21* subgroup contains the largest number of known elements. General characteristics of the Tn*3* family have been reviewed by Sherrat (19), and those of the Tn*21* subgroup have been reviewed by Grinsted et al. (7). Transposons belonging to the Tn*3* family typically consist of terminal inverted repeat (IR) sequences of about 38 bp, genes for a transposase (*tnpA*) and a resolvase (*tnpR*), and an approximately 130-bp *res* region at which the resolvase acts. In the case of the Tn*21* subgroup, the *tnp* genes and *res* are arranged in the order *res-tnpR-tnpA* (7, 19). Transposons belonging to this subgroup occur widely among members of the γ subgroup of proteobacteria such as *Escherichia coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *Yersinia enterocolitica*, *Enterobacter cloacae* and species belonging to the genera *Shigella*, *Salmonella*, and *Pseudomonas*. Transposons of the Tn*21* subgroup also have a variable region which frequently contains genes that encode for resistance to mercury and antibiotics such as aminoglycosides, β-lactams, sulfonamides, chloramphenicol, and tetracycline (7).

Although several plasmids from *T. ferrooxidans* have been cloned and at least two have been sequenced (14), no phenotype has been ascribed to any *T. ferrooxidans* plasmid studied to date. In this paper, we report on the structure, sequence, and biological activity of a 3.5-kb, Tn*21*-like transposon from the plasmid pTF-FC2. We show that a gene located within the transposon-like element is expressed in *E. coli* and encodes a protein which is able to complement *E. coli* thioredoxin mutants.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and general techniques. The strains and plasmids used in this study are described in Table 1. *E. coli* strains were grown in Luria broth (LB), on Luria agar (LA) plates, or on minimal medium plates

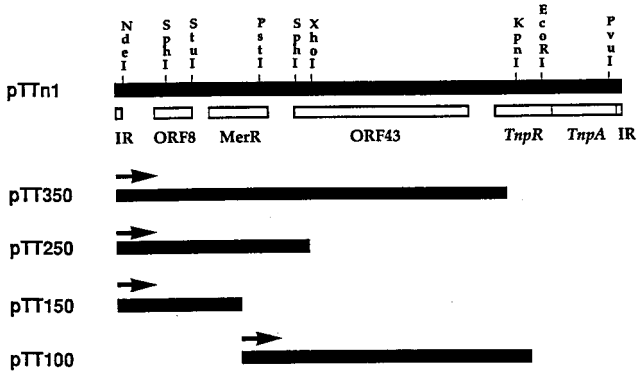
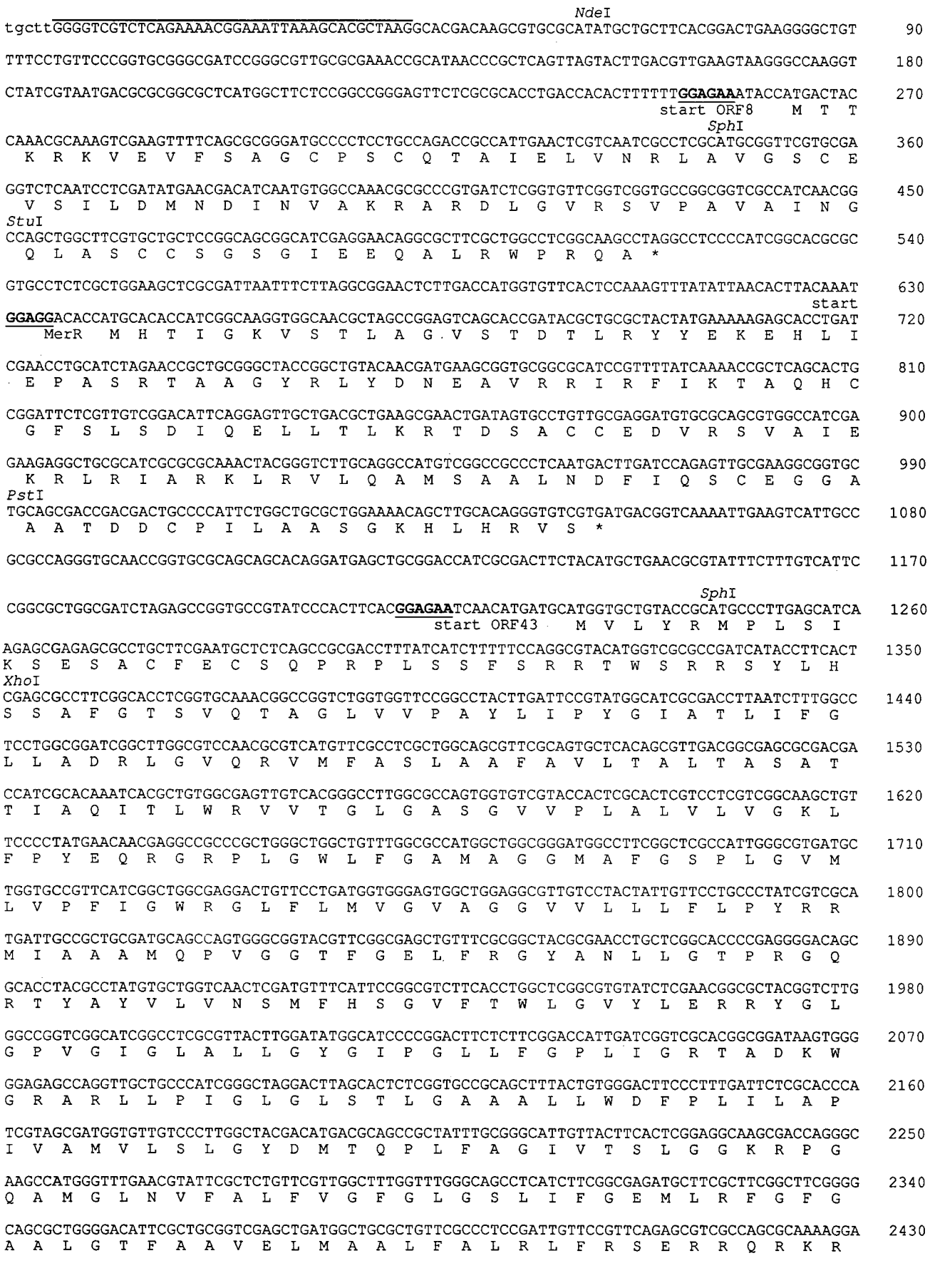


FIG. 1. A restriction endonuclease cleavage map of Tn*5467* showing the location of potential ORFs and the subclones used in this study. The arrows indicate the direction of transcription from the *lacZ* promoter of the pBluescript vector.

* Corresponding author. Phone: 27 21 650 3261. Fax: 27 21 650 4023. Electronic mail address: Doug@molbiol.uct.ac.za.



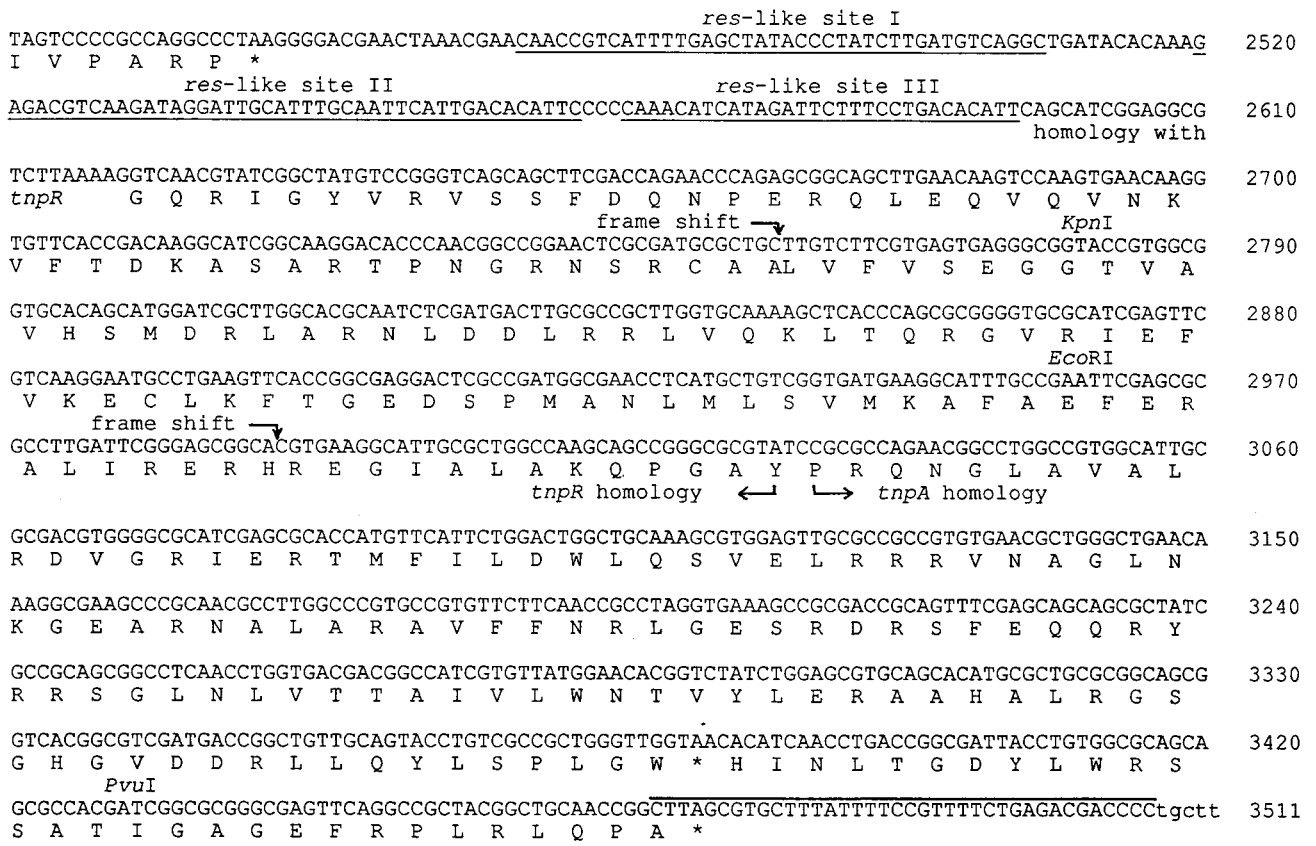


FIG. 2. Nucleotide sequence of Tn*5467* and the predicted amino acid sequences of ORF8, *merR*, ORF43, and regions which show homology to the resolvase and transposase of Tn*21*. Potential ribosome binding sites have been underlined and indicated in bold type. The 38-bp terminal IR sequences are overlined, and the *res*-like sites are underlined. The 5-bp target duplication sequences are shown in lowercase type.

(18). Glutathione and methionine sulfoxide (both from Sigma Chemical Co., St. Louis, Mo.) were added at 25 μg/ml when required. Where necessary, the following antibiotics were added at the indicated concentrations: ampicillin, 100 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 30 μg/ml; streptomycin, 100 μg/ml; and trimethoprim, 100 μg/ml. Plasmid preparation, restriction digests, gel electrophoresis, and ligations were carried out by standard methods (18).

Nucleotide sequencing and DNA analysis. Nucleotide sequence determination was done by the dideoxy chain termination method with a Sequenase DNA sequencing kit (U.S. Biochemical Corporation, Cleveland, Ohio). The entire sequence of the 3,511-bp transposon and target sites was determined from both strands. DNA sequence data were analyzed with the Genetics Computer Group Inc. software package and the BLAST subroutines (National Center for Biotechnology Information [NCBI], New Bethesda, Md.). The transposon was registered with E. Lederberg (Plasmid Reference Center, Stanford University, Stanford, Calif.) and assigned the number Tn*5467*.

Protein gels. Translation products were identified with a DNA-directed transcription-translation kit (code L4500 *E. coli* system; Promega Corp., Madison, Wis.). Polypeptides were separated on sodium dodecyl sulfate (SDS)–12 or 20% polyacrylamide gels.

Assay for transposition. The nonmobilizable plasmid pTfTn2 (Tc^r) was transformed into an *E. coli* strain (Sm^s) containing the self-transmissible plasmid R388 (Tp^r) or into a strain containing R388 plus pUB2413 (Cm^r *tnpA tnpR*) or pUB2584 (Cm^r *tnpA*). Ten transformants were picked, inoculated into 10 ml of LB, and incubated overnight at 30°C to allow transposition into R388 to take place. Transposition was detected by mating the transformants with *E. coli* HB101 (Sm^r) followed by plating on LA plus streptomycin (selection against the donor) and tetracycline (selection for the transposon). Matings were carried out by mixing equal volumes of donor and recipient strains, inoculating the mixture onto the surface of an LA plate, and incubating the plate overnight. The growth was scraped off the plate and resuspended, and suitable dilutions were spread

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or phenotype	Source or reference
E. coli		
LK112	*supE44 hsr leu-6 recA thi-1 thr-1 tonA21 lacI*^{q} *lacZ*ΔM15	M. Zabeau, Ghent, Belgium
HB101	*supE44 hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*	18
BH2012	*araD139*? *galU galK hsr rpsL metA46 argH1 trxA7004 ilvC*::Tn*5*	12
BH5262	*araD139*? *galU galK hsr rpsL argH1 trxA7004 gshA srl*::Tn*10*	12
Plasmids		
R388	Tp^r Su^r Tra^+	3
pBluescriptKS$^+$	Ap^r Mob^-	Stratagene, San Diego, Calif.
pTTn1	Ap^r (Tn*5467* including the *repC* gene of pTF-FC2 cloned into pBluescript SK^+)	This work
pTTn2	Ap^r Tc^r (the Tc^r gene of pBR322 cloned into the *Pst*I-*Xho*I sites of pTTn1)	This work
pUB2413	Cm^r (pACYC184 containing the *tnpR* and *tnpA* genes of Tn*21*)	4
pUB2584	*tnpR* mutant derivative of pUB2413	1

```
Grdxn        VVKIEVFTSPTCPYCPMAIEVVDEAKKEFGDKIDVEKIDIMV  42
               :.::: :  :: :  :::.:.      .   ..  :: :
Tn5467    MTTKRKVEVF.SAGCPSCQTAIELVNRLACGSCEVSILDMNDINV  44

Grdxn     DREKAIDYGLMAVPAIAIDG.VVRFVGAPGREELFEAISDEI     83
           . .: : :   :::.::.: .    .. : ::
Tn5467    AK.RARDLGVRSVPAVAINGQLASCCSGSGIEEQALRWPRQA     85
```

FIG. 3. Alignment of the sequence of glutaredoxin redox-active protein from *Methanobacterium thermoautotrophicum* (13) and the predicted amino acid sequence of ORF8. Double dots and single dots indicate amino acid identity and similarity, respectively.

onto selective plates. Plasmid DNA was prepared from individual colonies and digested with the restriction enzyme *Sst*II, and the fragments were separated on agarose gel and examined for the presence of a pTTn2 insertion.

Assay for *res* activity. Transposition was carried out as described above except that the *E. coli recA* mutant LK112 was used as the donor strain and *E. coli recA* mutant HB101 was used as the recipient strain. After transposition, transconjugants were tested for the presence or absence of the Ap^r marker which is present on the pBluescriptSK vector portion of pTTn2. Colonies which tested tetracycline and ampicillin resistant indicated nonresolution of cointegrates, and those which tested Tc^r Ap^s indicated cointegrate resolution. Plasmid DNA was prepared from 10 Tc^r Ap^r colonies and 10 Tc^r Ap^s colonies and digested with *Sal*I to confirm the presence or absence of vector DNA.

Nucleotide sequence accession number. The sequence of Tn*5467* was deposited in the GenBank database and assigned the accession number L37364.

RESULTS

Structure of the transposon-like element. A diagram of the structure of Tn*5467* is shown in Fig. 1, and the nucleotide sequence is shown in Fig. 2. The element is bordered by two identical 38-bp IR sequences which have a 37- of 38-bp match to the *tnpA* distal and a 38- of 38-bp match to the *tnpA* proximal IR sequences of Tn*21* (7, 19), respectively. Two 5-bp directly repeated sequences (TGCTT) typical of transposition target-site duplications are located outside of the transposon-like element, immediately adjacent to the IR sequences. Within the IR sequence borders, several open reading frames (ORFs) were present. Three of the ORFs were preceded by strong predicted ribosome binding sites and, by use of *T. ferrooxidans* codon preference data and third-position GC bias statistics (codonpreference subroutine, Genetics Computer Group package), appeared to be likely to encode for proteins. Tn*5467* had two other regions which, if translated, would encode for proteins with amino acid sequence similarity to portions of the resolvase (*tnpR*) and transposase (*tnpA*) of Tn*21*. In addition, immediately upstream of the *tnpR*-homologous segment, a region which had sequence similarity to the three resolution sites (*res* sites) of Tn*21* and related transposons was found (see Fig. 7).

Analysis of the ORFs. A small ORF, previously termed ORF8 (17), which would encode for a predicted protein of 85 amino acids (calculated M_r, 9,052) was located close to the left-hand IR sequence (Fig. 1). When the amino acid sequence of ORF8 was compared for sequence similarity with the sequences of the GenEMBL nonredundant DNA and protein databases (NCBI) by the BLAST program, clear similarity to a redox-active glutaredoxin-like protein from *Methanobacterium thermoautotrophicum* (13) was found (Fig. 3).

A second ORF had sequence similarity to previously sequenced MerR proteins. These proteins are the regulators of mercury reductase operons and have been found in several different bacteria (21). A tree of the amino acid sequence relationship of the predicted Tn*5467* MerR polypeptide with those other MerR proteins is shown in Fig. 4. The MerR-like protein from pTF-FC2 was most closely related to the apparently silent *merR*-like gene (*yhdM*) from the chromosome of *E. coli* (2). Surprisingly, the predicted MerR protein from Tn*5467* was most distantly related to those found within transposons of other gram-negative bacteria. No other ORFs with similarity to mercury reductase or any other proteins usually associated with mercury resistance were found on the transposon.

A third ORF, ORF43, which could encode for a predicted protein of 406 amino acids (calculated M_r, 43,485) was found downstream of the MerR ORF. Analysis of the amino acid sequence indicated that the protein was a hydrophobic (35% hydrophobic residues), membrane-spanning protein. A search for homology of the ORF43 amino acid sequence with the GenEMBL protein and nucleotide databases by use of the NCBI BLAST subroutine showed that ORF43 had low, but clear, sequence similarity (49 to 54%) to a large group of membrane translocases. Several of these were proteins belonging to the large family of rather weakly homologous, multi-

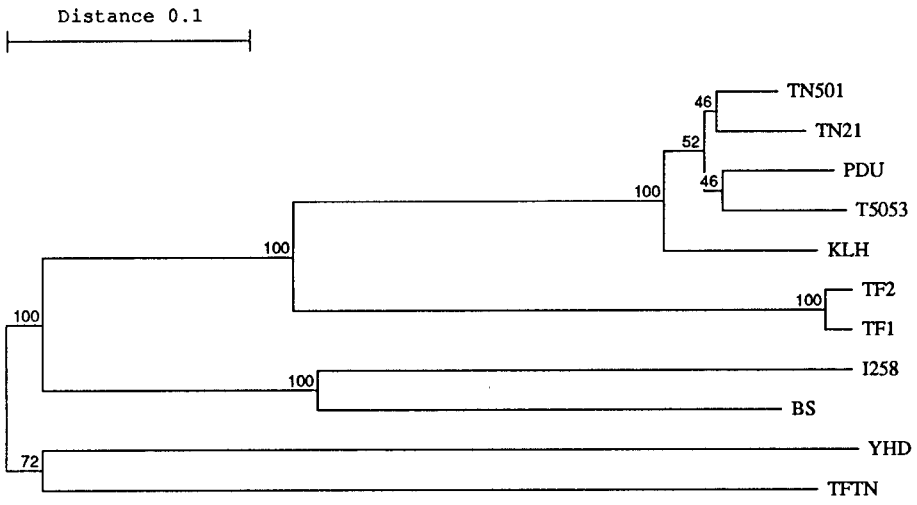


FIG. 4. Tree of the sequence relationships between the MerR regulatory proteins. Numbers at the branch points represent percentages from bootstrap analysis by the neighbor-joining method. Abbreviations of sequences (from the GenBank database) are as follows, with accession numbers shown in parentheses: TN501, transposon Tn*501* (K02503); TN21, transposon Tn*21* (J01730); PDU, plasmid pDU1358 (M24940); T5053, transposon Tn*5053* (L03729); KLH, plasmid pKLH2 (M54946); TF1 and TF2, *T. ferrooxidans* chromosome MerR1 and MerR2 (X57326); I258, plasmid I258 (L29436); BS, *Bacillus* spp. (M22708); YHD, predicted product of the *E. coli yhdM* gene (L29458); TFTN, transposon Tn*5467*.

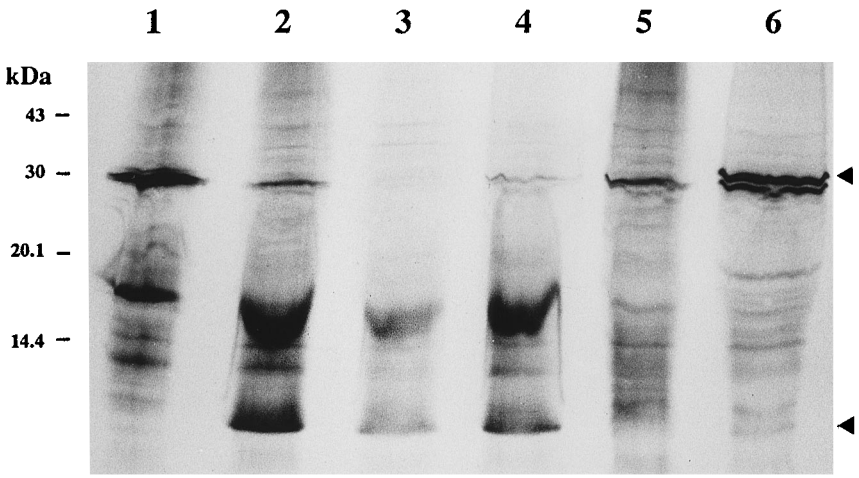


FIG. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of polypeptides translated in vitro from Tn*5467* and deletions. Lanes: 1, pBluescript SK control; 2, pTT350; 3, pTT250; 4, pTT150; 5, pTT100; 6, pTTn1.

drug-resistant membrane translocases (11). Examples of these translocases are the chloramphenicol resistance determinants from *Streptomyces lividans* (GenBank accession number X59968) and the In4 integron of Tn*1696* from plasmid R1033 (GenBank accession number M64556), the efflux-mediated flouroquinolone resistance protein (*norA* gene) from *Staphylococcus aureus* (GenBank accession number M97169), the *E. coli* bicyclomycin resistance protein (*bicR* gene; GenBank accession number X63703), and the imipenem-resistant associated protein (*opdE* gene) of *Pseudomonas aeruginosa* (GenBank accession number Z14064). Clear similarity (48%) was also found to other membrane proteins such as the protein involved in the transport of 4-hydroxybenzoate (*pcaF* gene) from *Pseudomonas putida* (GenBank accession number U10895).

Tn*5467* had a region which when translated had 88% amino acid identity to 85% of the N-terminal region of the Tn*21* resolvase (*tnpR*). This TnpR-homologous region had no apparent start codon (ATG, GTG, CTG, or TTG), and homology with the Tn*21* resolvase was maintained only by permitting two translational frame shifts (Fig. 2). The TnpR-like region was fused in frame to a region with 78% amino acid identity to 15% of the C terminus of the Tn*21* transposase (*tnpA*). The ORF with homology to the Tn*21* transposase was interrupted by a stop codon with regions of high homology located on either side (Fig. 2).

Expression of polypeptides from Tn*5467* in vitro. An *E. coli*-derived in vitro transcription-translation system was used to identify polypeptides produced from Tn*5467* (Fig. 5). A comparison between the proteins produced by vector pBluescriptKS and pTTn1 (Table 1; Fig. 1) indicated that two polypeptides of approximately 33 and 9 kDa were synthesized from the region of pTF-FC2 encompassing Tn*5467*. The 33-kDa protein is the product of the *repC* gene (5), which is located outside of Tn*5467* and is essential for DNA replication. This protein was not synthesized from Tn*5467* subclones pTT100, pTT150, pTT250, and pTT350. The 9-kDa protein was produced by pTTn1, pTT150, pTT250, and pTT350 but not pTT100 and corresponds in size and location to the glutaredoxin-like product of ORF8. No polypeptides of the predicted sizes of MerR (15 kDa) or ORF43 (43 kDa) could be detected, even though subclones pTT100, pTT150, pTT250, and pTT350 were constructed so that transcription from the *lacZ* promoter of the vector was in the same direction as that of MerR and ORF43.

Activity of the glutaredoxin-like protein. *E. coli* BH5262 is a *trxA* (thioredoxin) *gshA* (glutathione synthase) mutant which is unable to grow on minimal medium unless supplemented with glutathione (12). Derivatives of BH5262 that are Gsh^+ or $TrxA^+$ do not require glutathione for growth. Plasmids pTTn2, pTT350, pTT250, and pTT150 were transformed into *E. coli* BH5262, and strong growth on minimal medium lacking glutathione but no growth following transformation with vector pBluescriptKS was observed. The ability of the ORF8 product to serve as a cofactor for *E. coli* methionine sulfoxide reductase was tested with the *E. coli* BH2012 *trxA metA* mutant. This methionine auxotroph is unable to grow on minimal medium plus methionine sulfoxide unless this compound can be reduced to methionine (12). Upon transformation of *E. coli* BH2012 with a pTTn2 derivative, pTT350 or pTT150, good growth was obtained on minimal medium plus methionine sulfoxide, but there was no growth on transformation with vector DNA. This indicated that the product of ORF8 is a redox-active protein which is able to confer a $TrxA^+$ phenotype.

Transposition and resolution of Tn*5467*. We wished to determine whether the IR sequences, *res*-like sites, and partial resolvase and transposase sequences were biologically active in *E. coli*. Resolution of cointegrates might take place as a result of either *res* site-resolvase activity or RecA-mediated recombination (7, 19). To eliminate resolution by recombination, transposition and mating were carried out with *E. coli recA* mutant strains. No transposition to plasmid R388 was detected in *E. coli* LK112(pTTn2, R388) as no Tc^r recipient strains were isolated following mating (Table 2). However, when the Tn*21* transposase (*tnpA*) and resolvase (*tnpR*) genes present on pUB2413 were provided in *trans*, transposition occurred in each of eight *E. coli* LK112(pTTn2, R388, pUB2413) cultures tested. Of the 54 transconjugant colonies examined, 36 were Tc^r Ap^s, indicating transposon resolution, while the remaining 18 colonies were Tc^r Ap^r. When experiments were carried out in the presence of the Tn*21* transposase but in the absence of a resolvase, transposition occurred in each of the 10 *E. coli* LK112(pTfTn2, R388, pUB2584) cultures examined, and all 104 transconjugants tested were Tc^r Ap^r. This indicated nonresolution of cointegrates.

TABLE 2. Results of mating experiments to determine transposase and resolvase activity

Strain mated with *E. coli* HB101	No. of parallel expts	No. of expts giving colonies on[a]:		No. of colonies tested for Ap^r	No. of Ap^r colonies
		TP + SM	TC + SM		
LK112(pTTn2, R388)	10	10	0		
LK112(pTTn2, R388, pUB2413)	8	8	8	54	18
LK112(pTTn2, R388, pUB2584)	10	10	10	104	104

[a] TP, trimethoprim; SM, streptomycin; TC, tetracycline.

Transposition and resolution were confirmed by restriction enzyme analysis of plasmids from Tc^r Ap^s and Tc^r Ap^r colonies. Plasmid R388 has three *Sst*II restriction sites, and there are no *Sst*II sites on either Tn*5467* or pTTn2. Transposition of Tn*5467* into R388 could clearly be seen by an increase in the size of one of the R388 *Sst*II fragments (Fig. 6A). Digestion with *Sal*I was used to indicate resolution of cointegrates. Plasmid pTTn2 is cleaved into three *Sal*I fragments, two of approximately 4.1 kbp and one of 0.8 kbp, while R388 has a single *Sal*I site. On resolution, the 4.1-kbp *Sal*I fragments of pTTn2 would be lost, while the 0.8-kbp fragment which is located within the IR sequences will be retained. In the case of unresolved cointegrates, the 4.1-kbp as well as the 0.8-kbp fragments would be retained. Upon digestion with *Sal*I, plasmids from Tc^r Ap^s and Tc^r Ap^r colonies gave restriction fragments which were predicted from resolved and unresolved cointegrates, respectively (Fig. 6B). This indicated that the IR sequences and the *res* sites of Tn*5467* were functional provided that the transposase and resolvase of Tn*21* were supplied in *trans*.

DISCUSSION

The sequence of Tn*5467* completes the sequence of the 12.2-kbp plasmid pTF-FC2. It has two 38-bp terminal IRs, one of which is identical and one of which has a single-base-pair mismatch to those of Tn*21*. It was therefore not surprising that Tn*5467* was able to transpose in the presence of the Tn*21* resolvase. Like Tn*21*, the truncated Tn*5467* *tnpR*- and *tnpA*-like genes are orientated in the same direction and are preceded by resolution (*res*) sites. The Tn*5467* *res* sites were clearly similar to the resolvase-binding sites of Tn*21* and Tn*1721* (Fig. 7), and Tn*5467* cointegrates were resolved when a functional *tnpR* gene from Tn*21* was provided in *trans*. These features identify Tn*5467* as a member of the Tn*21* subgroup of the Tn*3* family of transposons. The Tn*21* subgroup is known to be among the most successful and widespread of all transposons. The finding of a Tn*21*-like transposon in an obligately chemolithotrophic, highly acidophilic (optimum pH, 1.5 to 2.5) bacterium like *T. ferrooxidans* extends the range of environmental niches from which this subgroup of transposons has been isolated.

The genes most commonly found on transposons of the Tn*21* subgroup are those associated with antibiotic and heavy metal resistance (7). In the era before the use of antibiotics, the occurrence of antibiotic resistance genes on plasmids was rare (3, 9). However, following the widespread use of antibiotics, many plasmids have acquired antibiotic resistance genes, and these genes are usually present on transposons which have become inserted into the plasmids. Since *T. ferrooxidans* grows in an inorganic, acidic environment, it would not have been exposed to antibiotics or to heterotrophic bacteria from which such genes could easily be acquired by horizontal transfer. We were therefore particularly interested to discover what genes would be found on Tn*5467*.

The discovery of a gene which encodes a glutaredoxin-like protein on Tn*5467* which is able to functionally complement *E. coli trxA* mutants was unexpected. Proteins of this type have important biosynthetic functions, and the genes encoding these proteins are not normally found on plasmids. There has also been no previous report of a redox-active protein of this type being located within a transposon. *T. ferrooxidans* has proteins which carry out similar functions on its chromosome, and the *T. ferrooxidans trxA* gene which encodes for thioredoxin and which is located on the chromosome has been cloned and sequenced recently (13a). The reason why *T. ferrooxidans* might require a gene for a plasmid-located redox-active protein is unclear.

The occurrence on Tn*5467* of what appears to be two potential genes, *merR* and ORF43, is interesting. Both genes have

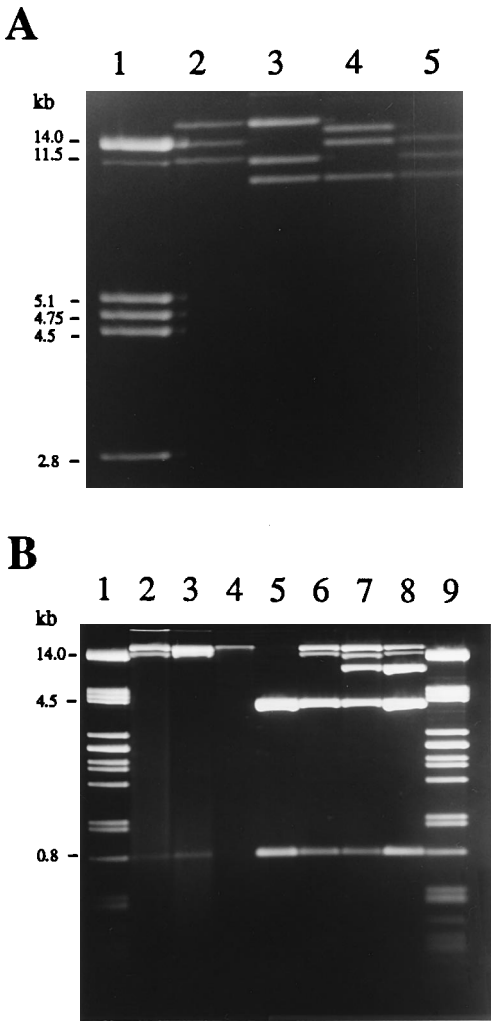


FIG. 6. Agarose gel analysis of transposition fusions between Tn*5467* and R388. (A) *Sst*II digests of R388 and R388::Tn*5467* fusions. Lanes: 1, λ *Pst*I molecular weight marker fragments; 2 to 4, independent R388::Tn*5467* fusions; 5, R388 control. (B) *Sal*I digests of resolved and unresolved R388::Tn*5467* fusion cointegrates. Lanes: 1 and 9, λ *Pst*I molecular weight markers; 2 and 3, resolved R388::Tn*5467* fusions; 4, R388 control; 5, pTTn2 control; 6 to 8, unresolved R388::Tn*5467* cointegrates.

```
SITE I    Tn5467      CAACCGTCATTTTGAGCTATACCCTATCTTGATGTCAG
          Tn21        CCGCCGTCAGGTTGAGGCATACCCTAACCTGATGTCAG
          Tn1721      CGCATGTCAATCTAGGCTATACCCTAACTTGATGTCAG
          consensus   C    GTCA   T  G  ATACCCTA C TGATGTCAG

SITE II   Tn5467      GAGACGTCAAGATAGGATTGCATTTGCAATTCATTGACACATT
          Tn21        ATTGCGTCAGGATAGGATTGAATTTTGAATTTATTGACATATC
          Tn1721      GCTTCGTCAGAATAGAGTCTGCTTTCCCATTTTTTGACACATG
          consensus       CGTCA  ATAG  T    TTT   ATT  TTGACA AT

SITE III  Tn5467      CAAACATCATAGATTCTTTCCTGACACATT
          Tn21        TTGAAGTCATAGAGTCTTCCCTGACATTTT
          Tn1721      CGAAGGTTATAGATTTCAGCCTGACAGAAA
          consensus     A  T ATAGA T    CCTGACA
```

FIG. 7. Sequence homology of Tn*5467* with the three resolvase binding sites of the *res* region of Tn*21* and Tn*1721* (16).

clearly identifiable ribosome binding sites and a codon-preference and third-base-pair position bias that suggest that they should be protein encoding. Although no protein product was detected for either potential gene in an *E. coli* in vitro system, it is possible that the genes may be expressed in *T. ferrooxidans*. The reason for the presence of a gene for a MerR-like regulator on Tn*5467* even though there is no *merA* gene to be regulated is uncertain. A similar situation exists in the case of the *ydhM* ORF of *E. coli* (2). The amino acid sequence of the *ydhM* ORF is clearly homologous to the sequence of MerR proteins (Fig. 4), but the adjacent ORFs have no similarity to any of the proteins usually associated with mercury resistance. Several *T. ferrooxidans* strains have been shown to have a *merA* gene on the chromosome (20), and one strain has been reported to have a *merC* and *merR* gene duplication (10). If expressed, it is possible that the *merR*-like gene could be involved in the regulation of a chromosomal *T. ferrooxidans mer* operon. A function for the potential ORF43 product is even more difficult to predict. The amino acid sequence has structural features which are clearly similar to those of a large family of membrane translocases, but the ORF43 product is approximately equally similar to all members of the family.

Plasmids based on the pTF-FC2 replicon have been found to be stably inherited by a number of host organisms. For example, Herrera et al. (8) reported that the pTF-FC2 derivative pDER405 was present in approximately 100% of *Pseudomonas flourescens* cells even after 100 generations in the absence of plasmid selection. Plasmid pTF-FC2 has been sequenced completely, but no features which are known in other plasmids to contribute to stable inheritance have been identified. We wondered whether the presence of the *res* region of Tn*5467* might permit multimer resolution which could contribute to plasmid stability in the same way as the multimer resolution system of plasmid RP4 (6). The finding that the *res* site-resolvase system of Tn*5467* was not functional unless a Tn*21* resolvase was provided in *trans* indicates that a function of Tn*5467* on pTF-FC2 is probably not to provide the plasmid with a multimer resolution system.

Several plasmids from different *T. ferrooxidans* strains have been isolated, but all *T. ferrooxidans* plasmids that have been investigated to date have been reported to be cryptic (14). The discovery of a functional gene for a glutaredoxin-like protein on Tn*5467* means that pTF-FC2 is the first *T. ferrooxidans* plasmid for which a function that is not associated with plasmid replication or mobilization has been reported.

ACKNOWLEDGMENTS

We are most grateful to J. Grinsted and F. de la Cruz for providing plasmids R388 and pUB2413 and to J. Fuchs for *E. coli* BH2012 and BH5262. We are also grateful to Ros Powles for help in testing the activity of the product of ORF8.

This work was supported by grants from the Foundation for Research Development and Gencor Process Research, Randburg, South Africa.

REFERENCES

1. **Avila, P., F. de la Cruz, E. Ward, and J. Grinsted.** 1984. Plasmids containing one inverted repeat of Tn*21* can fuse with other plasmids in the presence of Tn*21* transposase. Mol. Gen. Genet. **195:**288–293.
2. **Christie, G. E., T. J. White, and T. Goodwin.** 1994. A *merR* homologue at 74 min on the *Escherichia coli* genome. Gene **146:**131–132.
3. **Datta, N., and R. W. Hedges.** 1972. Trimethoprim resistance conferred by plasmids in *Enterobacteriaceae*. J. Gen. Microbiol. **72:**1141–1156.
4. **de la Cruz, F., and J. Grinsted.** 1982. Genetic and molecular characterization of Tn*21*, a multiple resistance transposon from R100.1. J. Bacteriol. **151:** 222–228.
5. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimal replicon of a broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
6. **Eberl, L., C. S. Kristensen, M. Givskov, E. Grohmann, M. Gerlitz, and H. Schwab.** 1994. Analysis of the multimer resolution system encoded by the *parCBA* operon of broad-host-range plasmid RP4. Mol. Microbiol. **12:**131–141.
7. **Grinsted, J., F. de la Cruz, and R. Schmidtt.** 1990. The Tn*21* subgroup of bacterial transposable elements. Plasmid **24:**163–189.
8. **Herrera, G., S. J. Snyman, and J. A. Thomson.** 1994. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugar cane borer, *Eldana saccharina*. Appl. Environ. Microbiol. **60:**682–690.
9. **Hughes, V. M., and N. Datta.** 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. Nature (London) **302:**725–726.
10. **Inoue, C., K. Sugawara, and T. Kusano.** 1991. The *merR* regulatory gene in *Thiobacillus ferrooxidans* is spaced apart from the *mer* structural genes. Mol. Microbiol. **5:**2707–2718.
11. **Lewis, K.** 1994. Multidrug resistance pumps in bacteria: variations on a theme. Trends Biol. Sci. **19:**119–123.
12. **Lim, C.-J., F. K. Gleason, and J. A. Fuchs.** 1986. Cloning, expression, and characterization of the *Anabaena* thioredoxin gene in *Escherichia coli*. J. Bacteriol. **168:**1258–1264.
13. **McFarlan, S. C., C. A. Terrell, and H. P. C. Hogenkamp.** 1992. The purification, characterization, and primary structure of a small redox protein from *Methanobacterium thermoautotrophicum*, an archaebacterium. J. Biol. Chem. **267:**10561–10569.

13a. **Powles, R., et al.** Unpublished data.

14. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:**39–55.
15. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **158:**737–738.
16. **Rogowsky, P., S. E. Halford, and R. Schmitt.** 1985. Definition of three resolvase binding sites at the *res* loci of Tn*21* and Tn*1721*. EMBO J. **4:**2135–2141.
17. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of the broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**6230–6237.
18. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Sherrat, D.** 1989. Tn*3* and related transposable elements: site-specific recombination and transposition, p. 163–184. *In* D. E. Berg and M. M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
20. **Shiratori, T., C. Inoue, K. Sugawara, T. Kusano, and Y. Kitagawa.** 1989. Cloning and expression of *Thiobacillus ferrooxidans* mercury ion resistance genes in *Escherichia coli*. J. Bacteriol. **171:**3458–3464.
21. **Silver, S., and M. Walderhaug.** 1992. Gene regulation of plasmid and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. **56:**195–228.

Molecular Microbiology (1997) **26**(5), 961–970

The poison–antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2

Anthony S. G. Smith and Douglas E. Rawlings*
Department of Microbiology, University of Cape Town, Rondebosch, 7700, South Africa.

Summary

In plasmid pTF-FC2, three small open reading frames (ORFs) are situated between the *repB* (primase) gene and the *repA* (helicase) gene of its IncQ-type replicon. Disruption of each of the three ORFs followed by tests for plasmid stability and host cell growth indicated that the ORFs encoded a poison–antidote plasmid stability system. The three genes were named *pasA*, *pasB* and *pasC* (plasmid addiction system), in which PasA is the antidote, PasB the toxin and PasC a protein that appears to enhance the ability of the antidote to neutralize the toxin. Disruption of the *pasA* gene resulted in two different spontaneous deletions, which inactivated the stability system but did not alter the host range or plasmid copy number. This indicated that the three small ORFs were not involved in plasmid replication. When placed behind a *tac* promoter, induction of *pasB* was found to be highly lethal to host cells, which suggests that the Pas system acts by killing plasmid-free host cells rather than by retarding the growth of plasmid-free segregants, as occurs in the ParD system of R1. In spite of this, the presence of the Pas poison–antidote system resulted in a relatively modest threefold stabilization of the pTF-FC2 host replicon and a similar increase in the stabilization of an unstable heterologous R1 plasmid replicon. The Pas system is a poison–antidote plasmid stability module, which appears to have become integrated within the pTF-FC2 replicon module.

Introduction

Thiobacillus ferrooxidans strain FC1 is a member of a consortium of bacteria that formed the inoculum of a bio-oxidation plant used for the pretreatment of gold-bearing arsenopyrite ores at the Fairview mine, South Africa (Rawlings and Silver, 1995). Plasmid pTF-FC2 is a 12.2 kb plasmid and was one of three plasmids resident in strain FC1 (Rawlings *et al.*, 1983). The nucleotide sequence of the whole of pTF-FC2 has been determined (GenBank accession numbers M64981 and M35249). Three regions of the plasmid have been identified: a replicon, a mobilization region and a transposon.

The pTF-FC2 replicon clearly resembles that of the IncQ plasmids, RSF1010, R300B and R1162 (Dorrington and Rawlings, 1990). Like the IncQ plasmids, pTF-FC2 has a broad host range and has been found to replicate in a large number of Gram-negative bacteria (Rawlings and Kusano, 1994). Nevertheless, plasmid pTF-FC2 is compatible with the IncQ plasmids in *Escherichia coli*, and the IncQ plasmid R300B was unable to complement *repA*, *repB* or *repC* mutants of pTF-FC2. The mobilization region most closely resembles the Tra1 region of the IncPα plasmids, RP4 or RK2, and the IncPβ plasmid, R751 (Pansegrau *et al.*, 1994), and pTF-FC2 is very efficiently mobilized by either type of IncP plasmid (Rohrer and Rawlings, 1992). A 3.5 kb transposon, Tn*5467*, which has 38 bp terminal repeat sequences that are identical to those of Tn*21* is present on pTF-FC2 (Clennel *et al.*, 1995). Tn*5467* is inactive but is capable of transposition and resolution if the Tn*21* transposase and resolvase genes are provided *in trans*. The transposon encodes for a functional glutaredoxin-like protein and has two ORFs, which resemble those of multidrug resistance efflux proteins and transcriptional regulators (Clennel *et al.*, 1995).

Plasmid pTF-FC2 has been reported to be remarkably stable in the absence of selection (Herrera *et al.*, 1994), in spite of the lack of an obvious stability mechanism. A number of plasmid-encoded toxin–antidote systems exist, which counteract the loss of plasmids by killing plasmid-free segregants selectively (Jensen and Gerdes, 1995). Phenotypically, this phenomenon leads to plasmid stability. Examples of poison–antidote systems are the sense–antisense *hok/sok* system of plasmid R1 (Gerdes *et al.*, 1990) and the proteic killer systems, such as the *ccd* system of F (Ogura and Hiraga, 1983), the *parD/pem* system of R1/R100 (Bravo *et al.*, 1987; Tsuchimoto *et al.*, 1988), the *parDE* system of RK2 (Roberts *et al.*, 1994) and the *phd/doc* system of prophage P1 (Lehnherr *et al.*, 1993).

We wished to investigate the function of the three small open reading frames (ORFs), which are located between the *repB* and *repA* genes of pTF-FC2 (Fig. 1). The small size and lack of clear homology to any known genes suggested that these ORFs served either a regulatory or some other non-enzymatic role. Furthermore, the two

Received 16 April, 1997; revised 1 September, 1997; accepted 11 September, 1997. *For correspondence. E-mail doug@molbiol.uct.ac.za; Tel. (21) 6503261; Fax (21) 6897573.

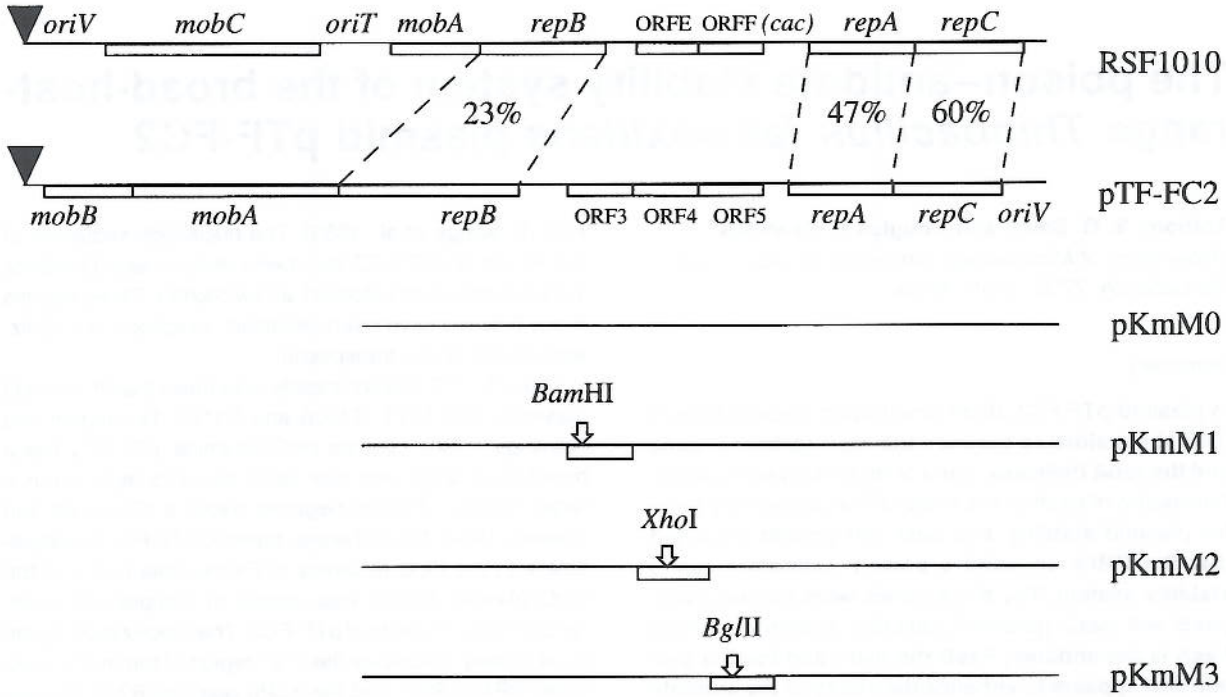


Fig. 1. A comparison of the IncQ plasmid RSF1010 and pTF-FC2, which has been simplified by excluding the transposon present in each plasmid, with the position of each transposon indicated by an inverted triangle. Broken lines between plasmids indicate similar genes, with the amino acid identity of the gene products shown as a percentage. The region of pTF-FC2 used to construct pKmM0 and the positions of the three frameshift mutations introduced into ORFs 3, 4 and 5 are shown below the map of pTF-FC2.

genes that are located in the equivalent position in the case of the IncQ plasmid, RSF1010, encode two small proteins (E and F), which have been implicated in the regulation of plasmid replication (Maeser *et al.*, 1990). In this work, we show that the products of ORFs 3, 4 and 5 serve as a proteic plasmid stabilization system and do not appear to be involved in the control of pTF-FC2 replication.

Results

Mutagenesis of ORFs 3,4 and 5 of pTF-FC2

In order to determine their function, site-directed mutagenesis was used to introduce a frameshift mutation into each of ORFs 3, 4 and 5. An unmutated replicon and each of the mutant replicons was excised from the pMa mutagenesis vector and ligated to a kanamycin resistance marker from Tn*5* to produce plasmids pKmM0 (unmutated), pKmM1 (ORF3 mutant), pKmM2 (ORF4 mutant) and pKmM3 (ORF5 mutant), as indicated in Fig. 1. The effect of the mutations in ORFs 3, 4 and 5 on the pTF-FC2 replicon was tested by transforming each construct into *E. coli* JM105 followed by determination of the plasmid copy number. Plasmids pKmM0, pKmM2 and pKmM3 were found to have a copy number of 10–14 per genome, which is similar to that for pTF-FC2 (Dorrington and Rawlings, 1990). However, the copy number of pKmM1 was reduced to one, and the host bacterium grew poorly on medium containing kanamycin. In order to test whether plasmid host range had been affected, the unmutated replicon and mutants were transformed into the *E. coli polA* mutant GW125a and *Pseudomonas putida*. Only plasmids pKmM0, pKmM2 and pKmM3 were able replicate. The region containing ORF3 was cloned into the broad-host-range vector pSUP106 to create pORF3 and, when this construct was present in *E. coli polA* mutant or *P. putida* cells, the ability of co-resident pKmM1 to replicate was restored, whereas co-resident pSUP106 vector controls were negative. Our initial assumption was that the frameshift mutation in ORF3 had produced a regulatory imbalance, which had resulted in a reduction in copy number and host range.

However, when pKmM1 was introduced by itself into either *P. putida* or the *E. coli polA* mutant, a small number of spontaneous mutants arose that were able to replicate in these hosts. Plasmids were extracted from 36 of these mutants and analysed by restriction enzyme mapping. This revealed that two types of plasmid deletion, which allowed the mutants to regain the ability to replicate in *P. putida* or *E. coli polA* mutants, had occurred. A representative of each type of deletion mutant was selected and

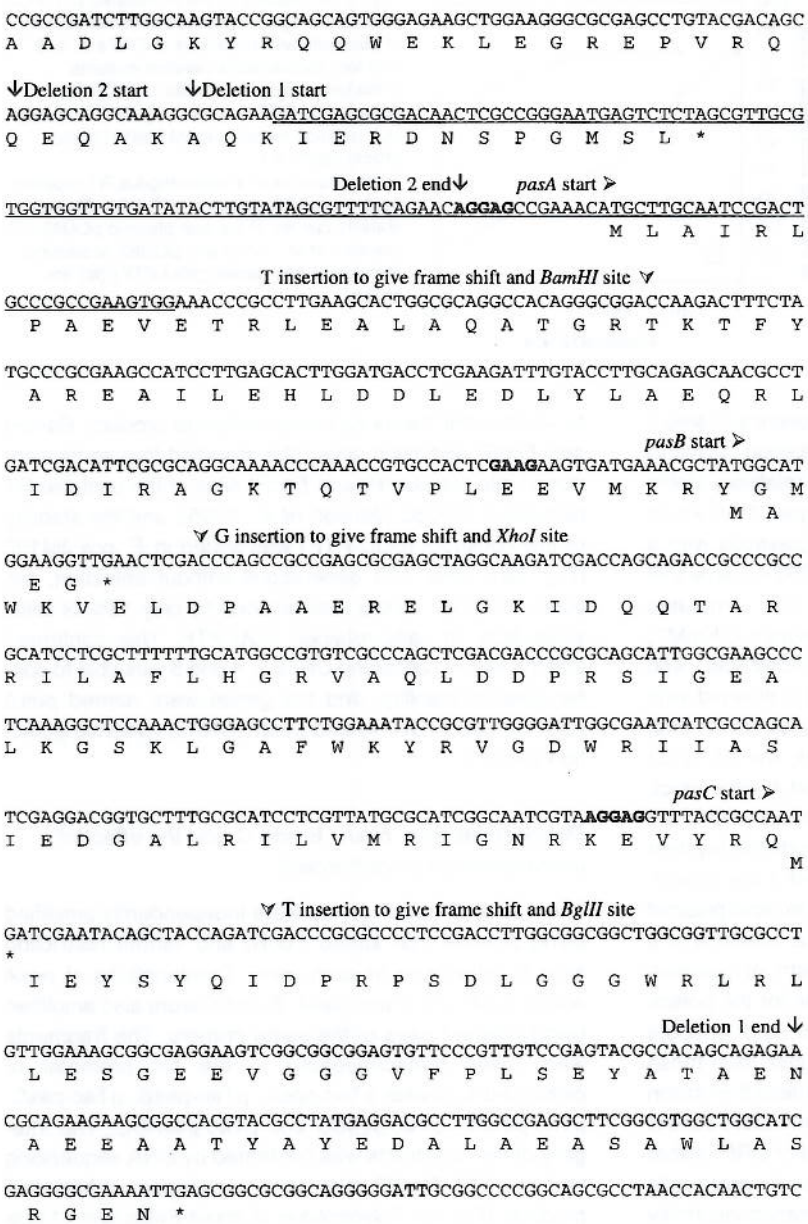


Fig. 2. Nucleotide sequence of the region of pTF-FC2 encoding ORFs 3, 4, 5 as well as the immediate flanking regions. The genes for each ORF have been renamed *pasA*, *B* and *C*, respectively, and the amino acid sequence of each gene product is indicated below the nucleotide sequence. Sites of insertion of bases into each gene, T insertion into *pasA* (creating pKmM1), G insertion into *pasB* (creating pKmM2) and T insertion into *pasC* (creating pKmM3), are shown above the sequence. The end positions of the spontaneous deletions 1 and 2 are indicated by vertical arrows (↓), and the repeated sequences that flank each spontaneous deletion are boxed. Potential ribosome-binding sites for each gene are indicated in bold. The sequence above overlaps two sequences that have been deposited in the GenBank database and extends from nt 1511 (M64981; Dorrington *et al.*, 1991) to nt 835 (M35249; Dorrington and Rawlings, 1990).

sequenced. One type of deletion mutant (pKmM1del1) had a 711 bp deletion bordered by a 9 bp repeated sequence (5′-CGCAGAAGA-3′), and the deletion had removed all of the DNA encoding ORFs 3 and 4 as well as the first 134 bp of ORF5 (Fig. 2). The other type of mutant (pKmM1del2) had a 104 bp deletion, which was bordered by a different 7 bp repeat sequence (5′-CAGGAGC-3′), and this deletion had removed 44 bp from the 5′ end of RepB and the region upstream of the start codon of ORF3. Both the deletion mutants had copy numbers that were indistinguishable from the unmutated replicon. As the deletion of the region upstream of the start codon of ORF3 had the same effect as a deletion of ORFs 3, 4 and part of 5, it is probable that this region contains a promoter responsible for the transcription of the three ORFs.

The affect of mutations in ORFs 3, 4 and 5 on plasmid stability

The observation that the copy number and host range of the deletion plasmids was the same as for the unmutated plasmid indicated that ORFs 3, 4 and 5 were not essential for plasmid replication nor involved in the regulation of pTF-FC2 replication. The stability of each of the mutants

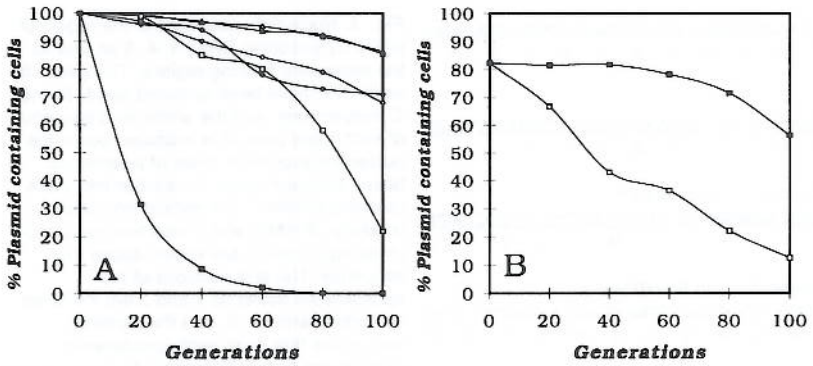


Fig. 3. A. Stability of the unmutated pTF-FC2 replication region compared with the stability of plasmids with mutations in ORFs 3, 4, 5 and two spontaneous deletion mutants. Unmutated plasmid pKmM0 (△); ORF3 mutant (■); ORF4 mutant (◇); ORF5 mutant (▲); deletion mutant pKmM1del1 (□) and pKmM1del2 (◆).
B. Stabilization of a heterologous R1 replicon by the *pasABC* genes of pTF-FC2. Plasmid stability curves of the test plasmid pOU82 (□) (Jensen *et al.*, 1995) and pOU82 containing the cloned *pas* genes (pOU-PTF) (■) are shown.

in *E. coli* JM105 was then tested. In the absence of selection, plasmids containing the unmutated replicon (pKmM0) and the mutation in ORF5 (pKmM3) were relatively stable and were maintained at rates that are higher than would be predicted by random segregation of plasmids with a copy number of 10–14 (Fig. 3A). The ORF4 frameshift (pKmM2) mutant was less stable than the unmutated plasmid, whereas the ORF3 frameshift mutant (pKmM1) was highly unstable. Deletion mutants, pKmM1del1 and pKmM1del2, were both unstable, with the plasmid with the smaller deletion (pKmM1del2) being less stable than the plasmid with the larger deletion. Thus, the pTF-FC2 replicon was stable only when ORFs 3 and 4 were intact. The results could be explained if the products of ORFs 3 and 4 were a poison–antidote plasmid addiction system with ORF3 encoding the antidote and ORF4 the poison. A mutation in ORF3 (pKmM1) would result in rapid plasmid loss because production of the toxin in the absence of a functional antidote would cause the death of plasmid-containing cells. Plasmids lacking the gene for the poison (pKmM2) or the genes for the poison and antidote (pKmM1del1) would be lost at a rate higher than those with an intact stability mechanism. The frameshift mutation in ORF5 (pKmM3) was as stable as the unmutated control (Fig. 3A). Based on this result, the product of ORF5 either did not play a role in plasmid stability or its role was subtle (confirmed later). The reason for the increased instability of pKmM1del1 above that of pKmM1del2 is more difficult to explain, and this will be addressed in the *Discussion*.

Stabilization of a heterologous replicon by the region encoding ORFs 3, 4 and 5

To confirm that the region encoding ORFs 3, 4 and 5 functions as a poison–antidote plasmid stability system, the ability of this region to stabilize a heterologous R1 plasmid replicon was investigated. The region of DNA from 174 bp upstream of the ORF3 start to 23 bp downstream of the stop of ORF5 (Fig. 2) was amplified by the polymerase chain reaction (PCR) using specific oligonucleotide primers to which extra bases had been added to produce *Bam*HI and *Eco*RI restriction sites. The amplified fragments were cloned into the *Bam*HI and *Eco*RI sites of the unstable R1 derivative, pOU82 (Jensen *et al.*, 1995), and the stability of this construct (pOU-PTF) was tested in *E. coli* JM105 (Fig. 3B). After 100 generations without selection, the plasmid pOU82 control was retained by only 13% of cells, while 56% of cells retained pOU-PTF. This confirmed that the region containing ORFs 3, 4 and 5 was able to confer plasmid stability, and the genes were named *pasA* (ORF3), *pasB* (ORF4) and *pasC* (ORF5) (plasmid addiction system).

Polypeptides from pasA, B *and* C *and the effect of overexpression on cell growth*

The *pas A*, *B* and *C* genes were independently amplified using primers that added *Eco*RI and *Hin*dIII restriction sites to either end of each gene. Combinations of *pasA* and *B*, *pasB* and *C* and *pasA*, *B* and *C* were also amplified using different pairs of the same primers. The fragments were fused transcriptionally to the *tac* promoter of pKK223-3 to create pTac-pasA, pTac-pasB, pTac-pasC, pTac-pasAB, pTac-pasBC and pTac-pasABC. The integrity of the constructs was confirmed by DNA sequencing and by SDS–PAGE of *in vitro* transcription–translation products (Fig. 4). Polypeptides of about 8 kDa and 11 kDa were produced from the pTac-pasA and pTac-pasC constructs respectively (Fig. 4A, lanes 2 and 4), but no protein band of the size predicted for PasB was observed in lane 3 despite *pasB* being cloned downstream of the *tac* promoter. The toxin protein of poison–antidote systems is synthesized in small amounts relative to the antidote (Roberts and Helinski, 1992; Tsuchimoto *et al.*, 1992; Lehnherr *et al.*, 1993), presumably because the protein is poorly translated. The start of the PasB ORF overlaps the end of the PasA ORF, and the ribosome-binding site of *pasB* is less homologous to a consensus site and further from the AUG start than is usual for highly expressed genes. When combinations of PasA, B and C were expressed

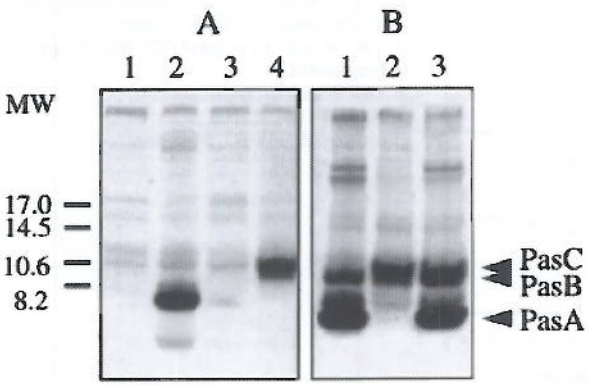


Fig. 4. Autoradiograph of an SDS–PAGE analysis of polypeptides produced from *pasABC* genes under the control of a *tac* promoter in an *E. coli*-derived *in vitro* transcription–translation system.
A. Lane 1, pKK223-3 control; lane 2, pTac-*pasA*; lane 3, pTac-*pasB*; lane 4, pTac-*pasC*.
B. Lane 1, pTac-*pasAB*; lane 2, pTac-*pasBC*; lane 3, pTac-*pasABC*.

from the vector *tac* promoter, a protein band of approximately 10.5 kDa was observed in addition to the 8 kDa band of PasA in the pTac-pasAB construct (Fig. 4B, lane 1). The 10.5 kDa band was also visible in the expression products of pTac-*pasABC* (Fig. 4B, lane 3) but not in those of pTac-pasBC (Fig. 4B, lane 2) in which the shadow band is similar to that produced from *pasC* (Fig. 4A, lane 4). It appears that translation of *pasA* is required for the efficient production of PasB. Polypeptides of higher molecular weight (25–27 kDa) were detected in the pTac-pasAB (Fig. 4B, lane 1) and pTac-pasABC (Fig. 4B, lane 3) constructs. These proteins could possibly represent PasA–PasB fusions caused by frameshift translational readthrough or covalently linked PasA–PasB complexes, as they were only detected in constructs in which PasA and PasB were both present (and PasB was not detectable when expressed alone).

On induction of the *tac* promoter with IPTG, growth was inhibited in *E. coli* cells containing pTac-pasB (Fig. 5B) but not in cells containing pTac-pasA (Fig. 5A) or pTac-*pasC* (Fig. 5C). This indicated that, in spite of the inability to detect PasB expression from pTac-pasB *in vitro*, on IPTG induction sufficient PasB was produced *in vivo* to inhibit the growth of pTac-pasB-containing *E. coli* cells. When pTac-pasAB was present, the toxic effect of the *pasB* gene product was moderated but not completely removed by the presence of *pasA*, while the presence of *pasC* (pTac-pasBC) had no affect on reducing toxicity (Fig. 5D). When all three genes (pTac-pasABC) were present, the toxic effect of *parB* was fully reduced to the same

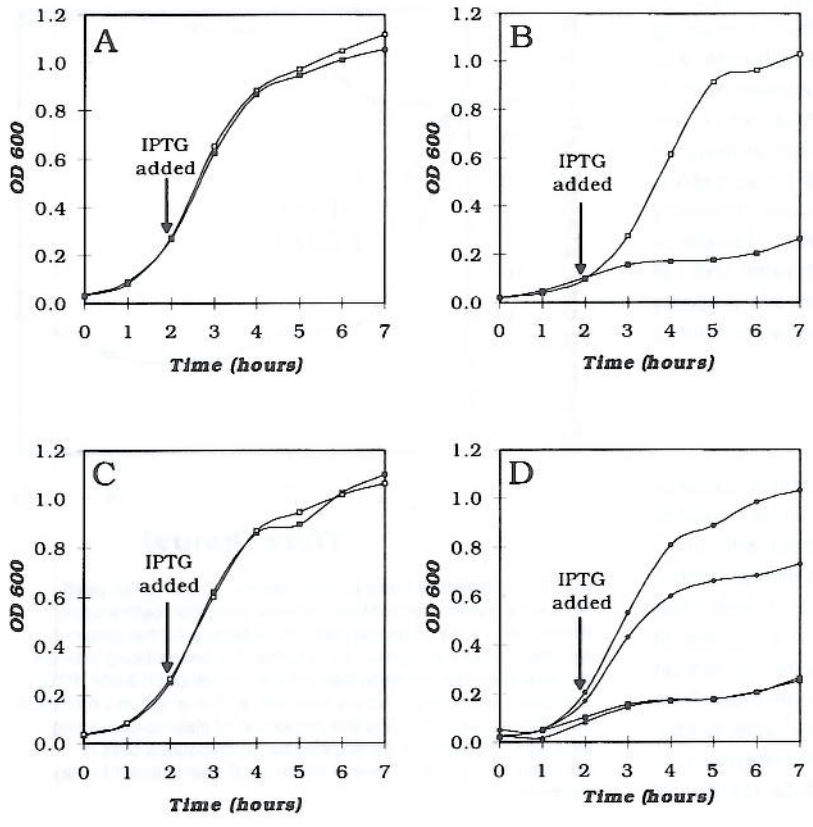


Fig. 5. Growth curves of *E. coli* cells containing one or more of the *pas* genes under the control of a *tac* promoter. Uninduced cells are indicated by curves marked (□) and induced cells are marked (■).
A. *E. coli* (pTac-pasA).
B. *E. coli* (pTac-pasB).
C. *E. coli* (pTac-pasC).
D. (▲) induced *E. coli* (pTac-pasB); (◆) induced *E. coli* (pTac-pasAB); (■) induced *E. coli* (pTac-pasBC) and (●) induced *E. coli* (pTac-pasABC) cells. Plasmid-containing *E. coli* JM105 cells were grown overnight with plasmid selection before being diluted 1:100 in prewarmed broth with plasmid selection. After 2 h growth, IPTG was added to 0.5 mM and OD_{600} measurements were made at hourly intervals.

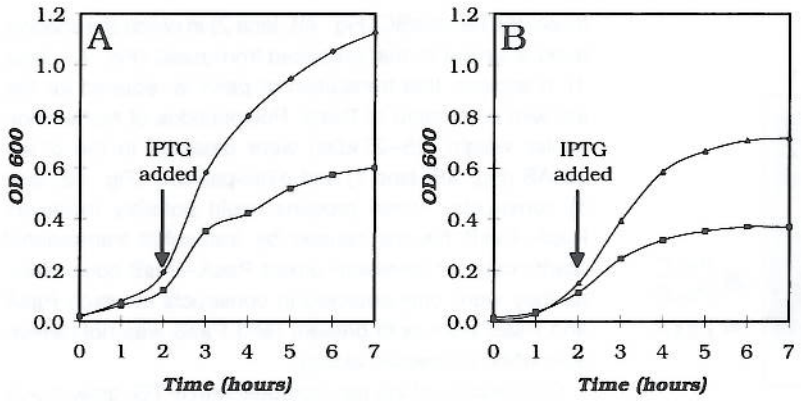


Fig. 6. The effect of *pasC* on the growth rate of *E. coli* cells expressing *pasAB*.
A. (♦), *E. coli* (pTac-pasABC); (■), *E. coli* (pTac-pasABC*).
B. (▲), *E. coli* (pTac-pasAB-pACYC, pKK223-3); (■), *E. coli* (pTac-pasAB-pACYC, pTac-pasC).

level as uninduced cells (Fig. 5D). From these results, it appeared that PasA was an antidote, PasB a toxin and that PasC enhanced the ability of ParA to neutralize the toxic effect of ParB.

The effect of PasC on cell growth

To explore further the role of PasC, the *pasABC* genes were amplified from the *pasC* mutant plasmid, pKmM3, and cloned into the *Eco*RI and *Hin*dIII sites pKK223-3 to produce pTac-pasABC*. This construct is isogenic to pTac-pasABC except that it contains a T inserted into *pasC* causing a frameshift and inactivation of *pasC*. On induction with IPTG, *E. coli* (pTac-pacABC*) containing an inactivated *pasC* had a reduced growth rate and reached a lower log-phase cell density compared with *E. coli* (pTac-pasABC) cells (Fig. 6A). A similar result was obtained when the *pasC* gene was supplied *in trans* on a co-resident compatible plasmid. *E. coli* (pTac-pasAB) cells grew more slowly and reached a lower stationary phase cell density than *E. coli* (pTac-pasAB, pTac-pasC-pACYC) cells (Fig. 6B). The lower growth rates and cell densities achieved in this latter experiment are probably due to the presence of two plasmids and the two antibiotics required to select for the plasmids.

PasB – bacteriostatic or bacteriocidal?

To determine whether PasB was bacteriocidal or bacteriostatic, the construct pTac-pasB was grown in the presence of antibiotic selection either with or without with IPTG induction, and the effect of PasB induction on the number of plasmid-containing cells was observed. If PasB was bacteriostatic, on the induction of *pasB*, the number of plasmid-containing cells would be expected to remain constant over time as cells would be prevented from dividing but would not be killed. If PasB was bacteriocidal, there would be a decrease in plasmid-containing cells on *pasB* induction. In induced cultures, the total number of plasmid-containing cells fell rapidly by approximately a million-fold within 3 h owing to cell death caused by PasB (Fig. 7). No noticeable toxic effect was observed in cells in which *parB* was not induced, and the number of plasmid-containing cells increased about 50-fold within 3 h.

Discussion

Unlike the proteic stability systems of other plasmids, which consist of two components (a poison and an antidote), the

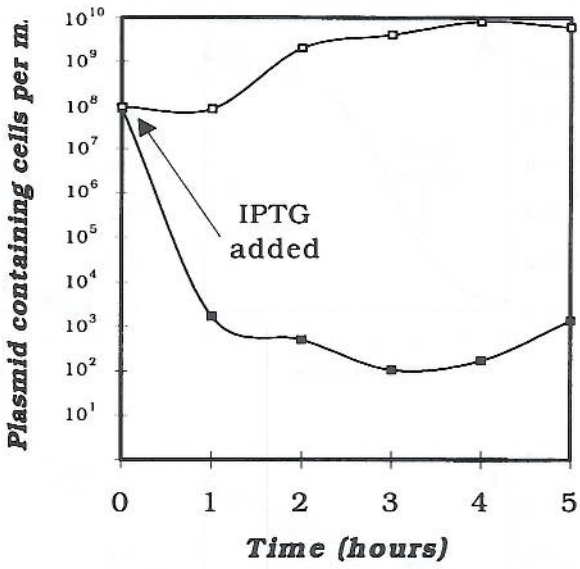


Fig. 7. The effect of PasB on cell viability. *E. coli* (pTac-pasB) cells were grown overnight with plasmid selection before being diluted into 100 ml of prewarmed LB containing further plasmid selection. Cells were grown for a further 2 h before being washed and resuspended in prewarmed LB with or without 0.5 mM IPTG. Samples were taken at hourly intervals and counted on LA and LA plus Ap (100 μg ml^{-1}), and the proportion of plasmid-containing cells was calculated. Plasmid retention in uninduced cells is indicated by (□) and in cells in which *parB* was induced by (■) symbols.

stability system of pTF-FC2 appears to have three components. Proteins of this plasmid addiction system have been named PasA, PasB and PasC. Site-directed mutagenesis of each ORF as well as the controlled expression of each protein from the regulated *tac* promoter clearly identified PasA as the antitoxin and PasB as the toxin. PasC appeared to enhance the ability of PasA to neutralize the PasB toxin (Figs 5D, 6A and B). This reduction in PasAB toxicity by PasC did not result in an increase in plasmid stability (Fig. 3A) over the 100 generations tested. The *pas* toxin–antidote system appears to be equally effective at stabilizing a plasmid in the presence or absence of PasC. However, since the presence of PasC reduced PasAB toxicity, *pasABC*-containing cells would be expected to outgrow cells that contained *pasAB* alone.

Amino acid sequences between different poison–antidote systems are generally poorly conserved. However, limited sequence homology has been detected between the Ccd antitoxin of plasmid F and the ParD antitoxin of plasmid R1 (Ruiz-Echevarrìa *et al.*, 1991). Alignment of the predicted amino acid sequences of the three Pas proteins with other plasmid proteic systems was carried out, and no clear homology was detected with either the PasB toxin or the PasC protein. However, a weak but clear similarity (31% amino acid sequence identity) was detected between the PasA antitoxin of pTF-FC2 and the ParD antitoxin of the RK2 RP4 plasmids (Fig. 8).

Frameshift mutations have an advantage over point mutations in that they are more certain to inactivate the protein into which they are introduced, but they have the disadvantage that some frameshift mutations are polar. The construct containing a frameshift mutation in *parA* was highly toxic and, therefore, the mutation did not reduce the expression of *parB* completely. In fact, the mutation in the gene for the antitoxin followed by its transformation into *E. coli* and selection for the plasmid antibiotic resistance marker resulted in a strong selection pressure for plasmids in which the toxin system had been eliminated. Two types of deletion mutant were isolated. In one type of mutant, the region upstream of the *pasA* gene was removed and, in the other type, *pasA*, *pasB* and about half of the *pasC* gene had been deleted. Interestingly, the deletions occurred between two different pairs of directly repeated nucleotide sequences. The smaller deletion occurred within the first two nucleotides of the presumptive ribosome-binding site (AGGAG) of *pasA* in such a way that the complete ribosome-binding site would have been reconstituted by the deletion. The reason that this mutation inactivated the *pas* system was most probably because of the deletion of one or more elements of the *pas* promoter. The reason why the plasmid with the smaller spontaneous deletion was more unstable than the larger deletion is difficult to explain. A possible explanation is that the deletion removing the presumptive promoter also removes the 19 C-terminal amino acids from RepB. The removal of the stop codon is predicted to result in a 6.5 kDa increase in the size for RepB, and this has been observed for *in vitro* transcription–translation products of pKmM1del1 (data not shown). Readthrough from the promoter of *repB* would result in transcription of *pasABC*. However, as the antitoxin has been inactivated as a result of site-directed mutagenesis, translation of the *pasB* in the absence of a functional *pasA* would have a toxic effect. The level of toxicity is lower than that seen for the pKmM1 construct as it is driven by the *repB* promoter, which is weaker than that of *pasABC* (data not shown).

```
PasA    M..LAIRLPAEVETRLEALAQATGRTKTFYAREAILEHLD..DLEDL
           : :          : :::   :.:   ::    :: :   :  :
ParD    MSRLTIDMTDQQHQSLKALAALQGKTIKQYA....LERLFPGDA.DA

PasA    ........YLAEQRLIDIRAGKTQTVPLEEVMKRY..GMEG     74
                .:. :  :::  :  . :.      :
ParD    DQAWQELKTMLGNRINDGLAGKVSTKSVGEILDEELSGDRA     83
```

Fig. 8. Alignment of the amino acid sequences of ParD of RK2 and PasA of pTF-FC2. Identical amino acids and conservative amino acid changes are indicated between the alignments by double and single dots respectively.

Proteic killer gene systems fall into two categories with respect to their lethality to the host cell. The *parD/pem* systems of plasmids R1/R100 are bacteriostatic and inhibit cell division rather than kill plasmid-free segregants. Inhibition of cell division is thought to place plasmid-free cells at a selective growth disadvantage, which results in a modest plasmid stabilization effect of about fivefold (Jensen *et al.*, 1995). In contrast, the ParDE proteic killer system of RK2/RP4 and the Phd/Doc system of prophage P1 are bacteriocidal, and the ParDE system results in a more efficient stabilization system than the bacteriostatic system, of about 100-fold (Jensen *et al.*, 1995). The PasABC system of pTF-FC2 falls into the bacteriocidal category, although plasmid stabilization based on a calculation of the loss frequency (Gerdes *et al.*, 1985) was increased by only approximately threefold in the case of pOU82 and by 2.5-fold in the case of pTF-FC2 in rich medium in *E. coli* JM105. The *pasABC* system may be more or less effective in the original *T. ferrooxidans* host, but it is not possible to carry out stability tests of this type in this difficult-to-grow obligately autotrophic and highly acidophilic chemolithotroph.

The observation that replication and copy number of the deletion mutants in *P. putida* and *E. coli* were unaffected indicated that, even though the *pas* genes are positioned between the *repB* and *repAC* genes, they are not involved in plasmid replication. It has been suggested that plasmids are naturally constructed from functional modules. Replication, conjugation and partition/stability modules appear to have been acquired by plasmids independently of each other. In the case of pTF-FC2, the module for plasmid stability appears to have become inserted between the *rep*

genes in such a manner that its insertion has not interfered with plasmid replication.

Experimental procedures

Bacterial strains, plasmids and media

Bacterial strains and plasmids used are shown in Tables 1 and 2. *E. coli* strains were grown on Luria broth (LB), Luria agar (LA) plates (Sambrook *et al.*, 1989) or on terrific broth (TB) containing 24 g of yeast extract, 12 g of tryptone and 4 ml of glycerol per 900 ml with 100 ml of sterile 0.17 M KH_2PO_4/0.72 M K_2HPO_4 added immediately before use. Antibiotics were added to the media at the following concentrations: ampicillin, 100 $\mu g\,ml^{-1}$; chloramphenicol, 30 $\mu g\,ml^{-1}$; and kanamycin, 50 $\mu g\,ml^{-1}$. *Pseudomonas putida* was grown on LNG broth or plates (Dorrington and Rawlings, 1990) and selected for with 300 $\mu g\,ml^{-1}$ chloramphenicol and 250 $\mu g\,ml^{-1}$ kanamycin.

General DNA and other techniques

Standard methods were used for plasmid DNA isolation, digestion, ligation, agarose gel electrophoresis and the preparation and transformation of competent *E. coli* cells (Sambrook *et al.*, 1989). *Pseudomonas putida* was transformed by electroporation using a Bio-Rad Gene Pulser according to the method recommended by the manufacturers. Translation products were detected using a DNA-directed transcription–translation kit (Promega, catalogue number L1020), and the products were analysed by electrophoresis using a 15% polyacrylamide gel (Maeser *et al.*, 1990). DNA sequencing was performed on a Pharmacia ALF express automated DNA sequencer for 12 h at 60 W and 25 mA at 55°C using templates prepared with the Autoread system (Pharmacia, catalogue number 27-2690-02).

The minimal replicon of pTF-FC2 was cloned on a *Bam*HI–*Eco*RI fragment from pTF200 into the mutagenesis vector pMa. Three separate frameshift mutants were constructed. A *Bam*HI site was introduced into *pas*A, a *Xho*I site into *pas*B and a *Bgl*II site into *pas*C; in each case, the mutation caused the protein to shift frame and become a nonsense protein. The mutants and an unmutated copy of the minimal replicon were then excised from the mutagenesis vector on *Eco*RI–*Sal*I fragments and ligated to *Eco*RI–*Sal*I fragments containing a kanamycin resistance gene from pSKm1. The kanamycin-resistant constructs were designated, pKmM0 (unmutated minimal replicon), pKmM1 (*pas*A nonsense mutation), pKmM2 (*pas*B nonsense mutation) and pKmM3 (*pas*C nonsense mutation).

Table 1. Bacteria and plasmids vectors used in this study.

Strains	Genotype	Source or reference
E. coli JM105	*thi, rpsL, endA, sbcB15, hspR4,* Δ(*lac-proAB*), [F′, *tra36, proAB lacI*q ΔM15]	Yanisch-Perron *et al.* (1985)
P. putida	Prototrophic	Franklin *et al.* (1981)
E. coli GW125a	*recA99, thr1, leu6, thi1, lacY1, galK2, ara14, xyl5, mtl1, proA2, his4, argE3, str31, tsx33, polA1*	Dorrington and Rawlings (1989)
Plasmids		
pACYC184	p15a replicon, Cm, Tc	Chang and Cohen (1978)
pUC19	ColEI replicon, Ap	Yanisch-Perron *et al.* (1985)
pMa	ColE1 replicon, Ap, Cm*	Stanssens *et al.* (1989)
pMc	ColEI replicon, Ap*, Cm	Stanssens *et al.* (1989)
pKK223-3	ColE1 replicon, Ap, *tac*	Brosius and Holy (1984)
pSUP106	RSF1010 and p15a replicons, Ap, Cm	Puhler *et al.* (1985)
pOU82	R1 replicon, Ap, *lacZYA*	Gerdes *et al.* (1985)

Ap, ampicillin resistance; Km, tetracycline resistance; Cm, chloramphenicol resistance; Tc, tetracycline; *lacZYA**, deletion of *lacZ* promoter and ATG; Ap* and Cm*, amber mutation in *bla* or *cat* genes respectively; *tac*, *trp-lac* hybrid promoter.

Plasmid stability assays

Stability assays were performed by growing plasmid containing *E. coli* JM105 cells for 100 generations without selection in TB. Aliquots were taken at 20 generation intervals, plated onto LA and incubated at 37°C overnight in the absence of selection. One hundred colonies were pricked onto LA plates with plasmid selection, and the percentage survival was used to calculate plasmid loss. The stability assay for pOU82 and derivatives was performed as above except that aliquots taken at 20 generation intervals were plated to LA containing 40 $\mu g\,ml^{-1}$ Xgal. Plasmid-containing cells grow as blue colonies, whereas plasmid-free cells are white.

Copy number determination

Copy number determination was performed in a dot-blot experiment by hybridizing plasmid DNA to known amounts of total DNA isolated from plasmid-containing cells and known amounts of plasmid DNA. By comparing the amounts of DNA giving equivalent hybridization signals from total DNA and plasmid DNA, it was possible to determine the amount of plasmid in a sample of total DNA. Given the relationship shown below it was therefore possible to determine plasmid copy number:

(copy number × plasmid size)/amount of plasmid DNA = genome size/amount of total DNA.

Site-directed mutagenesis

Site-directed mutagenesis was carried out using the pMa and pMc vector system according to the method of Stanssens *et al.* (1989). Primers (5′-CCACAGGGCGGATCCAAGACTTTCT-3′), (5′-GGTTGAACTCGAGCCCAGCCGCCGA-3′) and (5′-CTAGGCAAGATCTGACCAGCAGACC-3′) were used to introduce a *Bam*HI site, a *Xho*I site and a *Bgl*II site into *pasA*, *B* and *C* respectively.

Table 2. Plasmid constructs used in this study.

Plasmid	Position relative to *Cla*I–*Pst*I fragment of pTV100 (Dorrington and Rawlings, 1991)	Vector	Antibiotic resistance marker[a]	Reference
pTV400	1240–4441	pUC19	Ap	Dorrington and Rawlings (1989)
pTac-pasA	1316–1559	pKK223-3	Ap	This work
pTac-pasB	1518–1816	pKK223-3	Ap	This work
pTac-pasC	1789–2028	pKK223-3	Ap	This work
pTac-pasAB	1316–1816	pKK223-3	Ap	This work
pTac-pasBC	1518–2028	pKK223-3	Ap	This work
pTac-pasABC	1316–2028	pKK223-3	Ap	This work
pTac-pasABC*	1316–2028	pKK223-3	Ap	This work
pTac-pasAB-pACYC	T insertion at 1833 1316–1816	pACYC184	Cm	This work
pKmM0	1–4910	–	Km	This work
pKmM1	1–4911	–	Km	This work
pKmM2	T insertion at 1402 1–4911	–	Km	This work
pKmM3	G insertion at 1563 1–4911	–	Km	This work
pKmM1del1	T insertion at 1833 Δ1217–1321	–	Km	This work
pKmM1del2	Δ1232–1943	–	Km	This work
pORF3	1240–1559	pSUP106	Cm	This work
pOU-PTF	1158–2027	pOU82	Ap	This work

Ap, ampicillin resistance; Km, tetracycline resistance; Cm, chloramphenicol resistance.

Fragment amplification and modification

Primers PTF-ECO (5′-ATATGAATTCTTGGCAAGTACCG-GCAGC-3′) and PTF-BAM (5′-TATTAGGATCCGCAATCC-CCTGCCGCGCCGC-3′) were used to amplify *pasABC* for cloning into pOU82. Primers 3F (5′-TATTGAATTCGAACA-GGAGCCGAAACATGC-3′) and 3R (3′-TAATGAAGCTTA-GTTCAACCTTCCATGCCATAGC-5′) were used to amplify *pas*A. Primers 4F (5′-ATACGAATTCCTCGAAGAAGTGAT-GAAACGC-3′) and 4R (5′-TCGCCAAGCTTCGATCATTGG-CGGTAAACCTCC-3′) were used to amplify *pas*B. Primers 5F (5′-TCCGGAATTCCGTAAGGAGGTTTACCGCC-3′) and 5R (5′-TTTCTAAGCTTCGCTCAATTTTCGCCCCTCG-3′) were used to amplify *pas*C. Appropriate pairs of the above primers were used to amplify combinations of *pasA* and *B*, *pas B* and *C* as well as *pasA*, *B* and *C*. All amplification products were digested with *Eco*RI and *Hin*dIII and cloned into the vector pKK223-3.

The Tac–*pas* fusions were excised from their respective constructs on *Pvu*I (blunted)–*Bam*HI fragments and cloned into pACYC184, which had been cut with *Bam*HI and *Cla*I (blunted). The constructs produced were pTac-*pasA*-pACYC, pTac-*pasB*-pACYC, pTac-*pasC*-pACYC, pTac-*pasAB*-pACYC and pTac-*pasABC*-pACYC. DNA sequencing was used to confirm the integrity of all constructs following the cloning of PCR-amplified fragments.

Acknowledgements

We are most grateful to Kenn Gerdes for the gift of pOU82 and to Rasmus Jensen for advice on using it. This work was supported by grants from Gencor Process Research, Randburg and the Foundation for Research Development, Pretoria, South Africa.

References

Bravo, A., de Torrontegui, G., and Díaz, R. (1987) Identification of the components of a new stability system of plasmid R1. ParD, that is close to the origin of replication of this plasmid. *Mol Gen Genet* **210:** 101–110.

Brosius, J., and Holy, A. (1984) Regulation of ribosomal RNA promoters with a synthetic lac operator. *Proc Natl Acad Sci USA* **81:** 6929–6933.

Chang, A.C.Y., and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. *J Bacteriol* **134:** 1141–1156.

Clennel, A.-M., Johnston, B., and Rawlings, D.E. (1995) Structure and function of Tn*5467*, a Tn*21*-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid pTF-FC2. *Appl Environ Microbiol* **61:** 4223–4229.

Dorrington, R.A., and Rawlings, D.E. (1989) Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. *J Bacteriol* **171:** 2735–2739.

Dorrington, R.A., and Rawlings, D.E. (1990) Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. *J Bacteriol* **172:** 5697–5705.

Dorrington, R.A., Bardien, S., and Rawlings, D.E. (1991) The

broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. *Gene* **108:** 7–14.

Franklin, F.C.H., Bagdasarian, M., Bagdasarian, M.M., and Timmis, K.N. (1981) Molecular and functional analysis of the TOL plasmid pWW0 from *Pseudomonas putida* and cloning of the genes for the entire regulated aromatic ring *meta*-cleavage pathway. *Proc Natl Acad Sci USA* **78:** 7458–7462.

Gerdes, K., Larsen, J.E.L., and Molin, S. (1985) Stable inheritance of plasmid R1 requires two different loci. *J Bacteriol* **161:** 292–298.

Gerdes, K., Poulsen, L.K., Thirstead, T., Nielsen, A.K., Martinussen, J., and Andreasen, J.H. (1990) The *hok* killer gene family in Gram-negative bacteria. *New Biol* **4:** 1807–1818.

Herrera, G., Snyman, S.J., and Thomson, J.A. (1994) Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugar cane borer, *Eldana saccharina*. *Appl Environ Microbiol* **60:** 682–690.

Jensen, R.B., and Gerdes, K. (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. *Mol Microbiol* **17:** 205–210.

Jensen, R.B., Grohmann, E., Schwab, H., Diaz-Orejas, R., and Gerdes, K. (1995) Comparison of *ccd* of F, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. *Mol Microbiol* **17:** 211–220.

Lehnherr, H., Maguin, E., Jafri, S., and Yarmolinsky, M.B. (1993) Plasmid addiction genes of plasmid P1: *doc*, which causes cell death on curing of the prophage, and *phd*, which prevents host death when the prophage is retained. *J Mol Biol* **233:** 414–428.

Maeser, S., Scholz., P., Otto, S., and Scherzinger, E. (1990) Gene F of plasmid RSF1010 codes for a low-molecular-weight repressor protein that autoregulates expression of the *repAC* operon. *Nucleic Acids Res* **18:** 6215–6222.

Ogura, T., and Hiraga, S. (1983) Mini-F plasmid genes that couple host cell division to plasmid proliferation. *Proc Natl Acad Sci USA* **80:** 4784–4788.

Pansegrau, W., Lanka, E., Barth, P.T., Figurski, D.H., Guiney, D.G., Haas, D., Helinski, D.R., Schwab, H., Stanisich, V.A, and Thomas, C.M. (1994) Complete nucleotide sequence of the Birmingham IncPα plasmids: compilation and comparative analysis. *J Mol Biol* **239:** 623–663.

Puhler, A., Simon, R., and Priefer, U.B. (1985) Extension of the host range of *Escherichia coli* vectors by incorporation of RSF1010 replication and mobilization functions. *J Bacteriol* **163:** 324–330.

Rawlings, D.E., and Kusano, T. (1994) Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol Rev* **58:** 39–55.

Rawlings, D.E., and Silver, S. (1995) Mining with microbes. *BioTechnology* **13:** 773–778.

Rawlings, D.E, Gawith, C., Petersen, A., and Woods, D.R. (1983) Characterisation of plasmids and potential genetic markers in *Thiobacillus ferrooxidans*. In *Progress in Biohydrometallurgy*, Rossi, G., and Torma, A.E. (eds). Cagliari: Associazione Mineraria Sarda, pp. 555–570.

Roberts, R.C., and Helinski, D.R. (1992) Definition of a minimal plasmid stabilization system from the broad-host-range plasmid RK2. *J Bacteriol* **174:** 8119–8132.

Roberts, R.C., Ström, A.R., and Helinski, D.R. (1994) The *parDE* operon of the broad-host-range plasmid RK2 specifies growth inhibition associated with plasmid loss. *J Mol Biol* **237:** 35–51.

Rohrer, J., and Rawlings, D.E. (1992) Sequence analysis and characterization of the mobilization region of the broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. *J Bacteriol* **174:** 6230–6237.

Ruiz-Echevarria, M.J., Berzal-Herranz, A, Gerdes, K., and Diaz-Orejas, R. (1991) The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. *Mol Microbiol* **5:** 2685–2693.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Stanssens, P., Opsomer, C., McKeown, Y.M., Kramer, W., Zabeau, M., and Fritx, H.-J. (1989) Efficient oligonucleotide-directed construction of mutations in expression vectors by the gapped duplex DNA method using alternating selectable markers. *Nucleic Acids Res* **17:** 4441–4453.

Tsuchimoto, S., Ohtsubo, H., and Ohtsubo, E. (1988) Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. *J Bacteriol* **170:** 1461–1466.

Tsuchimoto, S., Nishimura, Y., and Ohtsubo, E. (1992) The stable maintenance system *pem* of plasmid R100: degradation of PemI protein may allow PemK protein to inhibit cell growth. *J Bacteriol* **174:** 4205–4211.

Yannisch-Perron, C., Vieira, J., and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. *Gene* **33:** 103–119.

JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5458–5462 Vol. 180, No. 20
0021-9193/98/$04.00+0

Efficiency of the pTF-FC2 *pas* Poison-Antidote Stability System in *Escherichia coli* Is Affected by the Host Strain, and Antidote Degradation Requires the Lon Protease

ANTHONY S. G. SMITH AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 13 February 1998/Accepted 22 June 1998

The stabilization of a test plasmid by the proteic, poison-antidote plasmid addiction system (*pas*) of plasmid pTF-FC2 was host strain dependent, with a 100-fold increase in stability in *Escherichia coli* CSH50, a 2.5-fold increase in *E. coli* JM105, and no detectable stabilization in *E. coli* strains JM107 and JM109. The lethality of the PasB toxin was far higher in the *E. coli* strains in which the *pas* was most effective. Models for the way in which poison-antidote systems stabilize plasmids require that the antidote have a much higher rate of turnover than that of the toxin. A decrease in host cell death following plasmid loss from an *E. coli lon* mutant and a decrease in plasmid stability suggested that the Lon protease plays a role in the rate of turnover of PasA antidote.

Proteic plasmid stabilization systems have been discovered on a number of plasmids and include the *ccd* system of plasmid F (7), the identical *parD*/*pem* and *kis*/*kid* systems of plasmids R1 and R100 (1, 16, 19), the *parDE* system of plasmids RP4/ RK2 (14) and the *phd*/*doc* system of phage P1 (10). These systems consist of a long-lived toxin which is expressed at low levels and a short-lived, highly expressed antidote (8). On cell division, if a progeny cell fails to inherit the plasmid, it loses the ability to make the shorter-lived and more abundantly produced antidote and is unable to counter the toxic effects of the poison. As a result, plasmid-free cells are killed or their cell division is inhibited, depending on the type of poison-antidote system.

The 12.2-kb mobilizable, broad-host-range plasmid pTF-FC2 (GenBank accession nos. M64981 and M35249 [13]) was originally isolated from *Thiobacillus ferrooxidans*. This natural hybrid plasmid has a replicon clearly related to those of the IncQ plasmids (e.g., R1162 and RSF1010) (4) and a mobilization region with low but clear similarity to those of the IncP plasmids (e.g., R751 and RK2/R68/RP4) (15). Situated within the IncQ-like replicon and between the *repB* and *repA* genes (Fig. 1) is a proteic poison-antidote system named *pas* (for plasmid addiction system). This system is unusual in that it consists of three genes; *pasA* encodes an antidote, *pasB* encodes a toxin (which is bacteriocidal rather than bacteriostatic), and *pasC* encodes a protein that appears to enhance the neutralizing effect of the antidote (17).

Efficiency of the *pas* stability system in *Escherichia coli* is strain dependent. The ability of the pTF-FC2 *pasABC* system to stabilize a heterologous plasmid in *E. coli* JM105 had previously been shown (17) by cloning the *pasABC* genes into the unstable, low-copy-number, test plasmid pOU82 (6). We repeated the stability assays in *E. coli* CSH50 to compare the efficiency of the pTF-FC2 *pas* to those of other poison-antidote systems in a host strain background identical to that used by other workers (9). It was observed that *pas* varied in its ability to act as a plasmid stabilization system depending upon which strain (Table 1) was used as host. Plasmid stability was determined by growing plasmid-containing *E. coli* cells in batch culture for 100 generations without selection in TB (24 g of yeast extract, 12 g of tryptone, and 4 ml of glycerol per 900 ml with 100 ml of sterile 0.17 M KH_2PO_4–0.72 M K_2HPO_4 added immediately before use). Aliquots were taken at 20-generation intervals and grown at 37°C overnight in the absence of selection. One hundred colonies were transferred to Luria agar (LA) plates with plasmid selection (ampicillin [100 μg ml^{-1}], chloramphenicol [30 μg ml^{-1}], or kanamycin [50 μg ml^{-1}], as required), and the percentage survival was used to calculate plasmid loss. The stability assay for pOU82 and derivatives was performed as described above, except that aliquots were plated on LA containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml. Plasmid-containing cells form blue colonies, whereas plasmid-free cells are white. At least three stability tests were performed for each strain, and the loss frequency was calculated by the method of Gerdes et al. (6). After 100 generations, a test plasmid containing the *pasABC* genes (pOU-pasABC [Table 1]) was stabilized approximately 2.5-fold in an *E. coli* JM105 host (Table 2). However, in an *E. coli* CSH50 host, the *pas* enhanced plasmid stability about 100-fold. In contrast, the *pas* was ineffective in enhancing the stability of the test plasmid in an *E. coli* JM107 host strain or its *recA* derivative, *E. coli* JM109. Surprisingly, the base level of pOU82 stability in *E. coli* JM109 was 10-fold higher than in strain JM107 (Table 2). The fact that *E. coli* strains JM107 and JM109 are isogenic except for the *recA* gene implies that the *recA* system has an effect on the stability of the pOU82 test plasmid. The reason for this increased stability is unknown, but the finding is similar to the finding that mini-RK2 plasmids were threefold more stable in *E. coli* JM109 than in JM107 (14).

In previous work (17), we showed that inactivation of PasC (through the introduction of a frameshift mutation in the *pasC* gene) resulted in an increase in the toxicity of the PasA-PasB poison-antidote complex (see also Fig. 2). We therefore examined how inactivation of PasC affects the ability of the *pas* to stabilize the test plasmid in different *E. coli* hosts. In *E. coli* JM105 (pOU-pasABC*), plasmid stability was about the same (loss frequency of 3×10^{-2}) as that of the pOU82 test plasmid and considerably less than that of pOU-pasABC (loss fre-

* Corresponding author. Present address: Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa. Phone: 27 21 808 4866. Fax: 27 21 808 3611. E-mail: der@maties.sun.ac.za.

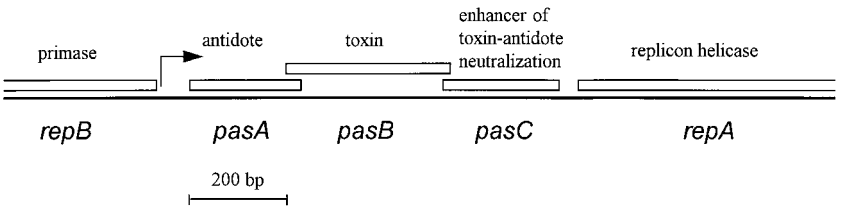


FIG. 1. Layout of the pTF-FC2 *pas* showing its location within the plasmid replicon. The positions of the genes are relative to the *Cla*I site of pTF-FC2 (5).

quency of 9×10^{-3}), whereas in *E. coli* CSH50, inactivation of *pasC* reduced the ability of *pas* to stabilize the test plasmid from about 100- to 2.5-fold. Inactivation of *pasC* had little effect in *E. coli* JM107 or JM109.

Pas toxicity varies between *E. coli* host strains. To investigate the reason for host-strain dependent variation in stability, combinations of the *pas* genes were cloned behind the *tac* promoter of a pKK223-3 vector (Table 1). In *lacI*^{q} strains containing these constructs, *tac*-controlled expression of the *pas* genes is induced by isopropyl-β-D-thiogalactopyranoside (IPTG). Since *E. coli* CSH50 does not contain *lacI*^{q}, the F′ *lacI*^{q}-containing episome was transferred from *E. coli* 71/18 to CSH50 by conjugation. Conjugation was carried out overnight on the surface of a LA plate followed by plating on minimal medium plus streptomycin (100 μg/ml) to select for streptomycin resistance and proline independence. The stability of the test plasmid in this *E. coli* CSH50-I^q strain was indistinguishable from that of the test plasmid in CSH50. The effect of IPTG-induced *pas* gene expression on the growth of *E. coli* host strains JM105, JM107, JM109, and CSH50-I^q is shown in Fig. 2. Not all of the strains were equally sensitive to the PasB toxin. Growth of strain CSH50-I^q was the most severely inhibited by induction of pTac-pasB, with JM105 less inhibited and strains JM107 and JM109 the least inhibited. Expression of the *pasA* gene (encoding the antidote) relieved the toxic effect of *pasB*, although in *E. coli* strain CSH50-I^q, growth inhibition was relieved only slightly. When all three *pasABC* genes were expressed, the growth rates of all strains increased further, although in no strain did the growth rate reach that of the vector control. IPTG-induced expression of the *pasABC* system was toxic to all strains, but toxicity was most severe in *E. coli* CSH50-I^q, which was also the strain in which the *pas* plasmid stabilization system was most effective.

TABLE 1. Bacteria, plasmid vectors, and *pas* constructs used in this study

Strain, plasmid, or construct	Genotype or description[a]	Source or reference
E. coli strains		
JM105	*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
JM107	*thi endA gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
JM109	*recA1 thi endA gyrA96 hsdR17 supE44 relA1* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
71/18	*thi supE* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	20
CSH50	*rpsL* Δ(*lac-pro*)	12
CSH50-I^q	*rpsL* Δ(*lac-pro*) [F′ *traD36 proAB lacI*^{q}ΔM15]	This work
SG22025	Δ*lac rcsA166*::mini-*kan* parent of SG22093 and SG22095	S. Gottesman (11)
SG22093	Δ*lac rcsA166*::mini-*kan clpP1*::*cat*	S. Gottesman (11)
SG22095	Δ*lac rcsA166*::mini-*kan lon-146*::mini-Tn*10*	S. Gottesman (11)
Plasmids		
pACYC184	p15a replicon, Cm^r Tc^r	3
pKK223-3	ColE1 replicon, Ap^r *tac*	2
pOU82	R1 replicon, Ap^r *lacZYA*	6
pKG399	pSC101 replicon, Tc^r (see Fig. 3)	9
pKGCm	pACYC184 replicon, Cm^r (see Fig. 3)	This work
pas-containing constructs		
pTac-pasB	pKK223-3 replicon, Ap^r, *pas* region 1518–1816[b]	17
pTac-pasAB	pKK223-3 replicon, Ap^r *pas* region 1316–1816	17
pTac-pasABC	pKK223-3 replicon, Ap^r *pas* region 1316–2028	17
pOU-pasABC	pOU82 replicon (R1), Ap^r, *pas* region 1158–2027	17
pOU-pasABC*[c]	pOU82 replicon (R1), Ap^r, *pas* region 1158–2027	17

[a] Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; *tac*, *trp-lac* hybrid promoter.
[b] The *pas* region numbers are the nucleotide positions relative to those of the *Cla*I-*Pst*I fragment of pTV100 (5).
[c] C* indicates a T insertion at 1833 bp to inactivate the *pasC* product (17).

The effects of combinations of *pas* genes expressed under the control of a *tac* promoter on the host strains provided some insight into why *pas*-mediated plasmid stability varied in strains. The lower growth rate and cell density of *E. coli* CSH50-I^q containing different combinations of *pas* genes indicated that in this strain the PasA antidote did not effectively neutralize the toxic effect of PasB toxin even in the presence of PasC. It may be that the greater toxicity of the *pas* in *E. coli* CSH50-I^q was why the test plasmid was best stabilized by *pas* in this strain. Possible reasons for increased PasB toxicity in this strain are that the as yet unidentified cytoplasmic target may be more susceptible to the PasB toxin and that the protease which degrades the antidote may be particularly effective in *E. coli* CSH50-I^q. Variations in the levels of *pas* gene expression between strains may also play a role in the efficiency of plasmid stability, but this is less likely. Since the *pas* is autoregulated (18), differences in *pas* promoter strength between strains

TABLE 2. Loss frequency of plasmids from *E. coli* host strains after 100 generations of growth

Strain	Loss frequency		Approximate fold increase in stability
	No stability genes present (pOU82)	Stability genes present (pOU-pasABC)	
JM105	2×10^{-2}	9×10^{-3}	2.5
JM107	5×10^{-2}	5×10^{-2}	1
JM109	5×10^{-3}	4×10^{-3}	1
CSH50	2×10^{-2}	3×10^{-4}	100

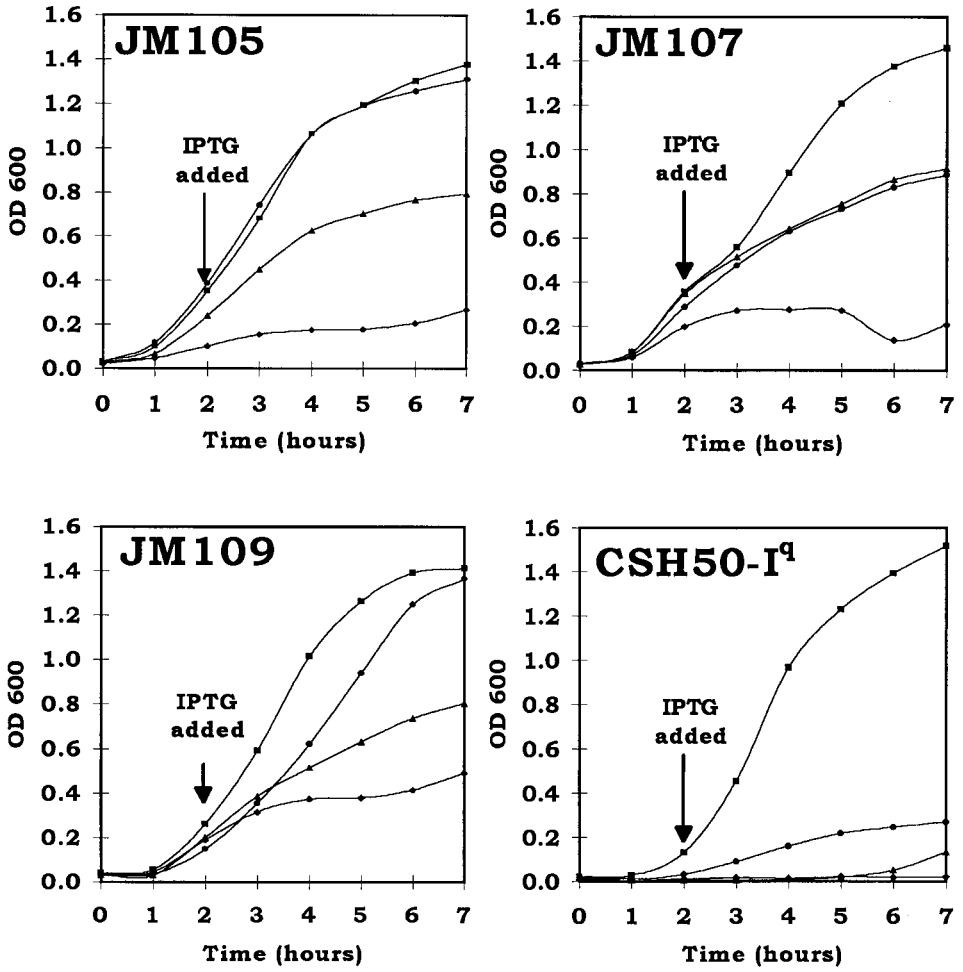


FIG. 2. Growth curves of *E. coli* strains overexpressing the *pas* genes. Each graph shows pKK223-3 (control) (■), pTac-pasB (◆), pTac-pasAB (▲), and pTac-pasABC (●). Datum points are the means of three separate experiments. OD 600, optical density at 600 nm.

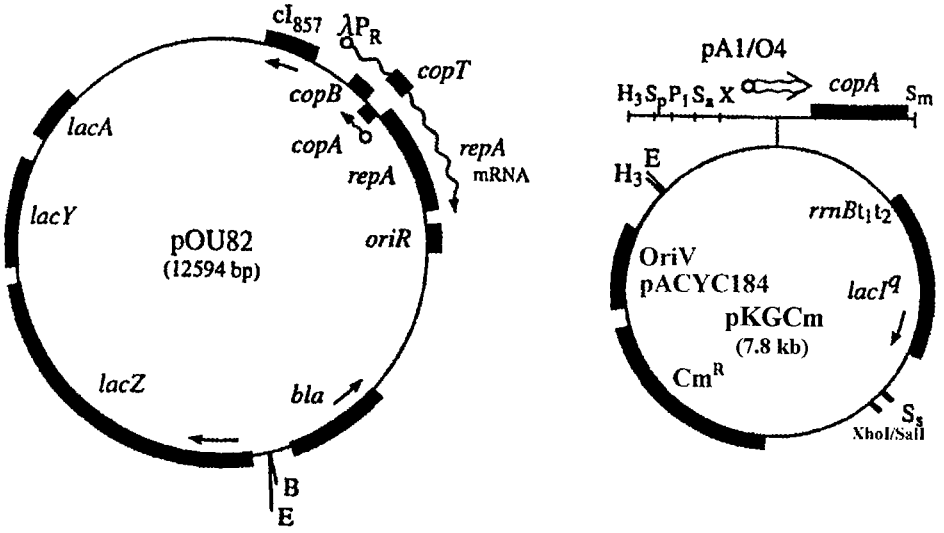


FIG. 3. pOU82-pKGCm conditional replication system based on the pOU82-pKG339 system (modified from reference 9 with permission of the publisher). The pACYC replicon and chloramphenicol resistance markers of pKGCm have replaced the pSC101 replicon and tetracycline resistance marker of pKG339. Addition of IPTG results in expression of *copA* from the pA1/O4 promoter, and CopA inhibits replication of the pOU82 R1 replicon. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; H_3, *Hin*dIII; Sp, *Sph*I; P_1, *Pst*I; Sa, *Sal*I; X, *Xha*I; Sm, *Sma*I; Ss, *Ssp*I.

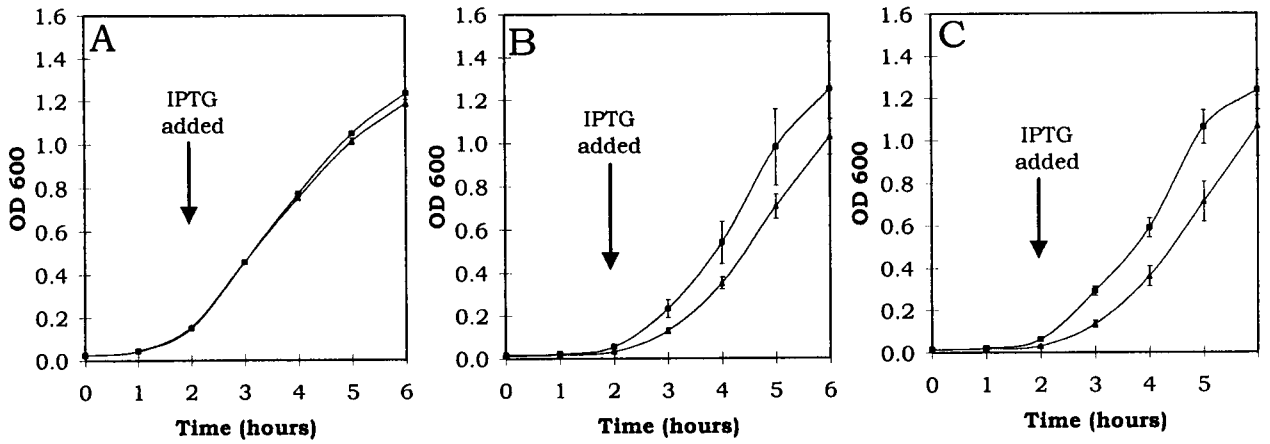


FIG. 4. Growth curves of protease mutants with different plasmids. Growth curves of *E. coli lon* mutant SG22095 (A), *E. coli clpP1* mutant SG22093 (B), and *E. coli* protease mutant parent strain SG22025 (C) containing pOU82 and pKGCm (control) (■) or pOU-pasABC and pKGCm (▲) are shown. OD 600, optical density at 600 nm.

would not be expected to be as important as in a non-self-regulated system. Variations in the rate of transcription would alter the time required to reach self-regulating levels of antidote and toxin but probably not the actual levels reached.

Role of Lon protease in plasmid stabilization. Plasmid proteic stabilization systems operate on the principle that the antidote is subject to a much higher rate of turnover than the toxin (8). A pOU82-pKG339-based conditional replication system (9) was used to determine which *E. coli* protease is involved in the selective degradation of the PasA antidote. Since one of the *E. coli* protease mutants was resistant to tetracycline (Table 1), the tetracycline resistance marker and pSC101 replicon of pKG339 were replaced by the chloramphenicol resistance marker and the p15a replicon of pACYC184 to produce plasmid pKGCm. When the *copA* gene of pKGCm is provided in *trans* to pOU82, replication of pOU82 can be halted by IPTG induction of the pA1/O4 promoter (Fig. 3). Therefore, addition of IPTG to *E. coli* (pOU-pasABC) cells will result in arrest of plasmid replication, and plasmid-free cells containing the toxin-antidote complex will result. The protease responsible for selective degradation of the antidote will digest the antidote, which will no longer inhibit the toxin, resulting in cell death. Functional antidote will persist in *E. coli* mutants deficient in the antidote-degrading protease, and cell death will not occur. Plasmid pOU82 and the *pas*-containing pOU82-based plasmid (pOU-pasABC) were transformed into *E. coli* SG22093 *clpP1* and SG22095 *lon* mutants and into *E. coli* SG22025, a protease-proficient parental strain of these mutants, each of which contained a coresident pKGCm plasmid. The growth of all strains following the addition of 2 mM IPTG is shown in Fig. 4. Growth of the *E. coli* (pOU-pasABC) *lon* mutant strain was similar to that of the same strain containing pOU82 (Fig. 4A), suggesting that in this strain the antidote was long-lived. In contrast, growth of the *E. coli* (pOU-pasABC) *clpP* mutant and *E. coli* (pOU-pasABC) protease-proficient strains was reduced relative to that of the same strains containing the pOU82 control plasmid (Fig. 4B and C). The observation that growth was not reduced by forced plasmid loss in an *E. coli lon* mutant, whereas there was a decrease in growth following the loss of plasmids expressing the *pasABC* genes in *lon*-proficient strains, suggests that the antidote protein was stable in the *lon* mutant and that the Lon protease is involved in the degradation of the PasA antidote.

To obtain additional evidence for the involvement of the Lon protease, the stability of pOU-pasABC was tested in each strain in the absence of pKGCm. Strains in which the protease required for selective degradation of the antidote is not present would cause pOU-pasABC to be less stably inherited. In the *E. coli clpP* mutant and *lon*-proficient parental strains, the pOU-pasABC loss frequencies were 1.5×10^{-2} and 1.4×10^{-2}, respectively, whereas the loss frequency was increased to 3.5×10^{-2} in the *E. coli lon* mutant. This 2.5-fold decrease in plasmid stability seen in the *E. coli lon* mutant strain supports the notion that the Lon protease plays a role in the degradation of PasA. The level of stabilization by the *pas* in the *E. coli lon* parental strain was comparable to that in *E. coli* JM105 but less than that found in *E. coli* CSH50-I^q. This is additional evidence that the Lon protease plays a role in *pas*-mediated plasmid stability.

We are most grateful to Kenn Gerdes for the gift of plasmids pOU82 and pKG339 and to Susan Gottesman for *E. coli* SG22025, SG22093, and SG22095.

This work was supported by grants from Gencor (now Billiton) Process Research (Randburg, South Africa), the Foundation for Research Development (Pretoria, South Africa), and the THRIP programme of the Department of Trade and Industry.

REFERENCES

1. **Bravo, A., G. de Torrontegui, and R. Dìaz.** 1987. Identification of the components of a new stability system of plasmid R1, ParD, that is close to the origin of replication of this plasmid. Mol. Gen. Genet. **210:**101–110.
2. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
4. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
5. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene **108:**7–14.
6. **Gerdes, K., J. E. L. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
7. **Jaffé, A., T. Ogura, and S. Hiraga.** 1985. Effects of the *ccd* function of the F plasmid on bacterial growth. J. Bacteriol. **163:**841–849.
8. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
9. **Jensen, R. B., E. Grohmann, H. Schwab, R. Dìaz-Orejas, and K. Gerdes.** 1995. Comparison of *ccd* of F, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. **17:**211–220.
10. **Lehnherr, H., E. Maguin, S. Jafri, and M. B. Yarmolinsky.** 1993. Plasmid

addiction genes of plasmid P1: *doc*, which causes cell death on curing of the prophage, and *phd*, which prevents host death when the prophage is retained. J. Mol. Biol. **233:**414–428.
11. **Lehnherr, H., and M. B. Yarmolinsky.** 1995. Addiction protein Phd of plasmid prophage P1 is a substrate of the ClpXP serine protease of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **92:**3274–3277.
12. **Miller, J. H.** 1983. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:**39–55.
14. **Roberts, R. C., and D. R. Helinski.** 1992. Definition of a minimal plasmid stabilization system from the broad-host-range plasmid RK2. J. Bacteriol. **174:**8119–8132.
15. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**6230–6237.
16. **Ruiz-Echevarrìa, M. J., A. Berzal-Herranz, K. Gerdes, and R. Díaz-Orejas.** 1991. The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. Mol. Microbiol. **5:**2685–2693.
17. **Smith, A. S. G., and D. E. Rawlings.** 1997. The poison antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. **26:**261–270.
18. **Smith, A. S. G., and D. E. Rawlings.** 1998. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (*pas*) is essential for plasmid stabilization. J. Bacteriol. **180:**5463–5465.
19. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, *pemK* and *pemI*, responsible for stable maintenance of resistance plasmid R100. J. Bacteriol. **170:**1461–1466.
20. **Yannisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

JOURNAL OF BACTERIOLOGY, Oct. 1998, p. 5463–5465 Vol. 180, No. 20
0021-9193/98/$04.00+0

Autoregulation of the pTF-FC2 Proteic Poison-Antidote Plasmid Addiction System (*pas*) Is Essential for Plasmid Stabilization

ANTHONY S. G. SMITH AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

Received 13 February 1998/Accepted 22 June 1998

The *pasABC* genes of the proteic plasmid addiction system of broad-host-range plasmid pTF-FC2 were autoregulated. The PasA antidote was able to repress the operon 25-fold on its own, and repression was increased to 100-fold when the PasB toxin was also present. Autoregulation appears to be an essential requirement for *pas*-mediated plasmid stabilization because when the *pas* genes were placed behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-regulated *tac* promoter, they were unable to stabilize a heterologous test plasmid.

Plasmid pTF-FC2 is a 12.2-kb, mobilizable, broad-host-range plasmid that was originally isolated from the biomining bacterium *Thiobacillus ferrooxidans* (9). The plasmid contains a proteic poison-antidote plasmid addiction system (*pas*) located between the *repB* and *repA* genes (Fig. 1) of its IncQ-like replicon (4). This stability system is unusual in that it consists of three genes rather than the two-gene systems identified in other plasmids (7). The *pasA* gene encodes an antidote, *pasB* encodes a toxin, and *pasC* encodes a protein that appears to enhance the neutralizing effect of the antidote (12). Autoregulation is a general property of proteic stabilization systems in which regulation has been studied. For example, the *ccd* system of plasmid F is autoregulated by a 69-kDa complex of CcdA and CcdB (14), and neither CcdA nor CcdB alone is capable of autorepression. In contrast, the *parDE* system of plasmid RK2 is autoregulated solely by the ParD antidote protein (10). The *parD* locus of plasmid R1 is repressed only 30 to 40% by Kis on its own, and the complete Kis-Kid complex is required for maximal repression (11).

We investigated whether the *pas* of pTF-FC2 is autoregulated and whether the third component of the *pas*, PasC, plays a role in regulation. Furthermore, we investigated whether autoregulation is a necessary requirement for *pas* stabilization. It is conceivable that differences in the half-lives of the antidote and toxin proteins together with differences in the levels of toxin and antidote translation may by themselves be sufficient to increase plasmid stability. The bacteria, plasmids, and constructs we used in this study are given in Table 1.

Autoregulation of *pasABC*. Regulation of the *pasABC* genes was investigated by the construction of an in-frame translational fusion of *pasA* to a *lacZ* reporter gene. A translational fusion would indicate the cumulative effect of transcriptional and translational regulation. An in-frame translational fusion of *pasA* to a *lacZ* reporter gene was constructed by cloning a PCR amplification product which extended for 124 bp upstream of the *pasA* start into the vector pMC1403. The primers 1212F (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and FP2 (5′-AGTAGGGATCCACTTCGGCGGGCAGTCGG-3′) (shown in Fig. 1), were used to amplify the *pasA* promoter from pTV400 (4). The PCR was carried out by using DynazymeII (Finnenzymes Oy) in a JDI2500 thermocycler (denaturation step of 2 min at 95°C and then 30 cycles, with 1 cycle consisting of 30 s at 95°C, 30 s at 52°C, and 60 s at 72°C). Primer FP2 introduced a *Bam*HI site which allowed in-frame cloning of the fragment into vector pMC1403 to create construct pP2H. DNA sequencing with a Pharmacia ALF express automated DNA sequencer was used to confirm the integrity of the construct. β-Galactosidase assays were performed by the method of Miller (8) on log-phase cultures grown with the appropriate antibiotic selection. The *pas-lacZ* fusion, pP2H, when placed in *Escherichia coli* CSH50-I^q gave moderate levels of β-galactosidase activity (252 Miller units) (Table 2). When plasmid pKmM0, which has the PasABC system situated within its natural, broad-host-range, pTF-FC2 replicon, was placed in *trans* to pP2H, expression of β-galactosidase activity was reduced to 12 Miller units. To identify the repressor of gene expression, β-galactosidase activity was measured in strains in which pKmM0 was replaced by the pKmM0-based *pas* mutant plasmids pKmM1 (*pasA*), pKmM2 (*pasB*), and pKmM3 (*pasC*) (12). However, the pKmM1 *pasA* mutant was lethal to *Escherichia coli* CSH50-I^q, and inactivation of *pasB* or *pasC* relieved the repression of *lacZ* reporter gene expression to a small extent (from 12 to 31 and 14 Miller units, respectively). When two spontaneous pKmM0 *pas* deletion mutants in which the *pasA* promoter region (pKmM1del1) or most of *pasABC* (pKmM1del2) had been deleted (Fig. 1) (12) were placed in *trans* to pP2H, reporter gene expression was restored to near unrepressed levels. This indicated that PasA was the primary repressor.

To confirm regulation by the *pas* gene products, constructs of each of the *pas* genes cloned individually and in combination behind the non-*pas*-regulated *tac* promoter of vector pKK223-3 were used. Since both the pMC1403 reporter gene vector and pKK223-3 use ColE1 origins of replication and both are ampicillin resistant, the *tac*-regulated *pas* genes were subcloned into the pACYC184 vector. *tac-pas* fusions were excised from their respective pKK223-3 constructs as *Pvu*I (blunted)-*Bam*HI fragments and cloned into pACYC184 which had been cut with *Bam*HI and *Cla*I (blunted). These constructs, pTac-pasA-pACYC, pTac-pasB-pACYC, pTac-pasC-pACYC, pTac-pasAB-pACYC, and pTac-pasABC-pACYC were transformed into *E. coli* CSH50-I^q cells containing pP2H. The level of β-

* Corresponding author. Present address: Department of Microbiology, University of Stellenbosch, Stellenbosch 7600, South Africa. Phone: 27 21 808 4866. Fax: 27 21 808 3611. E-mail: der@maties.sun.ac.za.

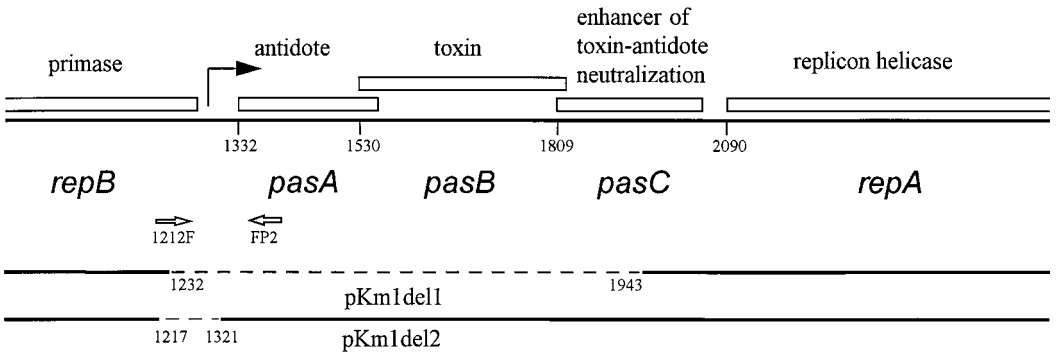


FIG. 1. Layout of the pTF-FC2 *pas* showing its location within the plasmid replicon. Numbers below the thick line indicate the positions of the genes relative to that of the *Cla*I site of pTF-FC2 (5). The positions of the PCR primers used to amplify the *pas* promoter region and the regions missing from the spontaneous deletions following PasA antidote inactivation (12) (broken lines) are shown below the layout.

galactosidase expression decreased from 252 to 10 Miller units when only *pasA* was provided in *trans* (Table 2). This repression was enhanced when *pasAB* (2 Miller units) or *pasABC* (3 Miller units) was present. The *pasABC* promoter therefore appears to be autorepressed (25-fold) by PasA, and this repression increased when both PasA and PasB were present (100-fold). Regulation by *pasB* or *pasBC* could not be tested due to the lethal effects of PasB in the absence of PasA. PasC alone had little effect on the expression of the *pasA* promoter.

Levels of reporter gene activity varied substantially between strains and experiments. Only the data for *E. coli* CSH50-I^q, in which reporter gene activity was less variable than in some of the other strains, are presented. Similar experiments with *E. coli* JM105 showed the same trends, although these data were unreliable because of the greater variability in reporter gene expression (data not shown). PasA was clearly the primary repressor, and this negative regulation was enhanced in the presence of PasB. The pTF-FC2 *pas* is therefore similar to the *par*D locus of R1 in that *parD* is only partially repressed by Kis (30 to 40%) and the complete Kis-Kid complex is required for maximal repression (11).

Effect of expression from a heterologous promoter on *pas* function. To investigate whether autoregulation is important for the proper functioning of the *pas* proteic plasmid stability system, we examined the stability of the pOU82 test plasmid containing the *pasABC* genes under the control of a *tac* promoter (pOU-tac-pasABC) in *E. coli* CSH50-I^q. The *tac-pas* fusions in pOU82 were constructed by excising the *pasABC* genes

TABLE 1. Bacteria, plasmid vectors, and *pas*-containing constructs used in this study

Strain, plasmid, or construct	Genotype or description[a]	Reference
E. coli strains		
JM105	*thi rpsL endA sbcB15 hspR4* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ΔM15]	15
CSH50-I^q	*rpsL* Δ(*lac-pro*) [F′ *traD36 proAB lacI*^{q}ΔM15]	13
Plasmids		
pMC1403	ColE1 replicon, Ap^r *lacZYA**	2
pACYC184	p15a replicon, Cm^r Tc^r	3
pKK223-3	ColE1 replicon, Ap^r *tac*	1
pOU82	R1 replicon, Ap^r *lacZYA*	6
Constructs containing *pas* genes		
pP2H	pMC1403 vector, Ap^r, *pas* region 1240–1362[b]	This work
pTac-pasA-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–1559	This work
pTac-pasB-pACYC	pACYC184 vector, Cm^r, *pas* region 1518–1816	This work
pTac-pasC-pACYC	pACYC184 vector, Cm^r, *pas* region 1789–2028	This work
pTac-pasAB-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–1816	This work
pTac-pasABC-pACYC	pACYC184 vector, Cm^r, *pas* region 1316–2028	This work
pKmM0	pTF-FC2 replicon, Km^r, 1–4910[c]	12
pKmM1	pTF-FC2 replicon, Km^r, 1–4911 *pasA*	12
pKmM2	pTF-FC2 replicon, Km^r, 1–4911 *pasB*	12
pKmM3	pTF-FC2 replicon, Km^r, 1–4911 *pasC*	12
pKmM1del1[d]	pTF-FC2 replicon, Km^r, 1–4911, Δ1217–1321[e]	12
pKmM1del2[d]	pTF-FC2 replicon, Km^r, 1–4911, Δ1232–1943[e]	12
pOU-pasABC	pOU82 R1 replicon, Ap^r, *pas* region 1158–2027	12
pOU-tac-pasABC	pOU82 R1 replicon, Ap^r, *tac*, *pas* region 1158–2027	This work

[a] Ap^r, ampicillin resistance; Km^r, kanamycin resistance; Cm^r, chloramphenicol resistance; Tc^r, tetracycline resistance; *lacZYA**, *lacZYA* genes with deletion of *lacZ* promoter and ATG; *tac*, *trp-lac* hybrid promoter.
[b] The *pas* region numbers are the nucleotide positions relative to those of the *Cla*I-*Pst*I fragment of pTV100 (5).
[c] Entire pTF-FC2 replicon from the *Cla*I site to the *Pst*I site of pTV100 including the *pas* region (5).
[d] Spontaneous deletions of the toxic plasmid pKmM1 (12).
[e] Regions missing from the spontaneous deletions after PasA antidote inactivation (12).

TABLE 2. Regulation of the *pasABC* genes in *E. coli* CSH50-I^q containing the *pas-lacZ* reporter construct, pP2H

Coresident plasmid	Avg β-galactosidase activity[a] (Miller units) ± SD	% Activity
pACYC184 (control)	252 ± 6	100
pKmM0	12 ± 1	5
pKmM2	31 ± 4	12
pKmM3	14 ± 1	6
pKmM1del1	185 ± 70	73
pKmM1del2	206 ± 9	82
pTac-pasA-pACYC	10 ± 1	4
pTac-pasB-pACYC	NA[b]	NA
pTac-pasC-pACYC	226 ± 37	90
pTac-pasAB-pACYC	2 ± 1	1
pTac-pasABC-pACYC	3 ± 2	1

[a] β-Galactosidase activity was measured three times on each of three independently selected colonies.
[b] No assay possible because of the lethality of the PasB toxin in CSH50-I^q.

linked to the *tac* promoter from the construct pTac-pasABC on a *Bam*HI-*Pvu*I (blunted) fragment and ligating them into pOU82 which had been cut with *Eco*RI (blunted) and *Bam*HI. Stability of the pOU-tac-pasABC construct was tested in the presence and absence of *pas* induction by 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 2). This was compared with the stability of the pOU82 control and pOU82-pasABC containing the *pas* genes under the control of the natural promoter. The pOU82-tac-pasABC construct was less stable than the pOU82 control even without induction of the *pas* genes from the *tac* promoter. On IPTG induction of the *pas* genes, the pOU82-tac-pasABC construct was even less stable than without induction. Lower levels of IPTG (0.5 and 1.0 mM) were also used, but the result was similar to that for 2 mM (data not shown). Autoregulatory feedback by PasA-PasB would therefore appear to be an essential feature of the proteic poison-antidote *pas* for it to stabilize a heterologous test plasmid in *E. coli*.

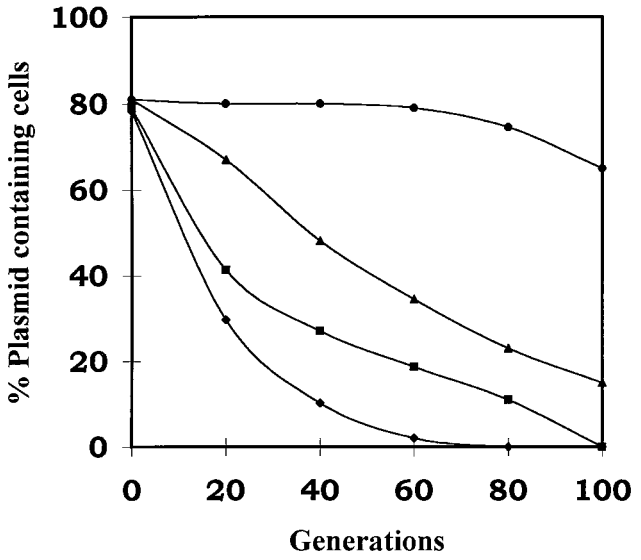


FIG. 2. Stability of plasmids in *E. coli* CSH50-I^q host cells. Cells containing pOU82 (▲), pOU82-pasABC (●), pOU-tac-pasABC without IPTG induction (■), or pOU-tac-pasABC with 2 mM IPTG (◆) are shown.

We are most grateful to Kenn Gerdes for the gift of plasmid pOU82.
This work was supported by grants from Gencor (now Billiton) Process Research (Randburg, South Africa), the Foundation for Research Development (Pretoria, South Africa) and the THRIP programme of the Department of Trade and Industry.

REFERENCES

1. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
2. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. In vitro gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **142:**971–980.
3. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
4. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
5. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli.* Gene **108:**7–14.
6. **Gerdes, K., J. E. L. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
7. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
8. **Miller, J. H.** 1983. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
9. **Rawlings, D. E., and T. Kusano.** 1994. Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:**39–55.
10. **Roberts, R. C., C. Spangler, and D. R. Helinski.** 1993. Characteristics and significance of the DNA binding activity of plasmid stabilization system protein ParD from the broad-host-range plasmid RK2. J. Biol. Chem. **268:** 27109–27117.
11. **Ruiz-Echevarrìa, M. J., A. Berzal-Herranz, K. Gerdes, and R. Díaz-Orejas.** 1991. The *kis* and *kid* genes of the *parD* maintenance system of plasmid R1 form an operon that is autoregulated at the level of transcription by the coordinated action of the Kis and Kid proteins. Mol. Microbiol. **5:**2685–2693.
12. **Smith, A. S. G., and D. E. Rawlings.** 1997. The poison antidote stability system of the broad-host range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. **26:**261–270.
13. **Smith, A. S. G., and D. E. Rawlings.** 1998. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. J. Bacteriol. **180:**187–98.
14. **Tam, J. E., and B. C. Kline.** 1989. The F plasmid ccd autorepressor is a complex of CcdA and CcdB proteins. Mol. Gen. Genet. **219:**26–32.
15. **Yannisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

ELSEVIER

FEMS Microbiology Letters 176 (1999) 269–277

FEMS MICROBIOLOGY LETTERS

Minireview

Proteic toxin-antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2

Douglas E. Rawlings *

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Received 1 April 1999; received in revised form 14 May 1999; accepted 17 May 1999

Abstract

Genes encoding toxin-antitoxin proteins are frequently found on plasmids where they serve to stabilize the plasmid within a bacterial population. The toxin-antitoxin proteins do not increase the likelihood of a progeny cell receiving a plasmid but rather function as post-segregational killing mechanisms which decrease the proportion of cells that survive after losing the plasmid. These toxin-antitoxin couples therefore act as plasmid addiction systems. Several new proteic toxin-antitoxin systems have been identified and these systems appear to be ubiquitous on the chromosomes of bacteria and archaea. When placed on plasmids, these chromosomal systems also have the ability to stabilize plasmids and in at least one case, chromosomal- and plasmid-based toxin-antitoxin systems have been shown to interact. Recent findings regarding toxin-antitoxin systems and questions that have arisen as a result of these findings are reviewed.

Keywords: Plasmid stability; Plasmid addiction; Toxin-antitoxin couple; Plasmid evolution

1. Introduction

Bacterial plasmids are extrachromosomal elements which are not essential for the survival of their host cells and therefore may become lost at cell division. Many plasmids possess mechanisms which help to reduce their loss to well below the rate predicted from their copy number. Several mechanisms that reduce the plasmid loss have been discovered. For example, the *par* region of prophage P1 and the *sop* of plasmid F depend on the presence of a centromere-like region that appears to result in the pairing of plasmids that share this region [1,2]. During cell division, there is an active distribution of one member of the pair into each of the progeny cells. Site-specific recombination systems such as the *parCBA* system of RK2 (*mrs*/*par* locus) or the *cer-xer* of ColE1 ensure that plasmid multimers that arise during replication are resolved [2]. This maximizes the number of independent units for segregation at cell division. Another type of mechanism are the plasmid addiction systems which result in the killing of progeny cells that have lost the plasmid. These post-segregational killing systems consist of two essential

* Tel.: +27 (21) 808 4866; Fax: +27 (21) 808 3611; E-mail: der@land.sun.ac.za

0378-1097/99/$20.00
PII: S0378-1097(99)00256-6

components, a stable toxin (or poison) and an unstable antitoxin (antidote). The toxins are usually proteins whereas the antidotes may be either proteins or antisense RNA [3–5]. Examples of systems where both toxin and antitoxin are proteins are the *ccd* system of plasmid F, the *parDE* system of RK2/RP4, the identical *parD* and *pem* systems of plasmids R1 and R100 and the *phd/doc* system of P1 (reviewed in [3]). The best studied example where the antitoxin is an antisense RNA molecule is the *hok/sok* system of plasmid R1 (reviewed in [5,6]). An unusual RNA-toxin RNA-antisense antidote system has been reported for the Gram-positive *Enterococcus faecalis* plasmid pAD1 [7].

2. Common features of the proteic toxin-antidote plasmid addiction systems

Proteic toxin-antidote plasmid stability systems usually consist of only two proteins, a long-lived toxin which is expressed at low levels and a short-lived, highly expressed antidote [3]. On cell division, if a progeny cell fails to inherit a plasmid, it is nevertheless likely to inherit some of the cytoplasmic toxin-antitoxin complexes. Plasmid-free cells are unable to synthesize the unstable antitoxin and are therefore not able to counter the toxic effects of the stable toxin (Fig. 1). Cell killing is dependent on the differences in stability of the antitoxin relative to the toxin. A high turnover of antitoxins is due to their degradation by cellular proteases such as Lon or Clp [7,8]. Cellular targets of two of the toxins are known. The CcdB toxin (also called LetD) of plasmid F inactivates the *Escherichia coli* host gyrase by trapping the gyrase in an inactive DNA complex [9,10]. The crystal structure of CcdB has been determined and it has been proposed that the CcdB dimer binds to the central hole of the N-terminal portion of GyrA [11]. CcdA (LetA) antidote is able to displace the CcdB toxin from the gyrase subunit A forming a CcdA-CcdB (LetA-LetD) complex and the gyrase is rejuvenated [12]. The PemK toxin of plasmid R100 is believed to function as an inhibitor of DnaB preventing the initiation of DNA replication [13]. Evidence has been obtained to suggest that the primary effect of the *pem* system is to inhibit cell division rather than killing of plasmid-free segregants [14]. The most important features of the toxin and antidote proteins of the *ccd* system of plasmid F, the *parDE* system of RK2/RP4, the *parD* and *pem* systems of plasmids R1 and R100 and the *phd/doc* system of P1

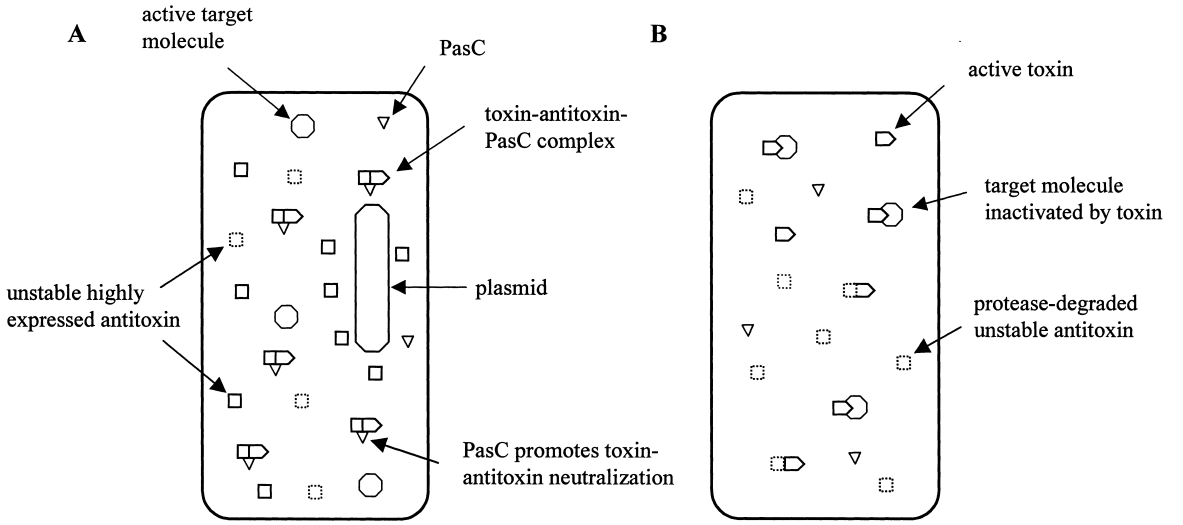


Fig. 1. Schematic diagram illustrating the proposed mechanism of action of proteic plasmid addiction systems. (A) Plasmid-containing progeny cells in which the toxin is prevented from binding to the target molecule by binding to the highly expressed but unstable antitoxin. PasC (absent in most plasmids) assists in toxin-antitoxin neutralization of the plasmid addiction system of pTF-FC2. (B) Non-viable progeny cells which have lost the plasmid and hence the ability to replace the protease-degraded antitoxins.

have been reviewed by Jensen and Gerdes [3]. Other plasmid toxin-antitoxin systems have been found on plasmid R485 from *Morganella morganii* [15] and virulence plasmid pMYSH6000 of *Shigella flexerni* [16]. In the absence of plasmid addiction systems, it is conceivable that cells which have become free of the metabolic burden imposed by the plasmid may outgrow plasmid-containing cells and the plasmid could become lost from the population.

Several workers have pointed out that DNA restriction-modification systems also constitute a proteic toxin-antitoxin couple. When present on plasmids, these toxin-antitoxin couples have the effect of stabilizing the plasmid within a population because loss of the methylase 'antitoxin' results in a loss of DNA modification and lethal attack by the restriction enzyme [17,18]. Likewise, bacteriocins (such as colicins) and their immunity proteins are found on many plasmids and constitute a toxin-antitoxin couple which kills or inhibits related plasmid-free cells [19]. A major difference between the colicins and other toxins is that the colicins are exported from cells and destroy plasmid-free cells from the outside. The effect of these systems is also to stabilize the plasmids on which they are present within a bacterial community [20].

3. The plasmid addiction system of plasmid pTF-FC2

Plasmid pTF-FC2 is a 12.2-kb broad host range, mobilizable plasmid that was isolated from a strain of the biomining bacterium *Thiobacillus ferrooxidans* [21]. This strain formed part of the inoculum used for the pretreatment of a gold-bearing arsenopyrite concentrate from the Fairview mine (Barberton district, Mpumalanga, South Africa) [22]. The plasmid has been sequenced and contains a replicon related to the IncQ plasmids, a mobilization region with a strong similarity to IncP plasmids and a transposon which is clearly Tn*21*-like [21]. Located within the replicon between the genes for the RepB primase and the RepA helicase are three small genes, *pasABC* (see Fig. 2), which encode a plasmid addiction system [23].

The TF-FC2 *pas* is unique among toxin-antitoxin plasmid stability systems analyzed to date in that it consists of three rather than two proteins. PasA is an antitoxin, PasB is a toxin and PasC is a protein that appears to enhance the ability of PasA to neutralize the toxic effects of PasB. The size of the PasA antitoxin (74 amino acids (aa)) is of the same order as other antitoxin proteins (72–84 aa) and the PasB toxin (90 aa) is slightly smaller than other toxins (101–126 aa). In general, proteins of the toxin-antitoxin systems show a large amount of sequence variation and none of the toxin proteins has been reported to have a detectable sequence similarity. Antitoxin sequences are slightly more conserved with the PasA antitoxin providing the best example of this. The pTF-FC2 PasA antitoxin is poorly but clearly related (31% aa identity) to the ParD antidote of the *parDE* system of plasmid RK2 [23]. The only other conserved plasmid-located antitoxins are the CcdA antitoxin of plasmid F and the Kid antitoxin of the ParD system of plasmid R1 which share 21% aa identity [24]. PasC, the third protein of the pTF-FC2 plasmid addiction system is a 71-aa polypeptide

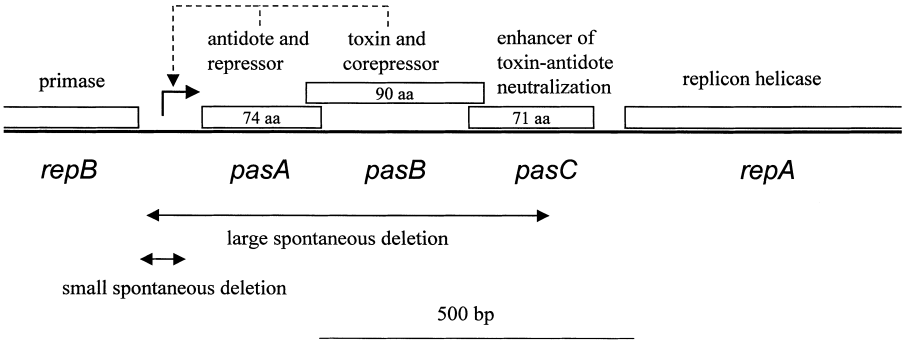


Fig. 2. Location, layout and regulation of the *pas* of pTF-FC2. The extent of the spontaneous deletion mutants which are isolated following the introduction of a frame-shift mutation within the gene for the PasA antidote are shown.

with no detectable sequence similarity to any other protein. Inactivation of PasC by the introduction of a frame-shift mutation did not have a marked effect on the stability of pTF-FC2 in *E. coli* JM105 [23]. An indication of the role of PasC came from a comparison of the toxicity of *pasAB* and *pasABC* constructs in which the *pas* genes were placed under the control of an IPTG-inducible *tac* promoter [25]. The ability of the PasA antidote to neutralize the PasB toxin was greatly enhanced when PasC was present either in *cis* or in *trans* (on a co-resident plasmid). The conclusion was that *pasAB* antitoxin-toxin genes were on their own sufficient for plasmid stability but that *E. coli* cells containing only these genes would be slow-growing compared to plasmid-free cells. The function of PasC was to reduce the overall toxicity of the system and permit cells containing *pas* to compete with plasmid-free cells.

4. Efficiency of addiction systems and strain-dependence

In general, the active partitioning systems are far more effective for plasmid stabilization (1000-fold for *sop* of F) than the toxin-antitoxin systems [3]. A comparison of four systems using a mini-R1 conditional replication system was carried out in an *E. coli* CSH50 host strain. A >100-fold stabilization was conferred by *parDE* of RP4 (RK2), 100-fold by the *hok/sok* system of R1 and 10-fold by the *ccd* of F and *parD* of R1 [14]. Using the same system and the same *E. coli* host strain, a 100-fold stabilization was achieved by the *pas* of pTF-FC2. However, if different *E. coli* host strains were used, stabilization by *pas* was also different. There was only a 2.5-fold increase in stability in *E. coli* JM105 with no increase detected in *E. coli* strains JM107 and JM109 [25]. The reason for strain variation in the stability is unknown. However, the strains in which *pas* was most effective were also the strains in which PasB was most toxic.

5. Operon structure and autoregulation

The genetic organization of antitoxin-toxin systems is usually similar to the genes for antidote and toxin located in an operon with the gene for antidote preceding that for the toxin. An exception to this is the *higB-higA* system of plasmid Rts1 where the order of toxin-antitoxin is reversed [26]. Autoregulation at the level of transcription is a general property of toxin-antidote sytems. For example, the *ccd* system of plasmid F is regulated by a CcdA-CcdB complex of 69 kDa and neither CcdA or CcdB alone is capable of autorepression [27]. In contrast, the *parDE* system of RK2 is autoregulated solely by the ParD antitoxin [28]. In other cases, although the antitoxin can serve as a repressor on its own, a combination of toxin and antitoxin is required for full repression. The *parD* locus of plasmid R1 is repressed only 30–40% by Kis on its own, but full repression occurs with the complete Kis-Kid complex [29]. Regulation of the pTF-FC2 *pas* genes was found to be similar. The PasA antidote was able to repress the operon 25-fold but when PasA and PasB were both present, repression was enhanced to 100-fold. PacC had no detectable regulatory activity [30].

A question posed by Jensen and Gerdes [3] is whether autoregulation is essential for the function of plasmid addiction systems. It is conceivable that differences in half-lives of the antitoxin and toxin proteins together with differences in the level of toxin and antitoxin translation may on their own be sufficient to increase the plasmid stability. To test this, the promoter of the *pas* system of pTF-FC2 was removed and the *pas* genes were placed behind an IPTG-inducible *tac* promoter [30]. The modified *pas* system was placed in a low copy number mini-R1 (pOU82) test plasmid system developed in the Gerdes laboratory [14]. The stability of the *tac*-regulated *pas*-containing test plasmid with and without induction by IPTG was compared with a pOU82 control and pOU82 containing the unmodified autoregulated *pas* genes. Even without IPTG induction, the *tac*-regulated *pas*-containing plasmid was less stable than the test plasmid which lacked a plasmid addiction system. On IPTG induction, this instability increased still further. The conclusion was that an autoregulated control circuit was required for the proper functioning of the pTF-FC2 *pas*.

6. Relationship between plasmid toxin-antitoxin addiction systems

Gross similarities in the operon structure, function and regulation of plasmid addiction systems have led to the suggestion that they originated from a common ancestor [3]. The discovery of a weak amino acid sequence similarity between the CcdA and Kid antitoxins of plasmids F and R1 as well as between the ParD and PasA antitoxins of plasmids RK2 and pTF-FC2 (see earlier) supports this view. More noticeable than the low levels of similarity is the great amount of toxin and antitoxin amino acid sequence divergence between addiction systems of different plasmids. Magnuson and Yarmolinsky [31] have suggested that this diversity could be explained by multiple origins of toxin-antitoxin genes, great age or a fast rate of change. The argument for a fast rate of change resulting in rapid sequence divergence is as follows. Once a toxin-antitoxin system has been acquired by a plasmid, it is likely to increase the fitness of that plasmid when competing for survival with other plasmids, especially related incompatible plasmids. Under such conditions, there is likely to be selective pressure for each plasmid to acquire its own copy of a toxin-antitoxin system. However, the value of such a system would decrease with an increasing frequency of occurrence. This would place strong selection for a rapid divergence in the components of plasmid addiction systems. A similar strong positive selection for divergence has been proposed for colicins [32].

7. Location of addiction systems on plasmids

It is possible that plasmid addiction systems may be more effective when located at a particular position on a plasmid relative to important functions like the origin of replication or the origin of conjugal transfer (*oriT*). For example, an advantage for an addiction system to be located in the leading region following the origin of transfer is that during conjugation, the addiction genes would enter the recipient first. This would enhance the possibility of an addiction system being expressed early in the transfer process and minimize the ability of the host to survive should it be successful in eliminating the plasmid on entry (for example though the use of a restriction endonuclease). Anti-restriction proteins have been found to occur in the leading region of certain plasmids so that during conjugal transfer, their early expression assists in plasmid establishment [33]. Interestingly, the *hok-sok* protein-antisense RNA addiction system of plasmid R1 (R100, NR1) and an equivalent system of plasmid F occupy a similar position to the anti-restriction proteins [34]. Examination of the location of proteic plasmid addiction systems has not revealed a pattern of placement. For example, the *parDE* genes of the IncPα plasmids RP4/RK2 are positioned close to the multimer resolution active partition system. This is 15 kb from the *oriT* and 17 kb from the origin of replication of the 60-kb plasmid [35]. In at least three plasmids, the toxin-antidote system is located close to the origin of replication. The *ccd* system of plasmid F is positioned between *ori-1* and *ori-2* and about 20 kb from the *oriT* site [36], while the genes for the Kis/Kid (PemI/PemK) proteins are within 2 kb of the origin of replication of plasmid R1 (also called NR1) [34,37].

In the case of pTF-FC2, the *pas* is also closely associated with the IncQ-like replicon and occurs between the *repB* (primase) and the *repA* (helicase) genes (Fig. 2). It may be questioned whether the *pas* is situated in this position by chance or whether there is an advantage to this placement. Circumstantial evidence suggests that the location of *pas* on pTF-FC2 may be by chance. When the *pasA* gene (antitoxin) was inactivated through the introduction of a frame-shift mutation, *E. coli* cells containing the construct produced small, slow-growing colonies presumably because of the effects of the unneutralized toxin [23]. However, occasionally, large strong-growing colonies were produced from these mutants and the region containing the *pas* system of plasmids isolated from these cells was sequenced. Two types of deletion were detected, one in which *pasAB* and most of *pasC* was missing and the other in which the promoter region of *pas* was deleted. In both cases, the plasmid copy number was unchanged. Therefore, in spite of their presence between *repB* and *repA*, the *pas* genes play no role in plasmid replication. This is in contrast to the IncQ plasmids (RSF1010, R1162) where one of the two small genes (*cac*) situated between *repB* and *repA* has been

shown to be a regulator of plasmid replication [38]. This pair of genes does not also function as a toxin-antitoxin plasmid addiction system [39]. It has been suggested that plasmids are composed of modules that have been acquired independently of each other. The location of the pTF-FC2 *pas* supports this view as the toxin-antitoxin module appears to have become inserted within the replicon in a way that has not interfered with plasmid replication.

8. Chromosomally located proteic toxin-antidote genes

Several toxin-antitoxin genes of which both products are proteins have been identified on the chromosome of *E. coli*. These include the *chpAI–chpAK* genes located downstream of the *relA* locus and the *chpBI–chpBK* genes found in the *ppa* region [40]. When cloned into plasmids, these genes result in plasmid stabilization by post-segregational killing of plasmid-free cells in a manner similar to plasmid-based systems. Not only are the chromosomal *chpA* and *chpB* systems related to the *parD*/*pem* systems of plasmids R1/R100 but it has also been shown that these systems can functionally interact [41]. The *chpAI–chpAK* gene products appear to be homologues of the Kis-Kid (PemI–PemK) proteins of plasmid R1 (R100). Cells containing a temperature sensitive Kis antitoxin mutant were unable to grow due to the inability to neutralize the Kid toxin at 42°C. In the presence of the ChpAI antitoxin, growth at 42°C was partially restored. This partial functional complementation occurred even though the sequences of the antitoxin proteins have only 22% amino acid identity. In vitro mutagenesis was used to isolate mutants with an improved ability to neutralize the Kid toxin. These mutations were all located in the N-terminal region of the antitoxin and did not alter the amino acid sequence identity between the ChpAI and Kis antitoxins [41]. This observation indicates that although proteins of the plasmid and chromosomal toxin-antitoxin systems might appear to be dissimilar, they may nevertheless be able to functionally react with each other. This may be taken as evidence of a common ancestry for chromosomal and plasmid toxin-antitoxin systems which is not apparent from direct sequence comparisons.

More recently, it has been demonstrated that the *relBE* genes present on the chromosome of *E. coli* K-12 belong to a new family of toxin-antitoxin genes [42]. These authors demonstrated that *relE* encodes a toxin and that *relB* encodes an antitoxin. *relBE* stabilized a mini-R1 test plasmid when present in an *E. coli relBE* chromosomal mutant and *relBE* was autoregulated with the RelB antitoxin serving as repressor and the RelE toxin as co-repressor. Database searching revealed that a second copy of related genes (*dinJ* and *yafQ*) was present on the chromosome of *E. coli* K-12 and that *relBE* gene equivalents were present on the chromosomes of *Haemophilus influenzae*, *Vibrio cholerae* and an enterotoxin encoding plasmid p307 [42]. As with other toxin-antitoxin systems, the degree of amino acid identity of the *relBE* family was rather low with the degree of RelB amino acid sequence identity ranging from 20 to 48% and that of RelE from 14 to 55%. Besides the Gram-negative bacteria, RelBE-like systems have been found to be widespread on the chromosomes of Gram-positive bacteria and archaea [43].

9. The role of toxin-antitoxin systems

As described earlier, poison-antidote plasmid stability mechanisms are rather ineffective compared to active plasmid stability mechanisms [3]. Furthermore, the killing of plasmid-free segregants would represent a reproductive loss for the plasmid and therefore be less favorable than an active partitioning system. This raised the question whether plasmid stability is their only function. Discovery of the role of toxin-antitoxin systems on the chromosomes of bacteria may indicate alternate roles for toxin-antitoxin couples and possible origins of the plasmid toxin-antitoxin systems.

There are clear indications that at least some chromosomal proteic poison-antitoxin systems may play a role in the stringent-relaxed response which occurs when bacteria such as *E. coli* face amino acid starvation. In their discussion on the role of RelBE, Gotfredsen and Gerdes [42] describe previous studies which demonstrated that *E. coli relB* mutants exhibit the so-called delayed relaxed response. This is based

on the observation that during amino acid starvation, stable RNA synthesis stops, but then resumes after a delay of about 10 min. It was speculated that the RelE toxin may be a protein synthesis inhibitor and that inactivation of RelB antitoxin may allow active RelE to decrease protein synthesis. This would in turn lead to recharging of tRNA and result in the delayed relaxed response observed.

Expression of the *E. coli relA*-associated chromosomal *chpA* locus (also called *mazEF*) has also been linked to the *E. coli* stringent response [44]. The promoter of *mazEF* is inhibited by a (p)ppGpp-dependent mechanism. Amino acid starvation might lead to the induction of *mazEF* and cell killing [45]. Killed cells would release nutrients which would permit other members of the culture to survive until conditions improved. It is interesting that the product of the bacteriophage λ *rexB* gene, one of the few to be expressed by λ when it is in the lysogenic state, inhibits degradation of the MazE antitoxin [46]. This allows phage λ to counter the effects of the *mazEF* system and to survive under conditions of nutrient stress.

10. Evolution of plasmid addiction systems

Given current information, one may speculate on how plasmid toxin-antitoxin addiction may have evolved (Fig. 3). Whatever the function of chromosomal toxin-antitoxin systems is found to be, such systems could have provided the source of genes for plasmid addiction systems. It is well-established

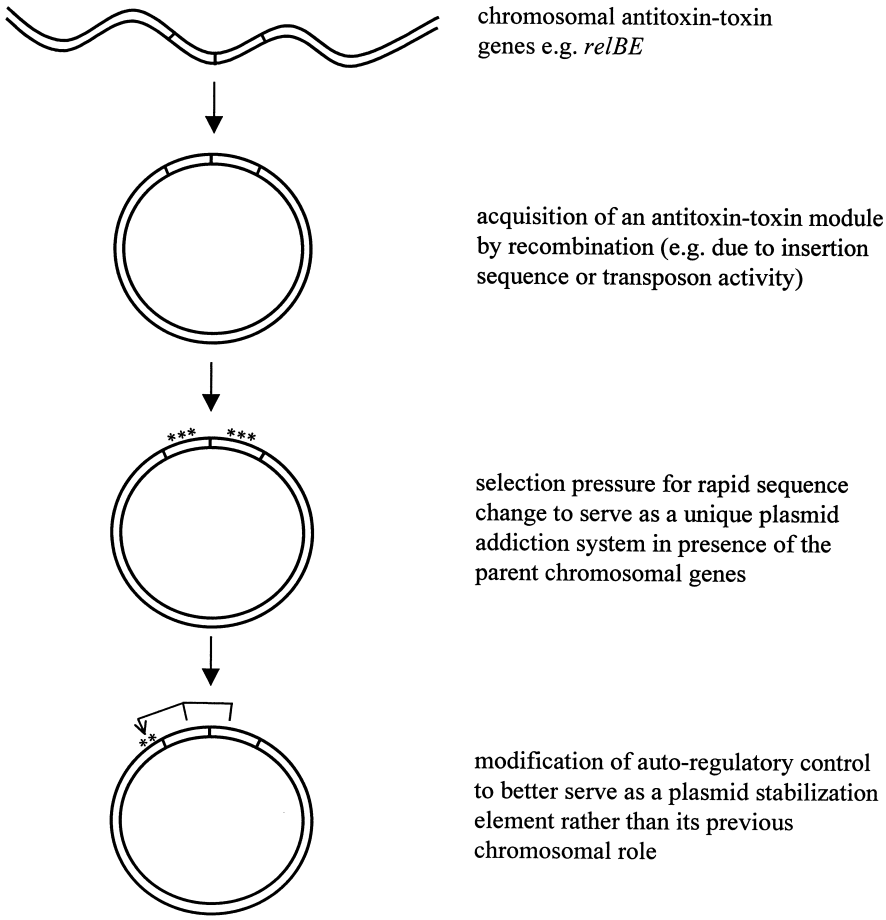


Fig. 3. A model for the evolution of proteic toxin-antitoxin plasmid addiction systems.

that as a result of transposon or insertion sequence activity, genes are able to move fairly readily between chromosomes and plasmids. It may be speculated that genes which have a physiological function on the chromosome have been captured by plasmids where they provide a selective advantage for the plasmid. Once captured on a plasmid, the addiction systems would be under a strong positive selective pressure to diversify to ensure that the advantages to be gained from the system were available to a particular plasmid and not to competing plasmids. The promoter regions may then have been modified so as to become independent of regulation by cellular functions such as the stringent response, thereby becoming more suited to their role in enhancing plasmid survival. It would be interesting to test whether any of the plasmid-based systems retains remnants of control by (p)ppGpp and stringent response regulators. Evidence that the plasmid Kis/Kid and chromosomal ChpAI-KI systems can interact supports this evolutionary model. It is possible that the acquisition of toxin-antitoxin genes occurred in the reverse direction from plasmids to the chromosome. However, since plasmid addiction systems are not very efficient in plasmid stabilization, this possibility is less likely.

References

[1] Nordström, K. and Austin, S.J. (1989) Mechanisms that contribute to the stable segregation of plasmids. Annu. Rev. Genet. 23, 37–69.

[2] Williams, D.R. and Thomas, C.M. (1992) Active partitioning of bacterial plasmids. J. Gen. Microbiol. 138, 1–16.

[3] Jensen, R.B. and Gerdes, K. (1995) Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. 17, 205–210.

[4] Gerdes, K., Gultyaev, A.P., Franch, T., Pedersen, K. and Mikkelsen, N.D. (1997) Antisense RNA-regulated programmed cell death. Annu. Rev. Genet. 31, 1–31.

[5] Holcík, M. and Iyer, V.N. (1997) Conditionally lethal genes associated with bacterial plasmids. Microbiology 143, 3403–3416.

[6] Weaver, K.E., Jensen, K.D., Colwell, A. and Sriram, S.L. (1996) Functional analysis of the *Enterococcus faecalis* plasmid pAD1-encoded stability determinant par. Mol. Microbiol. 20, 53–63.

[7] van Melderen, L., Bernard, P. and Couturier, M. (1994) Lon-dependent proteolysis of CcdA is the key control for activation of CcdB in plasmid-free segregant bacteria. Mol. Microbiol. 11, 1151–1157.

[8] Lehnherr, H. and Yarmolinsky, M.B. (1995) Addiction protein Phd of plasmid prophage P1 is a substrate the C1pXP serine protease of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 92, 3274–3277.

[9] Bernard, P., Kezdy, K.E., Van Melderen, L., Steyaert, J., Wyns, L., Pato, M.L., Higgins, P.N. and Couturier, M. (1993) The F plasmid CcdB protein induces efficient ATP-dependent DNA cleavage by gyrase. J. Mol. Biol. 234, 534–541.

[10] Couturier, M., Bahassiel, M. and Van Melderen, L. (1998) Bacterial death by gyrase poisoning. Trends Microbiol. 6, 269–275.

[11] Loris, R., Dao-Thi, M.-H., Bahassi el-, M., Van Melderen, L., Poortmans, F., Liddington, R., Couturier, M. and Wyns, L. (1998) Crystal structure of CcdB, a topoisomerase poison from *E. coli*. J. Mol. Biol. 285, 1667–1677.

[12] Maki, S., Takiguchi, S., Horiuchi, T., Sekimizu, K. and Miki, T. (1996) Partner switching mechanisms in inactivation and rejuvenation of *Escherichia coli* DNA gyrase by F plasmid proteins LetD (CcdB) and LetA (CcdA). J. Mol. Biol. 256, 473–482.

[13] Ruiz-Echevarría, M.J., Giménez-Gallego, G., Sabariegos-Jareño, R. and Díaz-Orejas, R. (1995) Kid, a small protein of the *parD* stability system of plasmid R1, is an inhibitor of DNA replication acting at the initiation of DNA synthesis. J. Mol. Biol. 247, 568–577.

[14] Jensen, R.B., Grohmann, E., Schwab, H., Díaz-Orejas, R. and Gerdes, K. (1995) Comparison of *ccd* of F, *par*DE of RP4, and *par*D of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. 17, 211–220.

[15] Hayes, F. (1998) A family of stability determinants in pathogenic bacteria. J. Bacteriol. 180, 6415–6418.

[16] Radnedge, L., Davis, M.A., Youngren, B. and Austin, S.J. (1997) Plasmid maintenance functions of the large virulence plasmid of Shigella flexerni. J. Bacteriol. 179, 3670–3675.

[17] Naito, T., Kusano, K. and Kobayashi, I. (1995) Selfish behavior of restriction-modification systems. Science 267, 897–899.

[18] Kulakauskas, S., Lubys, A. and Ehrlich, D. (1995) DNA Restriction-modification systems mediate plasmid maintenance. J. Bacteriol. 177, 3451–3454.

[19] Braun, V., Pilsl, H. and Groß, P. (1993) Colicins: Structures, modes of action, transfer through membranes, and evolution. Arch. Microbiol. 161, 199–206.

[20] Lauffenburger, D.A. (1987) Bacteriocin production as a method of maintaining plasmid-bearing cells in continuous culture. TIBTECH 5, 87–89.

[21] Rawlings, D.E. and Kusano, T. (1994) Molecular genetics of *Thiobacillus ferrooxidans*. Microbiol. Rev. 58, 39–55.

[22] Rawlings, D.E. and Silver, S. (1995) Mining with microbes. Bio/Technology 13, 773–778.

[23] Smith, A.S. and Rawlings, D.E. (1997) The poison-antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. 26, 961–970.

[24] Ruiz-Echevarría, M.J., de Torrontegui, G., Giménez-Gallego, G. and Díaz-Orejas, R. (1991) Structural and functional comparison between the stability systems ParD of plasmid R1 and Ccd of plasmid F. Mol. Gen. Genet. 225, 355–362.

[25] Smith, A.S.G. and Rawlings, D.E. (1998) Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. J. Bacteriol. 180, 5458–5462.
[26] Tian, Q.B., Ohnishi, M., Tabuchi, A. and Terawaki, Y. (1996) A new plasmid-encoded proteic killer gene system: cloning, sequencing, and analyzing *hig* locus of plasmid Rts1. Biochem. Biophys. Res. Commmun. 220, 280–284.
[27] Tam, J.E. and Kline, B.C. (1989) The F plasmid *ccd* autorepressor is a complex of CcdA and CcdB proteins. Mol. Gen Genet. 219, 26–32.
[28] Roberts, R.C., Spangler, C. and Helinski, D.R. (1993) Characteristics and significance of DNA binding activity of plasmid stabilization protein ParD from the broad host range plasmid RK2. J. Biol. Chem. 268, 27109–27117.
[29] Ruiz-Echevarria, M.J., Berzal-Herranz, A., Gerdes, K. and Diaz-Orejas, R. (1991) The *kis* and *kid* genes of the *par*D maintenance system op plasmid R1 form an operon that is autoregulated at the level of transcription by the co-ordinated action of the Kis and Kid proteins. Mol. Microbiol. 5, 2685–2693.
[30] Smith, A.S.G. and Rawlings, D.E. (1998) Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system *(pas)* is essential for plasmid stabilization. J. Bacteriol. 180, 5463–5465.
[31] Magnuson, R. and Yarmolinsky, M.B. (1998) Corepression of the P1 addiction operon by PhD and Doc. J. Bacteriol. 180, 6342–6351.
[32] Riley, M.A., Tan, Y. and Wang, J. (1994) Nucleotide polymorphism in colicin E1 and Ia plasmids from natural isolates of *Escherichia coli*. Proc. Natl. Acad. Sci. USA 91, 11276–11280.
[33] Althorpe, N.J., Chilley, P.M., Thomas, A.T., Brammar, W.J. and Wilkins, B.M. (1999) Transient transcriptional activation of the Incl1 plasmid anti-restriction gene *(ardA)* and SOS inhibition gene *(psiB)* early in conjugating recipient bacteria. Mol. Microbiol. 31, 133–142.
[34] Womble, D.D. and Rownd, R.H. (1988) Genetic and physical map of plasmid NR1: Comparison with other IncFII antibiotic resistance plasmids. Microbiol. Rev. 52, 433–451.
[35] Pansegrau, W., Lanka, E., Barth, P.T., Figurski, D.H., Guiney, D.G., Haas, D., Helinski, D.R., Schwab, H., Stanisich, V.A. and Thomas, C.M. (1994) Complete nucleotide sequence of Birmingham IncPα plasmids. J. Mol. Biol. 239, 623–663.
[36] Murakami, Y., Ohmori, H., Yura, T. and Nagata, T. (1987) Requirement of the *Escherichia coli dnaA* gene function for *ori*-2-dependent mini-F plasmid replication. J. Bacteriol. 169, 1724–1730.
[37] Bravo, A., Ortega, S., de Torrontegui, G. and Diaz, R. (1988) Killing of *Escherichia coli* cells modulated by components of the stability system ParD of plasmid R1. Mol. Gen. Genet. 215, 146–151.
[38] Maeser, S., Scholtz, P., Otto, S. and Scherzinger, E. (1990) Gene F of the plasmid RSF1010 codes for a low-molecular-weight repressor protein that autoregulates expression of the *repAC* operon. Nucleic Acids Res. 18, 6215–6222.
[39] Smith, A.S.G.S. (1997) Ph.D. thesis, University of Cape Town, Rondebosch.
[40] Masuda, Y., Miyakawa, K., Nishimura, Y. and Ohtsubo, E. (1993) *chpA* and *chpB*, *Escherichia coli* chromosomal homologues of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. 175, 6850–6856.
[41] Santos-Sierra, S., Giraldo, R. and Diaz-Orejas, R. (1997) Functional interactions between homologous conditional killer systems of plasmid and chromosomal origin. FEMS Microbiol. Lett. 152, 51–56.
[42] Gotfredsen, M. and Gerdes, K. (1998) The *Escherichia coli relBE* belong to a new toxin-antitoxin gene family. Mol. Microbiol. 29, 1065–1076.
[43] Grønlund, H. and Gerdes, K. (1999) Toxin-antitoxin systems homologous with *relBE* of *Escherichia coli* plasmid p307 are ubiquitous in prokaryotes. J. Mol. Biol. 285, 1401–1415.
[44] Aizenman, E., Engelberg-Kulka, H. and Glaser, G. (1995) An *Escherichia coli* chromosomal 'addiction module' regulated by 3′, 5′-bispyrophosphate: A model for programmed bacterial cell death. Proc. Natl. Acad. Sci. USA 93, 6059–6063.
[45] Nyström, T. (1998) To be or not to be: the ultimate decision of the growth-arrested bacterial cell. FEMS Microbiol. Rev. 21, 283–290.
[46] Engelberg-Kulka, H., Reches, M., Narasimhan, S., Schoulaker-Schwarz, R., Klemes, Y., Aizenman, E. and Glaser, G. *rexB* of bacteriophage λ is an anti-cell death gene. Proc. Natl. Acad. Sci. USA 95, pp. 15481–15486.

JOURNAL OF BACTERIOLOGY, June 2001, p. 3303–3309 Vol. 183, No. 11
0021-9193/01/$04.00+0 DOI: 10.1128/JB.183.11.3303–3309.2001

Isolation of a New Broad-Host-Range IncQ-Like Plasmid, pTC-F14, from the Acidophilic Bacterium *Acidithiobacillus caldus* and Analysis of the Plasmid Replicon

MURRAY N. GARDNER, SHELLY M. DEANE, AND DOUGLAS E. RAWLINGS*

Department of Microbiology, University of Stellenbosch, Matieland 7602, South Africa

Received 13 December 2000/Accepted 16 March 2001

A moderately thermophilic (45 to 50°C), highly acidophilic (pH 1.5 to 2.5), chemolithotrophic *Acidithiobacillus caldus* strain, f, was isolated from a biooxidation process used to treat nickel ore. Trans-alternating field electrophoresis analysis of total DNA from the *A. caldus* cells revealed two plasmids of approximately 14 and 45 kb. The 14-kb plasmid, designated pTC-F14, was cloned and shown by replacement of the cloning vector with a kanamycin resistance gene to be capable of autonomous replication in *Escherichia coli*. Autonomous replication was also demonstrated in *Pseudomonas putida* and *Agrobacterium tumefaciens* LBA 4404, which suggested that pTC-F14 is a broad-host-range plasmid. Sequence analysis of the pTC-F14 replicon region revealed five open reading frames and a replicon organization like that of the broad-host-range IncQ plasmids. Three of the open reading frames encoded replication proteins which were most closely related to those of IncQ-like plasmid pTF-FC2 (amino acid sequence identities: RepA, 81%; RepB, 78%; RepC, 74%). However, the two plasmids were fully compatible and pTC-F14 represents a new IncQ-like plasmid replicon. Surprisingly, asymmetrical incompatibility was found with the less closely related IncQ plasmid R300B derivative pKE462 and the IncQ-like plasmid derivative pIE1108. Analysis of the pTC-F14 *oriV* region revealed five direct repeats consisting of three perfectly conserved 22-bp iterons flanked by iterons of 23 and 21 bp. Plasmid pTC-F14 had a copy number of 12 to 16 copies per chromosome in both *E. coli,* and *A. caldus*. The *rep* gene products of pTC-F14 and pTF-FC2 were unable to functionally complement each other's *oriV* regions, but replication occurred when the genes for each plasmid's own RepA, RepB, and RepC proteins were provided in *trans*. Two smaller open reading frames were found between the *repB* and *repA* genes of pTC-F14, which encode proteins with high amino acid sequence identity (PasA, 81%; PasB, 72%) to the plasmid addiction system of pTF-FC2. This is the second time a plasmid stability system of this type has been found on an IncQ-like plasmid.

Plasmids of *Escherichia coli* incompatibility group Q and related IncQ-like plasmids have a very broad host range, being capable of replication in a wide variety of gram-negative and also gram-positive bacteria (10). Although none of the IncQ and related plasmids are self-transmissible, they are efficiently mobilized by IncPα (RK2, RP4, and R68) and IncPβ (R751) plasmids (5). As a result of their host range and mobility, these plasmids are highly promiscuous. The best-studied IncQ plasmids are RSF1010 (11), R1162 (20), and R300B (2), which are identical, or nearly identical, in spite of having been isolated from different hosts (10). RSF1010 (8,486 bp) has been completely sequenced, and the replication and mobilization functions of RSF1010 and R1162 have been extensively studied (30).

The discovery of several other plasmids which have replicons with clear similarity to IncQ plasmids has been reported. These are IncQ-like plasmids pTF-FC2 (12,180 bp) (7, 8), isolated from the biomining bacterium *Acidithiobacillus ferrooxidans* (previously *Thiobacillus ferrooxidans*); pIE1107 (8,520 bp), isolated from a mixture of bacteria present in pig manure (35); and pDN1 (5,112 bp), isolated from the sheep foot rot-causing pathogen *Dichelobacter nodosus* (37). In addition, sequences of at least two other IncQ-related plasmids, pIE1115 and pIE1130 (both 10,687 bp), appear in sequence databases although only research on the isolation of these has been published so far (31).

IncQ replicons contain three essential replication genes (*repA*, *repB* [*repB'*]), and *repC*) and an *oriV* region (28). The *repA* gene encodes a plasmid-specific helicase, *repB'* encodes a primase, and *repC* encodes a plasmid-specific DNA-binding initiation protein. The *oriV* region consists of 3.5 20-bp iterons and an approximately 500-bp flanking region containing G+C-rich and A+T-rich sequences and two plasmid-specific single-stranded initiation sites (*ssiA* and *ssiB*). The IncQ-related replicons have similar structures, although there are differences in some small replicon-associated proteins and the numbers and sequences of their iterons. The ability of IncQ-like plasmids to displace each other when coresident in *E. coli* has been tested, and it is clear that these plasmids may be divided into several distinguishable incompatibility subgroups.

Acidithiobacillus caldus (previously *Thiobacillus caldus*) is a sulfur-oxidizing, chemolithotrophic, obligately acidophilic (pH 1.5 to 2.5), and moderately thermophilic (45 to 50°C) bacterium (12). These bacteria, together with iron-oxidizing bacteria of the genus *Leptospirillum*, have been found to be the dominant organisms present in biooxidation tanks used in certain commercial processes for the recovery of gold from arsenopyrite ores (25). While investigating plasmids from strains of *A. caldus* isolated from biooxidation tanks, we discovered a 14-kb

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland, South Africa. Phone: 27 21 808 5848. Fax: 27 21 808 5846. E-mail: der@land.sun.ac.za.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description[a]	Reference or source
Strains		
E. coli DH5α	ϕ80d*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17*($r_K^- m_K^-$) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U169*	Promega Corp.
E. coli GW125a	*recA polA* mutant of AB1157	6
A. caldus strain f	Isolated from Billiton Process Research Bionic plant (Randburg, South Africa)	This study
A. tumefaciens LBA4404	Nononcogenic, T-DNA deletion, *vir* intact	13
P. putida	Prototrophic	9
Plasmids		
pTC-F14	Natural 14.4-kb plasmid from *A. caldus* strain f	This study
pTC-F14Cm	Cm^r (Cm^r gene cloned into pTC-F14)	This study
pIb	Ap^r (unique *Xba*I site of pTC-F14 interrupted by pBluescript)	This study
pK13	Ap^r (unique *Bam*HI site of pTC-F14 interrupted by pBluescript)	This study
pTF-FC2	Natural 12.2-kb plasmid from *A. ferrooxidans* FC2	26
pKE462	Tc^r As^r, R300B replicon	6
pIE1108	St^r Km^r, pIE1107 replicon with set of nonessential IncQ iterons deleted	35
pDER404	Cm^r, pTF-FC2 *Cla*I-*Pst*I fragment complete replicon	26
pTV4164	Ap^r, pTF-FC2 *oriV* fragment	8
pTC-F101	Km^r, pTC-F14 replicon (Fig. 1)	This study
pTC-F108	Ap^r, pUCBM21 vector (Fig. 1)	This study
pTC-F109	Ap^r, pGEM-T vector (Fig. 1)	This study
pACYC184	Tc^r Cm^r, p15A replicon, cloning vector	4
pBluescript (KS)	Ap^r *lacZ′*, ColE1 replicon, vector	Stratagene
pUCBM21	Ap^r *lacZ′*, ColE1 replicon, vector	Roche Molecular Biochemicals
pGEM-T	Ap^r, T-tailed PCR product cloning vector	Promega Corp.
Primers		
TACREPA (*Eco*RI)	5′-TATTGAATTCCCCGGCAGCGCC-3′	This study
TACREPAE (*Pst*I)	5′-TATTCTGCAGAGGGGGTGCGATAGC-3′	This study
SEQORI	5′-TATCGAGATGGCAGAGGTGCGAG-3′	This study
ORIR (*Hin*dIII)	5′-TGTCAAGCTTGGCACTCTCCTG-3′	This study

[a] Ap, ampicillin resistance; Cm, chloramphenicol resistance; Km, kanamycin resistance; St, streptothricin resistance; Tc, tetracycline resistance.

plasmid which was capable of replication in an *E. coli polA* mutant. Here we report on the characterization of this IncQ-related plasmid replicon and its biological relationship to other IncQ and IncQ-related plasmids.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *E. coli* cells were grown in Luria medium, and ampicillin (100 μg/ml), kanamycin (30 μg/ml), and tetracycline (20 μg/ml) were added as required. *Pseudomonas putida* was grown in Luria medium, and kanamycin (50 μg/ml) was added when appropriate. *Agrobacterium tumefaciens* LBA 4404 was grown in Luria medium which included rifampin (5 μg/ml) and, when required, kanamycin (30 μg/ml). The tetrathionate medium used to culture *A. caldus* strain f was made from a mineral salts solution containing the following (grams per liter): $(NH_4)_2SO_4$, 3.0; KCl, 0.1; K_2HPO_4, 0.5; $MgSO_4$, 0.7; H_2O, 0.5; $Ca(NO_3)_2$, 0.4; H_2O, 0.014; Na_2SO_4, 1.45. The pH was adjusted to 2.5 with H_2SO_4, and the mixture was autoclaved. The trace element solution used contained the following (milligrams per liter): $ZnSO_4$, 0.7; H_2O, 10.0; $CuSO_4$, 0.5; H_2O, 1.0; $MnSO_4$, 0.4; H_2O, 1.0; $CoCl_2$, 0.6; H_2O, 0.5; $Cr_2(SO_4)_3$, 0.15; H_2O, 0.5; $Na_2B_4O_7$, 0.10; H_2O, 0.5; $NaMoO_4$, 0.2; H_2O, 0.5. The mixture was autoclaved, and 1 ml was added per liter. Filter-sterilized $K_2S_2O_6$ was added to a final concentration of 10 mM, and the pH was adjusted to 2.5 with H_2SO_4. *A. caldus* was grown at 37°C with constant shaking.

DNA techniques, sequencing, and analysis. Plasmid preparation, restriction endonuclease digestions, gel electrophoresis, ligations, and Southern blot hybridization were carried out by standard methods (29). Labeling of probes, hybridization, and detection were done by using the digoxigenin-dUTP nonradioactive DNA labeling and detection system (Roche Molecular Biochemicals). Sequencing was done by the dideoxy-chain termination method, with an ABI PRISM 377 automated DNA sequencer, and the sequence was analyzed by using a variety of software programs but mainly the personal-computer-based DNAMAN (version 4.1) package from Lynnon BioSoft. Comparison searches were performed using the gapped-BLAST program of the National Center for Biotechnology Information (1). Homology trees were constructed using the Multiple Sequence Alignment tool in DNAMAN. The PCR was carried out with the primers described in Table 1. The reaction was performed in a PCR Sprint Temperature Cycling System (Hybaid) using the Expand High Fidelity PCR System DNA polymerase (Roche Molecular Biochemicals). After an initial denaturation of 60 s at 94°C, 25 cycles of 30 s at 94°C, 30 s at 63°C (for primers TACREPA and TACREPAE) or 55°C (for primers SEQORI and ORIR), and 90 s at 72°C were performed. A final extension step of 120 s at 72°C before cooling to 4°C completed the reaction.

Harvesting of bacteria and preparation of chromosomal DNA. Cells were recovered by centrifugation and washed three times in acidified water (pH 1.8), and extraneous sulfur species were removed by a process of low- and high-speed centrifugation. Washed cell pellets were resuspended in TE (0.01 M Tris, 0.001 M EDTA)–0.15 M NaCl (pH 7.6) buffer. Cells resuspended in TE-NaCl (pH 7.6) buffer were used for the preparation of chromosomal DNA as described by Breed et al. (3).

TAFE. Tetrathionate-grown cells were washed twice and resuspended in SET buffer (25% sucrose, 2 mM EDTA, 50 mM Tris, pH 8) to give an optical density at 600 nm of 1. The cells were set in an equal volume of 2% LMP agarose (SeaPlaque; FMC Bioproducts) in the presence of proteinase K at 1 mg/ml. The plugs were incubated in ESP buffer (0.5 M EDTA [pH 8], 1% sodium lauryl sarcosine, proteinase K at 1 mg/ml) for 16 h at 50°C (repeated twice) to lyse the cells. Proteinase K was inactivated by incubation of the plugs in TE buffer containing 5 mM Pefabloc (Roche Molecular Biochemicals) for 16 h at 4°C. The DNA-containing plugs were washed for 30 min at 4°C in 5 volumes of distilled water, pre-equilibrated, in restriction buffer for 1 h at 4°C, and digested according to the supplier's instructions, in 3 volumes of fresh restriction buffer containing restriction enzyme. Trans-alternating field electrophoresis (TAFE) was performed with a Beckman GeneLine apparatus. DNA fragments were separated in a 1% agarose (SeaKem LE; FMC Bioproducts) gel at 150 mA and 12°C for 16 h with a pulse interval of 13 s.

Plasmid copy number determination. Estimation of plasmid copy number was performed in a slot blot experiment by hybridizing plasmid DNA to known

amounts of total DNA isolated from plasmid-containing cells and known amounts of purified plasmid DNA. By comparing the amounts of total DNA and plasmid DNA giving equivalent hybridization signals, the amount of plasmid DNA in a sample of total DNA could be estimated. As the approximate sizes of the plasmid and chromosomal DNAs are known, it was possible to calculate the number of copies of each plasmid per chromosome.

Incompatibility assay. The ability of an incoming plasmid to displace a resident plasmid was used as the test for incompatibility. Transformation-competent, plasmid-containing *E. coli* DH5α host cells were transformed with a second plasmid and plated on antibiotic-containing medium which selected only for the incoming plasmid. Transformants were restreaked so as to obtain colonies derived from single cells on solid medium containing an antibiotic which again selected only for the newly acquired plasmid. Ten colonies were picked and plated onto two sets of solid medium containing a single antibiotic to separately test for the presence of the newly acquired plasmid or the plasmid which was resident at the start of the experiment. Controls to check for spontaneous loss of resident plasmids were carried out using the same procedure, except that plasmid-containing, competent *E. coli* cells were taken through two cycles of cell growth on solid medium without antibiotic selection before testing for retention of the resident plasmid.

Host range determination. Electroporation of *P. putida* was performed with a Gene Pulser electroporation apparatus (Bio-Rad Laboratories) by using the protocol described previously (16). *P. putida* was grown to mid-log phase (optical density at 600 nm, 0.4) at 30°C, harvested by centrifugation, and washed once in sterile cold water. The cells were then washed twice in sterile cold 300 mM sucrose (electroporation buffer). The washed cells were resuspended in the electroporation buffer, and 100 μl of this sample was placed in a prechilled electroporation cuvette with pTC-F101 plasmid DNA. The electrical settings were as follows: voltage, 12.5 kV/cm; capacitance, 25 μF; pulse controller parallel resistance, 200 Ω. Immediately after discharge, 900 μl of Luria medium was added to the electroporated cells, which were then incubated at 30°C for 2 to 3 h prior to plating on selective medium. Triparental mating (26) was used to transform *A. tumefaciens* LBA 4404.

Nucleotide sequence accession number. The nucleotide sequence determined in this study has been deposited in the GenBank database under accession number AF325537.

RESULTS

Isolation of pTC-F14 and identification of the plasmid replicon. *A. caldus* strain f was isolated from a pilot plant used to treat a nickel concentrate situated at the Billiton Process Engineering Laboratory (Randburg, South Africa). Pulsed-field gel electrophoresis of total *A. caldus* DNA indicated that strain f contained at least two plasmids, one of approximately 14 kb and one of about 45 kb (data not shown). Restriction endonuclease mapping of the mixed plasmid preparation suggested that the smaller plasmid contained unique *Xba*I and *Bam*HI sites. These sites were used to clone the 14-kb plasmid, called pTC-F14, into the *E. coli* pBluescript KS cloning vector using both the *Xba*I (plasmid pIb) and *Bam*HI (plasmid pK13) sites (Fig. 1). The resulting clones were used to transform an *E. coli polA* mutant (GW125a), but only pK13 produced transformants. As ColE1-based cloning vectors are unable to replicate in an *E. coli polA* mutant strain, this result was interpreted as indicating that pTC-F14 had a replicon which was capable of independent replication in *E. coli* and that disruption of the *Xba*I site had inactivated this replicon. This interpretation was confirmed by deleting the cloning vector inserted at the pTC-F14 *Bam*HI site and replacing it with a chloramphenicol resistance gene. The resulting construct retained the ability to replicate in both the *E. coli polA* mutant and *polA* wild-type cells. A 6.4-kb *Hin*dIII-*Sph*I fragment spanning the *Xba*I site was ligated to a kanamycin resistance gene, and the resulting construct (pTC-F101) was able to replicate in *E. coli*, confirming that cloning at the *Xba*I site had disrupted the pTC-F14 replicon.

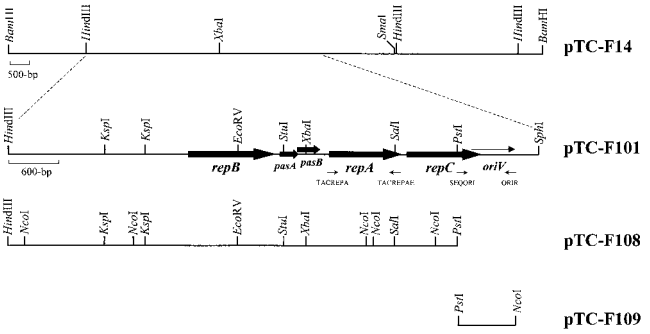


FIG. 1. Restriction enzyme and genetic maps of the pTC-F14 replicon and subclones constructed in this study. Genes are shown as solid black or grey arrows, and the *oriV* region is indicated by the long, thin arrow. The positions of the primers used to amplify *repA* and construct pTC-F109 are shown by small arrows.

Comparative analysis of the replication region. A 4-kb region incorporating the *rep* genes and *oriV* (Fig. 1) was sequenced in both directions. The G+C mole percent ratio of this region is 60%, which is typical for IncQ and IncQ-like plasmids (59 to 62%). It contains three open reading frames with high predicted amino acid sequence identity to the RepA, RepB, and RepC proteins of other IncQ-like plasmids (Fig. 2). The highest similarity was to the RepA, RepB, and RepC proteins of *A. ferrooxidans* plasmid pTF-FC2 (Table 2). Two small open reading frames with high amino acid sequence identity to the PasA and PasB proteins of pTF-FC2 (32) were located between the *repB* and *repA* genes (Fig. 1 and Table 2). The *pas* genes of pTF-FC2 have been shown to function as a plasmid addiction system which enhances plasmid stability through postsegregational killing of plasmid-free cells. The region of pTC-F14 which was required in *cis* for the plasmid to replicate was situated on a 716-bp fragment between the *Pst*I and *Nco*I sites (Fig. 1). This *oriV* region contained five iterons, with the central three 22-bp iterons being perfectly conserved while flanking iterons had either a single-base-pair insertion or a single-base-pair deletion (Fig. 3). The iterons overlapped the predicted C terminus of the RepC protein. IncQ plasmids are

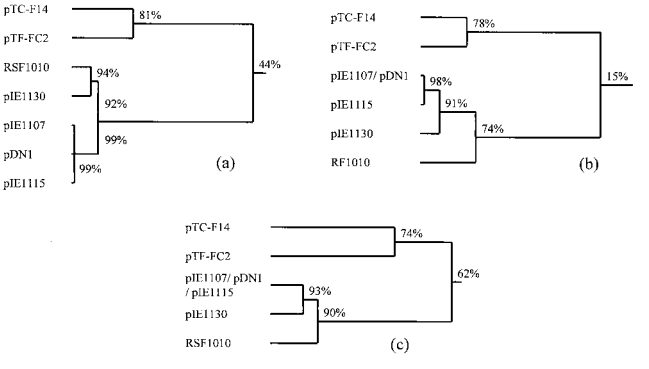


FIG. 2. Relationships between the replicons of plasmids of the IncQ-like family based on percent amino acid sequence identities of the replication proteins. a, RepA, b, RepB; and c, RepC. Accession numbers are as follows: pTC-F14, AF325537; pTF-FC2, M35249 and M64981; pIE1107, ACZ74787; pIE1115, AJ293027; pIE1130, AJ271879; RSF1010, M28829; pDN1, ACY19120.

TABLE 2. Replicon-associated proteins of pTC-F14 and comparison with pTF-FC2

Protein	Translational initiation sites	pTC-F14			pTF-FC2			% Amino acid identity
		No. of amino acids	Molecular mass (Da)	pI	No. of amino acids	Molecular mass (Da)	pI	
RepA	UCUGGAA**AGGAG**AACAGC*AUG*	291	31,289	5.92	290	31,227	6.21	81.0
RepB	C**AGGAG**AGGGCACAGGCG*AUG*	352	40,623	9.73	352	40,111	9.77	78.4
RepC	UACCCAG**GGAG**GCAAGCC*AUG*	303	33,712	9.28	299	33,740	8.99	74.2
PasA like	UUUGAGC**AGGAG**CUAAAC*AUG*	74	8,523	4.46	74	8,453	4.71	81.1
PasB like	AGGAAGU**GGAG**CGCGAUC*UUG*	90	10,483	10.36	90	10,307	10.4	72.2

[a] Putative ribosome binding sites are in bold, and start codons are in italics.

characterized by having a G+C-rich region (24 out of 28 bp) about 25 bp downstream of the iterons (28). This is followed by an A+T-rich region (23 out of 31 bp) which is believed to be the site of the DNA melting which takes place before replication. Plasmid pTC-F14 has no equivalent G+C-rich region but does have an extended A+T-rich region (29 out of 40 bp). However, a 28-of-28-bp G+C-rich region which contains two internal 12-bp directly repeated sequences is located approximately 100 bp downstream of the extended A+T-rich region. Other features within the *oriV* region are two inverted repeats, one of 8 bp and one of 12 bp, with predicted stem-loop ΔG values of −0.3 and −15.2 kJ/mol, respectively. The 12-bp, but not the 8-bp, inverted repeat is therefore likely to form a stem-loop structure. Stem-loop structures have been shown to play a role in the initiation of IncQ plasmid replication (14, 15, 19, 21), but whether the 12-bp direct repeats play a role in pTC-F14 replication is unknown.

Plasmid pTC-F14 appears to have a broad host range. As plasmid pTC-F14 was isolated from *A. caldus* and pTC-F14 with a chloramphenicol marker (pTC-F14Cm) was capable of replication in *E. coli*, it appears that, like its IncQ relatives, pTC-F14 has a broad-host-range replicon. To obtain additional evidence for the broad-host-range property of the pTC-F14 replicon, pTC-F101 (Fig. 1) was transformed into *P. putida* and *A. tumefaciens* LBA 4404 using electroporation and triparental mating, respectively. The presence of the pTC-F14 replicon was confirmed by PCR with primers specific for the *repA* gene of pTC-F14 (data not shown). The species identity of the transformants was confirmed by API 20E strips (bioMérieux sa, Marcy-l'Etoile, France). Furthermore, we tested for the presence of pTC-F101 in the absence of selection after 100 generations of growth in *E. coli*, *P. putida*, and *A. tumefaciens*. No plasmid loss was detected in *E. coli* DH5α or *P. putida*, but after 60 generations of growth in *A. tumefaciens* LBA 4404, the plasmid had been completely lost from the population.

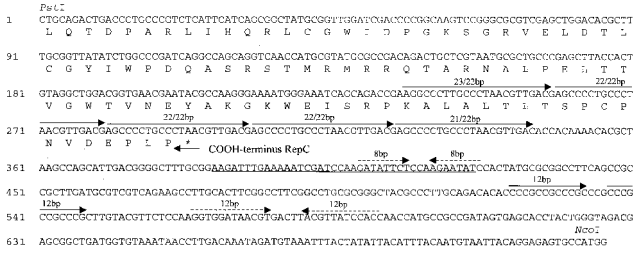


FIG. 3. Nucleotide sequence of the *Pst*I-*Nco*I fragment (716 bp) containing the functional *oriV* region of pTC-F14. The amino acid sequence of the C-terminal region is shown below the nucleotide sequence. Iterons and direct repeats are shown by arrows, and inverted repeats are shown by broken arrows. The A+T-rich region is underlined, and 12-bp direct repeats are located within the G+C-rich region.

Incompatibility among pTC-F14, IncQ, and IncQ-like plasmids. Replicon-associated plasmid incompatibility is believed to arise from the inability of the host cell to correct fluctuations in copy number between plasmids that have elements of their replication machinery in common (23). It has previously been established that the iterons of IncQ and related plasmids are able to exert incompatibility (6, 7, 18, 24). Since the IncQ and IncQ-like plasmids share iteron sequence similarity (Fig. 4), we wished to determine which members of the IncQ plasmid family were incompatible with each other. Plasmids pIE1107 and RSF1010 were previously found to be incompatible due to the presence of nonessential iterons present on pIE1107 that are identical to IncQ iterons. When these iterons were deleted, the resulting plasmid, pIE1108, was fully compatible with RSF1010 (35). We tested the functional replicon of pTC-F14 for incompatibility with the IncQ plasmid R300B replicon (pKE462) and the replicons of IncQ-like plasmids pIE1108 and pTF-FC2 (pDER404). The replicons which, according to Rep protein sequence identity, were most closely related to each other (pIE1108 and R300B, pTC-F14 and pTF-FC2) were fully compatible in *E. coli* (Table 3). However, the two pairs of less well-conserved replicons were asymmetrically (unidirectionally) incompatible. When pIE1108 was the resident plasmid and either pTC-F14 or pTF-FC2 was the incoming selected plasmid, neither plasmid displaced pIE1108. When either pTC-F14 or pTF-FC2 was the resident plasmid, incoming selected pIE1108 resulted in the displacement of both pTC-F14 and pTF-FC2. A resident IncQ R300B replicon could not be displaced by incoming pTC-F14 or pTF-FC2. However, an incoming selected IncQ R300B replicon displaced resident

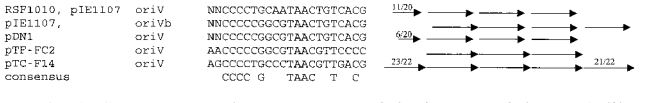


FIG. 4. Sequences and arrangement of the iterons of the IncQ-like plasmids tested in this study. The lengths and positions of partial iterons are indicated above the arrows. Iterons of RSF1010 and pDN1 *oriV* and pIE1107 *oriVa* and *oriVb* are 20 bp long with 2-bp spacers (designated NN and being either AA or CC). In the case of pIE1107 *oriVb*, the first 2-bp spacer is missing. Iterons of pTF-FC2 and pTC-F14 are 22 bp long (except where shown otherwise), and spacers are absent.

TABLE 3. IncQ-like plasmid displacement by other IncQ-like plasmids in *E. coli*

Incoming replicon (plasmid selected)	% Resident (unselected) plasmids remaining			
	R300B (pKE462)	pIE1108	pTF-FC2 (pDER404)	pTC-F14 (pTC-F101)
R300B (pKE462)		100	100	0
pIE1108	100		0	0
pTF-FC2 (pDER404)	100	100		100
pTC-F14 (pTC101)	NE[a]	100	100	

[a] NE, plasmid not established; pTC-F14 could not be transformed into *E. coli* (pKE462).

pTC-F14 but not resident pTF-FC2. Plasmid incompatibility testing should be treated with caution when using plasmids which have a postsegregational killing system. However, in the case of both pTC-F14 and pTC-FC2, it was the plasmid which possessed the postsegregational killing system that was displaced in an *E. coli* host. The four plasmids could therefore be distinguished from each other based on incompatibility testing with the plasmids which were originally isolated from heterotrophic bacteria having an incompatibility advantage over those isolated from autotrophic bacteria when tested for displacement in *E. coli*. As regions other than the *oriV* region of plasmids have been isolated and shown to exert plasmid incompatibility (6, 23, 35), plasmids pTC-F108 and pTC-F109 (Fig. 1) were used to search for further incompatibility elements in the pTC-F14 replicon. Plasmid pTC-F109 containing only the pTC-F14 *oriV* region demonstrated incompatibility with resident pTC-F101, while pTC-F108 was compatible. Therefore, within the pTC-F14 replicon region, only *oriV*-associated plasmid incompatibility could be detected.

Copy number of pTC-F14. We wished to determine the copy number of pTC-F14 in both *E. coli* and the original *A. caldus* strain, f, from which it was isolated. Plasmid copy number was determined by the Southern hybridization technique using genome sizes of 4.7 Mb for *E. coli* (www.tigr.org) and 2.8 Mb for *A. caldus*. The genome size of *A. caldus* was estimated by digesting chromosomal DNAs from several strains with the relatively rarely cutting restriction endonucleases *Xba*I and *Dra*I, followed by separation of the restriction fragments using a TAFE pulsed-field gel electrophoresis apparatus and summation of the sizes (data not shown). By using the relative sizes of the plasmid and chromosome DNAs, as well as the quantities of plasmid and total DNAs loaded onto the hybridization membrane, the copy number of pTC-F14 was calculated to be 12 to 16 copies per chromosome for both *E. coli* and *A. caldus*. This is approximately the same as the 12 to 15 copies (6) and 10 to 14 copies (32) estimated for plasmid pTF-FC2 in *E. coli*.

Specificity of the pTC-F14 *oriV* region for its own replication proteins. Since the replication proteins of plasmids pTC-F14 and pTF-FC2 are fairly closely related (Table 2), we wished to determine whether the pTC-F14 *oriV* region could be complemented in *trans* by the pTF-FC2 plasmid replicon and vice versa. To test this, *E. coli* GW125a (pDER404, pTF-FC2 replicon) and *E. coli* GW125a (pTC-F101, pTC-F14 replicon) were transformed with pTC-F109 containing the pTC-F14 *oriV* region cloned into pGEM-T (Promega Corp.) vector. Since the vectors in which the *oriV* regions were cloned do not replicate in strain GW125a (*polA*) and since the *oriV* regions cannot replicate unless the replication proteins are provided in *trans*, transformants would only be obtained if replication protein complementation had occurred. Transformants were obtained for *E. coli* GW125a(pTC-F101) and not *E. coli* GW125a(pDER404). This indicated that the replication proteins of pTC-F14, but not those of pTF-FC2, were able to complement the pTC-F14 *oriV* region. In a reciprocal experiment, *E. coli* GW125a(pDER404) and *E. coli* GW125a(pTC-F101) were transformed with pTV4164 containing the pTF-FC2 *oriV* region Transformants were obtained for *E. coli* GW125a(pDER404) but not *E. coli* GW125a(pTC-F101). Therefore, each *oriV* region could be complemented in *trans* by its own replication proteins but not by those of the other plasmid. We also attempted to transform pTC-F109 and pTV4164 into *E. coli* GW125a(pKE462) and *E. coli* GW125a(pIE1108) recipients to test whether the R300B IncQ or the pIE1108 replication proteins were able to complement either the pTC-F14 or the pTF-FC2 *oriV* region. No transformants were obtained, which suggested that cross-complementation did not occur.

DISCUSSION

Plasmid pTC-F14 is the second plasmid to be isolated from acidophilic, obligately chemolithotrophic iron- or sulfur-oxidizing bacteria which has an IncQ-like replicon. All other IncQ-like plasmids have been isolated from heterotrophic bacteria found in medical and animal-associated environments. Since the ecological niche occupied by acidiphilic chemolithotrophs is very different from that occupied by mammal-associated heterotrophs, it is likely that IncQ-like replicons will be found in other ecological niches and await discovery. How many groups of IncQ replicons exist is therefore uncertain, but those so far discovered fall into two major groupings. The existence of at least two replicon groups is supported by several observations. There is a fairly deep division based on amino acid sequence comparison of the RepA, RepB, and RepC proteins (Fig. 2). In addition, one group has replicons with 20-bp iterons and 2-bp spacers while the other group has 22-bp iterons without spacers. Furthermore, plasmids pTC-F14 and pTF-FC2 also both have plasmid addition system (*pas*) genes between the *repB* and *repA* genes whereas none of the other IncQ and IncQ-like plasmids have similar genes within the replicon. Interestingly, pTF-FC2 is unique in having three *pas* genes (*pasA*, *pasB*, and *pasC*) whereas pTC-F14 is more typical of other plasmid addition systems in having only two. Division into two groups is even more strongly supported by the observation that RSF1010, pIE1107, pIE1115, pIE1130, and pDN1 have IncQ-like mobilization proteins while pTF-FC2 and pTC-F14 have IncP-related mobilization proteins (27; pTC-F14 partial sequence data). The relatedness between the mobilization genes of pTC-F14 and pTF-FC2 raises many questions regarding whether the mobilization system of one plasmid can transfer the other, and this will be the subject of future studies.

Based on plasmid incompatibility testing, Tietze (35) has suggested that IncQ and IncQ-like plasmids should be named differently to distinguish between the different incompatibility groups. It was proposed that the IncQ plasmids (RSF1010, R1162, and R300B) should be named IncQα plasmids and that the plasmid pIE1107 replicon (and its incompatible relatives)

should be called IncQβ. Plasmid pDN1 and the recently discovered plasmid pIE1115 also belong to this group. Tietze and coworkers have recently discovered an IncQ-like plasmid, pIE1130, which belongs to a different incompatibility group which they propose to call IncQγ (31; E. Tietze and K. Smalla, Plasmid Biology 2000, p. 167). Plasmid pTC-F14 clearly belongs to an incompatibility group which is different from pTF-FC2 or any other previously reported IncQ-like plasmids. Furthermore, it is clear that there are at least two major groupings of IncQ-like plasmids, with the IncQα, IncQβ, and IncQγ plasmids belonging to one group and pTF-FC2 and pTC-F14 belonging to the other. We suggest that the former group be called IncQ-like group 1 plasmids, of which 1α, 1β, and 1γ are the currently known incompatibility subgroups. Plasmids pTF-FC2 and pTC-F14 should be called IncQ-like group 2 plasmids, and as these two plasmids are compatible, group 2 would consist of two incompatibility subgroups. We propose that the first discovered plasmid, pTF-FC2, should be designated a member of the IncQ-like 2α incompatibility subgroup, with pTC-F14 being a member of the IncQ-like 2β incompatibility subgroup.

The copy number of some iteron-containing replicons has been shown to be increased or decreased by the deletion or addition of iterons, respectively (33, 34, 36). Plasmid pTF-FC2 has three perfectly conserved 22-bp iterons, compared with the five iterons of pTC-F14, of which only the central three 22-bp iterons are perfectly conserved. We thought it possible that a reason for the increased number of iterons in pTC-F14 was to achieve a reduction in plasmid copy number with the concurrent reduction in metabolic burden to the host cell. However, the estimated copy numbers of plasmids pTC-F14 and pTF-FC2 appear to be similar and within the range of the 12 to 15 copies per chromosome reported for IncQ plasmids (10). In a study of the effects of mutations on the functionality of RSF1010 iterons, it was found that even a single-base-pair replacement in one of the iterons could result in a nonfunctional iteron-containing region and the inability of RSF1010 to replicate (22). It is therefore possible that the 23- or 21-bp iterons which flank the 22-bp iterons are nonfunctional and therefore not active in copy number control. This remains to be tested.

The functions of elements within the *oriV* regions of the IncQ plasmids have been extensively studied (see reference 28 for a review). The suggested model for initiation of replication is that the RepC proteins bind to the 20-bp iterons and this induces DNA melting and strand opening at the A+T-rich region (17). The A+T-rich region is about 60 bp from the iterons, and the two are separated by a G+C-rich region. The RepA helicase binds to the opened region and unwinds the DNA, probably mainly in the direction away from the iterons, until a pair of single-strand initiation sites (*ssiA* and *ssiB*) are reached. Each of these *ssi* sites has twofold symmetry, and they are situated within a region of extended twofold symmetry with a 40-bp stem and a 40-bp loop (19). The RepB primase is required to initiate replication at these *ssi* sites, which are arranged in such a way that replication in opposite directions takes place from each of the *ssi* sites by a strand displacement mechanism (14, 15, 38). Plasmid pTC-F14 clearly has the equivalent of the RepA, RepB, and RepC proteins, as well as an *oriV* region containing iterons, A+T-rich and G+C-rich regions, and a pair of sequences with twofold symmetry. However, several features, such as the number and sequence of the iterons, the relative positions of the A+T- and G+C-rich regions, the presence of two 12-bp direct repeats, and the lack of a region of extended twofold-symmetry, are unique to pTC-F14. Determination of whether these differences represent significant differences in the mechanism of pTC-F14 replication awaits further study. We found that the *oriV* regions of pTC-F14 and pTF-FC2 could be complemented only by their own replication proteins. The basis of this specificity presumably resides in the nucleotide sequences of the iterons and other features of the *oriV* region. For example, if each RepC protein binds specifically to its own iterons, the initiation of replication will be prevented by the lack of RepC-*oriV* binding. It is also possible that replicon specificity resides in the RepA or RepB protein and features of the *oriV* region with which they interact. We propose to investigate this by placing different combinations of *rep* genes expressed from non-IncQ-related replicons in *trans* with different features of the *oriV* regions to examine the basis of IncQ replication specificity.

ACKNOWLEDGMENTS

We are grateful to Erhard Tietze for providing plasmid pIE1108.

We are grateful to Billiton Process Research (Randburg, South Africa), the National Research Foundation (Pretoria, South Africa), and the University of Stellenbosch for funding support.

REFERENCES

1. **Altshul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein data base search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120:**618–630.
3. **Breed, A. W., C. J. N. Dempers, G. E. Searby, M. N. Gardner, D. E. Rawlings, and G. S. Hansford.** 1999. The effect of temperature on the ferrous iron oxidation kinetics of *Leptospirillum ferrooxidans* in continuous culture. Biotech. Bioeng. **65:**44–53.
4. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
5. **Derbyshire, K. M., and N. S. Willets.** 1987. Mobilization of the non-conjugative plasmid RSF1010: a genetic analysis of its origin of transfer. Mol. Gen. Genet. **206:**154–160.
6. **Dorrington, R. A., and D. E. Rawlings.** 1989. Identification and sequence of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171:**2735–2739.
7. **Dorrington., R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
8. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene **108:**7–14.
9. **Franklin, F. C. H., M. Bagdasarian, M. M. Bagdasarian, and K. N. Timmis.** 1981. Molecular and functional analysis of the TOL plasmid pWWO from *Pseudomonas putida* and the cloning of the genes for the entire regulated aromatic ring *meta*-cleavage pathway. Proc. Natl. Acad. Sci. USA **78:**7458–7462.
10. **Frey, J., and M. Bagdasarian.** 1989. The biology of IncQ plasmids, p. 79–93. *In* C. M. Thomas (ed.), Promiscuous plasmids of Gram-negative bacteria. Academic Press, London, England.
11. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117:** 619–630.
12. **Hallberg, K. B., and E. B. Lindström.** 1994. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology **140:** 3451–3456.
13. **Hoekema, A., P. R. Hirsch, P. J. J. Hooykaas, and R. A. Schilperoort.** 1983. A binary plant vector strategy based on separation of *vir*- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. Nature **303:**179–180.
14. **Honda, Y., H. Sakai, T. Komano, and M. Bagdasarian.** 1989. RepB' is required *in trans* for the two single-strand DNA initiation signals in *oriV* of

plasmid RSF1010. Gene **80:**155–159.
15. **Honda, Y., H. Sakai, H. Hiasa, K. Tanaka, and T. Komano.** 1991. Functional division and reconstruction of a plasmid replication origin: molecular dissection of the *oriV* of the broad-host-range plasmid RSF1010. Proc. Natl. Acad. Sci. USA **88:**179–183.
16. **Iwasaki, K., H. Uchiyama, O. Yagi, T. Kurabayashi, K. Ishizuka, and Y. Takamura.** 1994. Transformation of *Pseudomonas putida* by electroporation. Biosci. Biotech. Biochem. **58:**851–854.
17. **Kim, Y-J., and R. J. Meyer.** 1991. An essential iteron-binding protein required for plasmid R1162 replication induces localized melting within the origin at a specific site in AT-rich DNA. J. Bacteriol. **173:**5539–5545.
18. **Lin, L.-S., Y-J. Kim, and R. J. Meyer.** 1987. The 20-bp. Directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility in vivo and inhibits R1162 DNA replication in vitro. Mol. Gen. Genet. **208:** 390–397.
19. **Lin, L.-S., and R. J. Meyer.** 1987. DNA synthesis is initiated at two positions within the origin of replication of plasmid RSF1010. Nucleic Acids Res. **20:**8319–8331.
20. **Meyer, R., M. Hinds, and M. Brasch.** 1982. Properties of R1162, a broad-host-range, high-copy-number plasmid. J. Bacteriol. **150:**552–562.
21. **Miao, D-M., Y. Honda, K. Tanaka, A. Higashi, T. Nakamura., Y. Taguchi, H. Sakai., T. Komano, and M. Bagdasarian.** 1993. A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad-host-range plasmid RSF1010. Nucleic Acids Res. **21:**4900–4903.
22. **Miao, D-M., H. Sakai, S. Okamato, K. Tanaka, M. Okuda, Y. Honda, T. Komano, and M. Bagdasarian.** 1995. The interaction of RepC initiator with iterons in the replication of the broad-host-range plasmid RSF1010. Nucleic Acids Res. **23:**3295–3300.
23. **Novick, R. P.** 1987. Plasmid incompatibility. Microbiol. Rev. **51:**381–395.
24. **Persson, C., and K. Nordstrom.** 1986. Control of replication of the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated DNA sequences. Mol. Gen. Genet. **203:**189–192.
25. **Rawlings, D. E., N. J. Coram, M. N. Gardner, and S. M. Deane.** 1999. *Thiobacillus caldus* and *Leptospirillum ferrooxidans* are widely distributed in continuous flow biooxidation tanks used to treat a variety of metal containing ores and concentrates, p. 777–786. *In* R. Amils and A. Ballester (ed.), Biohydrometallurgy and the environment toward the mining of the 21st century. Part A. Elsevier, Amsterdam, The Netherlands.
26. **Rawlings, D. E., and D. R. Woods.** 1985. Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. **49:**1323–1325.
27. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of a broad host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**6230–6237.
28. **Sakai, H., and T. Komano.** 1996. DNA replication of IncQ broad host-range plasmids in Gram-negative bacteria. Biosci. Biotech. Biochem. **60:**377–382.
29. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
30. **Scholtz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad host-range plasmid RSF1010. Gene **75:**271–288.
31. **Smalla, K., H. Heuer, A. Götz, D. Niemeyer, E. Krögerrecklenfort, and E. Tietze.** 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl. Environ. Microbiol. **66:**4854–4862.
32. **Smith, A. G. S., and D. E. Rawlings.** 1997. The poison-antidote system of the broad host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. **25:**961–970.
33. **Thomas, C. M., D. M. Stalker, and D. R. Helinski.** 1981. Replication and incompatibility properties of segments of the origin of replication of the broad host range plasmid RK2. Mol. Gen. Genet. **181:**1–7.
34. **Thomas, C. M., M. A. Cross, A. A. K. Hussain, and C. A. Smith.** 1984. Analysis of copy number control elements in the region of the vegetative replication region of the broad host range plasmid RK2. EMBO J. **3:**57–63.
35. **Tietze, E.** 1998. Nucleotide sequence and genetic characterization of the novel IncQ-like plasmid pIE1107. Plasmid **39:**165–181.
36. **Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsubara.** 1983. Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bacteriol. **155:**337–344.
37. **Whittle, G., M. E. Katz, E. H. Clayton, and B. F. Cheetham.** 2000. Identification and characterization of a native *Dichelobacter nodosus* plasmid, pDN1. Plasmid **43:**230–234.
38. **Zhou, H., and R. J. Meyer.** 1990. Deletion of sites for initiation of DNA synthesis in the origin of broad host-range plasmid R1162. J. Mol. Biol. **214:**685–697.

MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS, Dec. 2001, p. 481–496 Vol. 65, No. 4
1092-2172/01/$04.00+0 DOI: 10.1128/MMBR.65.4.481–496.2001

Comparative Biology of IncQ and IncQ-Like Plasmids

DOUGLAS E. RAWLINGS[1]* AND ERHARD TIETZE[2]

Department of Microbiology, University of Stellenbosch, Matieland 7602, South Africa,[1] *and Robert Koch-Institut, Bereich Wernigerode, D38843 Wernigerode, Germany*[2]

INTRODUCTION

Plasmids of the *Escherichia coli* incompatibility group Q (IncQ) and related IncQ-like plasmids are characterized by their relatively small size, their ability to be mobilized by several self-transmissible plasmids, and their broad host range (25). This combination of characteristics has resulted in plasmids of this type being highly promiscuous. As a consequence, IncQ family plasmids are found in a variety of very different environments. Since there are very few families of truly broad-host-range, highly mobilizable plasmids, plasmids of the IncQ family are good candidates for research into fundamental aspects of plasmid biology. The importance of these studies is increased because IncQ plasmids have been used as a major source of broad-host-range replicons in the development of genetic systems for a wide range of bacteria (5, 85).

As will be described in this review, the promiscuous nature of plasmids of the IncQ family has resulted in the acquisition of these plasmids by microorganisms growing in a number of very different environments. The effect of this is that there appear to be several evolutionary lineages of these plasmids, making the study of these plasmids interesting from the points of view of evolutionary plasmid molecular biology and the ecological distribution of plasmids. The replication, regulation, and mobilization functions of some of the prototypic IncQ plasmids have been extensively studied, and good reviews on these aspects are available (19, 25, 59, 76). The aim of this review is not to repeat material that has been reviewed elsewhere but, rather, to compare the IncQ and IncQ-like plasmids in the light of several recently discovered plasmids which belong to the IncQ family.

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Private Bag XI, Matieland 7602, South Africa. Phone: 27 21 808 5848. Fax: 27-21-808 5846. E-mail: der@sun.ac.za.

ECOLOGICAL DISTRIBUTION AND DIVERSITY

The most extensively studied plasmids of the IncQ family are the IncQ plasmids RSF1010, R1162, and R300B, which were isolated independently from *E. coli* (32), *Pseudomonas aeruginosa* (12, 53), and *Salmonella enterica* serovar Typhimurium, respectively (6). Although originating from different hosts, these plasmids each contain streptomycin and sulfonamide resistance genes and appear to be nearly identical. During investigations into plasmids containing antibiotic resistance genes from *E. coli*, other enterobacteria, and a variety of other bacteria of medical importance, several IncQ plasmids which contain additional or different antibiotic resistance genes to RSF1010 have been isolated (94). Not all of these plasmids

TABLE 1. Characteristics of some naturally isolated IncQ and IncQ-like plasmids

Plasmid	Compatible with IncQ	Size (bp)	Source from which isolated	No. of copies per chromosome	Genes and ORFs in addition to backbone[a]	Reference(s)
RSF1010			*E. coli* strain 3			32
R1162	No	8,684	*P. aeruginosa*	12	*sulII, strAB*	12, 53
R300B			*S. enterica* serovar Typhimurium (United Kingdom)			6
R678	No	±14,000	*S. enterica* serovar Dublin (Denmark)	11	Sm, Su	6, 31
R684	No	±9,500	*Proteus mirabilis* (Germany)	11	Sm, Su	6, 31
PB165	NR	±11,900	*E. coli* (United Kingdom)	9	Sm, Su	6, 31
p89S	No	±8,180	*E. coli* (clinical)	NR	Su	75
pFM202	Yes	±7,100	*Neisseria gonorrhoeae* (Spain)	NR	Ap	73
pFM739	No	±9,450	*Neisseria sicca* (Spain)	NR	Ap, Sm, Su	74
pHD148	NR	±7,500	*Haemophilus ducreyi*	NR	Su	1
pHD8.1	NR	±8,100	*Actinobacillus pleuropneumoniae*	NR	Sm, Su	98
pAZ1	NR	±8,000	*S. enterica* serovar Typhimurium type 179	NR	Su, Tp (DHFR type III)	24
pIE639	No	±11,100	*E. coli* O20:H$^-$	±12[e]	*aph*(3′)-*Id*, *sat3*, *strAB*, *sulII*	94
pIE723	No	±9,500	*E. coli* O147:K88	±12[e]	*ant*(2″)-*Ia*, *strAB*, *sulII*	94
pIE1107	No[d]	8,520	Piggery manure	±12[e]	*aph*(3′)-*Id*, *sat3*, *sulII*[b]	94
pIE1115	No[d]	10,687	Piggery manure	±12[e]	*linB*-like, *strAB*, *sulII*	86
pIE1120	No	±9,100	Piggery manure	±12[e]	*tetA*(Y), *strAB*	86
pIE1130	No[d]	10,687	Piggery manure	±12[e]	*aph*(3′)-*I*, *catIII*, *strAB, sulII*	86
pDN1	Yes	5,112	*Dichelobacter nodosus*	NR	None	96
pTF-FC2	Yes	12,190	*A. ferrooxidans*	12–15	*grx*, *merR*-like, ORF43, *tnpR*,[b] *tnpA*[b]	17
pTC-F14	No[c]	±14,000	*A. caldus*	12–16	Unknown	27

[a] The exact gene is given where known; otherwise, the type of antibiotic resistance expressed is given. Ap, ampicillin; Cl, clindamycin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Lm, lincomycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; To, tobramycin; Tp, trimethiprim. Cl/Lm conferred by *linB*; Cm conferred by *catIII*; Km/Nm/Tb conferred by *ant*(2″)-Ia; Km/Nm conferred by *aph*(3′)-Id; streptothricin resistance conferred by *sat3*; Sm conferred by *strAB*; Su conf erred by *sulII*; Tc conferred by *tetA(Y)*.

[b] Gene incomplete or truncated.

[c] Unidirectionally (asymmetrically) incompatible with RSF1010.

[d] Two *oriV*-like regions; the nonfunctional *oriV* is incompatable with IncQ.

[e] E. Tietze, unpublished estimate.

[f] NR, no report.

have been sequenced completely, but in many cases it was shown that they have replicons which are incompatible with IncQ plasmids. Some characteristics of these plasmids are summarized in Table 1. Initial discoveries of IncQ and IncQ-like plasmids were restricted mainly to bacteria encountered in a medical context.

Screening of Nonmedical Environments for IncQ-Like Plasmids

A study of the detection of IncQ plasmids in bacteria by using PCR amplification and a set of three pairs of IncQ-specific primers indicated that IncQ plasmids were prevalent in total DNA isolated from a variety of samples. These included soils from Germany and Holland and pig manure slurries from Germany (30). Although the presence of IncQ plasmids would have been detected in this investigation, the specific primers used would not have detected plasmids which are related to but different from IncQ plasmids. In another study, antibiotic resistance plasmids were captured in mating experiments in which the bacteria present in pig manure slurry were used as plasmid donors and selectable *E. coli* and *Pseudomonas putida* strains were used as recipients (86). From the sequence of plasmid pIE1107, which is IncQ like but different from IncQ plasmids, five sets of primers were designed from regions where plasmids RSF1010 and pIE1107 have conserved nucleotide sequence identity as well as from regions where they are different. These primers were used to detect whether IncQ and IncQ-like plasmids had been captured in the recipient bacteria. A high prevalence of the two groups of IncQ and IncQ-like plasmids (prevalence approximately equal to IncP plasmids) was found, and furthermore a large amount of diversity in genotypic and phenotypic properties was discovered (30, 86). These workers suggested that plasmids with replicons which were incompatible with RSF1010 should be called IncQα and those with compatible replicons, IncQβ.

In contrast to the above studies, other investigators failed to detect IncQ plasmids in DNA hybridization experiments when using bacteria cultured from marine sediment microbial communities (88). Several broad-host-range plasmids were isolated, but none were of the IncQ type (89). Although IncQ plasmids themselves were not detected, this study does not eliminate the possibility that other less highly conserved members of the IncQ plasmid family may have been present. The exact region of the 357-bp IncQ plasmid probe was not given (88), and, depending on conservation of this region among plasmids of the IncQ family and the stringency of the hybridization and washing reactions, IncQ-like members of the IncQ plasmid family may not have been detected by these hybridization experiments.

Types of IncQ-Like Plasmids

Besides the rather limited range of environments that have been screened for the presence of IncQ plasmids, several IncQ-like plasmids have been discovered serendipitously. Plas-

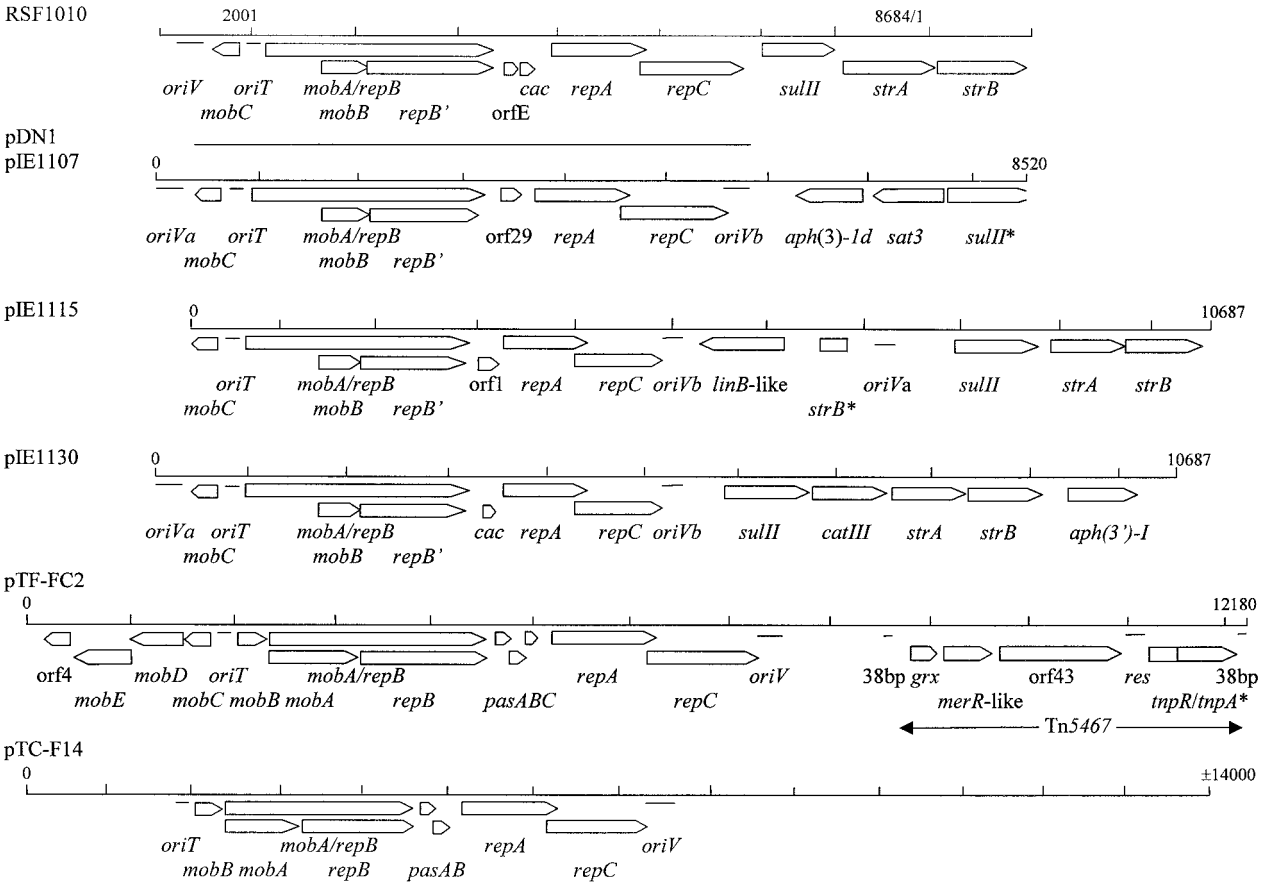


FIG. 1. Genetic maps of plasmids of the IncQ family showing the conserved replicon structure, the regions encoding the mobilization genes, and, where present, the accessory genes. Plasmid pDN1 is represented by a line since its gene map is identical to the backbone region of pIE1107. The genetic map of pTC-F14 is incomplete since only a partial nucleotide sequence is available. Genes and structural features: *aph*(3′)-I, kanamycin and neomycin aminoglycoside phosphotransferase; *cac*, control of *repA* and *repC* regulator; *catIII*, chloramphenicol acetyltransferase; *grx*, glutaredoxin-like gene; *linB*-like, *linB*-like lincosamide nucleotyltransferase; *merR*-like, *merR*-like regulator gene; *mobA*, *mobB*, *mobC*, *mobD*, and *mobE*, mobilization genes; *oriV*, origin of vegetative replication; orf1, orf4, orf29, orf43, and orfE, open reading frames of unknown function; *oriT*, origin of transfer; *pasA, pasB*, and *pasC*, plasmid addiction system genes; *repA*, *repB*, *and repC*, replication genes; *res*, site of cointegrate resolution by transposon resolvase; *strA* and *strB*, streptomycin aminoglycoside phosphotransferase; *sulII*, sulfonamide-resistant dihydropteroate synthase; *tnpA*, transposase; *tnpR*, resolvase. Genes which have been inactivated by deletions are indicated by asterisks.

mid pDN1 was isolated from the anaerobic bacterium, *Dichelobacter nodosus*, which is the principal causative agent of ovine footrot (96). This (5.1-kb) plasmid is closely related to pIE1107 but contains no antibiotic resistance genes and no other accessory genes. In contrast to the plasmids isolated from medical and animal environments, two IncQ-like plasmids have been isolated from bacteria present in inorganic biomining environments. Plasmid pTF-FC2 was isolated from a strain of *Acidithiobacillus ferrooxidans* (previously *Thiobaciilus ferrooxidans*) that was being prepared as an inoculum for a biooxidation plant to treat a gold-bearing arsenopyrite ore concentrate (68). *A. ferrooxidans* is an acidophilic (optimum pH, 1.5 to 2.5) iron- and sulfur-oxidizing bacterium which is obligately chemolithoautotrophic and has a very low tolerance for organic matter (40). Approximately 15 years later, plasmid pTC-F14 was isolated (27) from a strain of a related bacterium, the moderately thermophilic (50°C), acidophilic (pH 1.3 to 2.0), chemolithoautotrophic, sulfur-oxidizing bacterium, *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) (34). This *A. caldus* strain f was isolated from a pilot biooxidation plant being used to treat a nickel-containing ore concentrate. The nucleotide sequences of six IncQ and IncQ-like plasmids have been published. These are plasmids RSF1010, pIE1107, pIE1115, pIE1130 (last two also captured from piggery manure [86]), pDN1, and pTF-FC2. In addition plasmid, pTC-F14 (27) has been partially sequenced. The characteristics of a selection of IncQ-like plasmids are shown in Table 1, and complete or partial genetic maps of these plasmids are given in Fig. 1.

Based on available data from all sequenced members of IncQ-like plasmids, it is evident that the primer sets used would not have enabled the detection of IncQ-like plasmids such as pTF-FC2 or pTC-F14. The prevalence and variety of the IncQ family of plasmids in many environments is therefore unknown, and it is likely that many more types will be discovered.

Host Range

As described above, one of the most remarkable features of IncQ plasmids is their exceptionally broad host range. The list

TABLE 2. List illustrating the range of bacteria in which IncQ plasmids or IncQ-derived plasmid vectors can replicate

Host	Plasmid or vector replicon	Reference(s)
Acinetobacter sp.	pIE1120	85
Acidipilium cryptum	pSUP106	66
Acidithiobacillus ferrooxidans	pKMZ1, pKT230	44, 63
Actinobacillus actinomycetemcomitans	pBK1	28
Agrobacterium tumefaciens	RSF1010, pIE1120	8, 85
Alcaligenes sp. strain BR60	pMMB66HE	56
Arthrobacter sp.	pKT240, pML10	52
Bartonella henselae	IncQ vectors	18
Bdellovibrio bacteriovorus	IncQ vectors	16
Brevibacterium methylicum	RSF1010	57
Burkholderia pseudomallei	pKT230	50
Caulobacter crescentus	RSF1010	9
Chlorobium tepidum	pDSK519, pGSS33	95
Desulfovibrio desulfuricans	RSF1010 derivatives	4
Enterobacter agglomerans	RSF1010 vectors	84
Francisella novicida	RSF1010 derivative	2
Haemophilus parainfluenzae	RSF1010 derivatives	11
Legionella pneumophila	RSF1010	81
Mycobacterium smegmatis	RSF1010	29
Pseudoanabaena sp.	pSUP1021, pKT230	90
Pseudomonas putida	pIE1120	85
Ralstonia europha	pIE1120	85
Streptomyces lividans	RSF1010	29
Synechococcus sp.	pSUP1021, pKT230	90
Synechocystis sp.	pSUP1021	90

of organisms in which IncQ plasmids and IncQ plasmid-based cloning vectors have been reported to replicate is shown in Table 2. Although possibly not complete, the list includes a wide variety of hosts belonging to the kingdom *Bacteria* (including gram-negative bacteria, gram-positive bacteria, and cyanobacteria) but no members belonging to the kingdoms *Archaea* or *Eucarya*. It has been suggested that the broad-host-range properties of the IncQ plasmids occur because the plasmid possesses many of the genes required for its own replication and this allows it to be more independent of the host replication machinery than are most other plasmids. RSF1010 has genes encoding its own plasmid-specific DNA-binding protein, a helicase, and a primase (19, 80). Scherzinger et al. (77) reported that besides these three essential plasmid-encoded proteins, *E. coli* host-encoded single-strand-binding protein, DNA gyrase, and the γ subunit of the DNA polymerase III holoenzyme were required for RSF1010 replication in vitro. Plasmid replication was independent of the host RNA polymerase, initiator DnaA protein and primosome, DnaB, DnaC, DnaG, and DnaT proteins. This result was consistent with their in vivo results.

Investigations into the host range of the IncQ-like plasmids are less extensive. However, plasmids pIE1115 and pIE1130 can be mobilized to and replicate in (albeit with varying stability) *Pseudomonas putida*, *Acinetobacter* sp. strain BD14, *Ralstonia eutropha*, and *Agrobacterium tumefaciens* (86). Likewise, studies of the ability of pTF-FC2 to replicate in hosts other than *A. ferrooxidans* indicate that the host range of this IncQ-like plasmid may be as wide as that of RSF1010. Work in the author's laboratory has shown that pTF-FC2 is able to replicate in *A. tumefaciens*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, *P. putida*, *S. enterica* serovar Typhimurium, *Sinorhizobium meliloti*, and *Thiobacillus novellus* (67). Other workers have reported that plasmid pTF-FC2-based vectors are able replicate in *Acidiphilum facilis*, *Myxococcus xanthus* (67), and *Pseudomonas fluorescens* (37). It is possible that IncQ and IncQ-like plasmids are unable to replicate in certain bacteria but that these negative results have not been reported. For example, attempts to transfer pTF-FC2 into the strict anaerobe *Bacteriodes fragilis* using a clindamycin resistance gene as the selectable marker were unsuccessful (D. E. Rawlings, unpublished data).

COMPARISON OF PLASMID BACKBONE STRUCTURES

The concept of a plasmid backbone includes genes and sites required for typical plasmid-associated functions such replication, conjugation, and stability (61, 92, 97). The determination of the extent of this backbone may be assisted by comparing features shared by most members of a plasmid family. Plasmids of the IncQ family have a backbone structure consisting of a region required for plasmid mobilization and a region required for replication and maintenance. Not all members have a plasmid stability system, but in the members which do, this system is integrated within the replicon with elements which are essential for replication on either side (Fig. 1). The stability system has therefore also been considered to be part of the plasmid backbone.

Replicon Structure and Mechanism of Replication

A model of the mechanism for the replication of the IncQ plasmids has been reviewed in detail, and only a summary will be presented here (25, 76). The IncQ replicon consists of three genes, *repA*, *repB*, and *repC*, and an *oriV* region (80). The *oriV* region contains 3.5 20-bp iterons with 2-bp nucleotide spacers. The iterons exert incompatibility and serve as binding sites for the site-specific DNA-binding protein, RepC (35, 48, 65). Binding of RepC is essential for replication and is thought to introduce conformational changes leading to DNA unwinding in the adjacent AT-rich region (35, 42). This RepC-induced DNA melting serves as an entry site for RepA, a plasmid-specific helicase which unwinds the DNA in the flanking regions. One of these flanking regions contains a large inverted repeat which has two plasmid-specific, single-stranded DNA initiation sites, *ssiA* and *ssiB* (35, 39, 49). The *ssiA* and *ssiB* sites initiate the priming of single-stranded DNA synthesis on opposite strands in the leftward and rightward directions, respectively (54). None of the conserved amino acid sequence motifs associated with the four helicase superfamilies can be easily identified in the RepA helicases of the IncQ plasmid family (33). Initiation of DNA synthesis is dependent on the specific plasmid-encoded primase, RepB (RepB′) (38). The RepB primase occurs in two forms, a 78-kDa MobA-RepB fusion protein (Fig. 1) and a 36-kDa RepB′ form, which is translated from an initiation codon downstream of and in the same reading frame as the fusion protein (76, 80). The model for plasmid replication predicts that it takes place by a strand displacement mechanism.

The overall layout of the genes and many of the other features of the members of the IncQ-like plasmid family are very similar. All IncQ-like plasmids have genes which encode or-

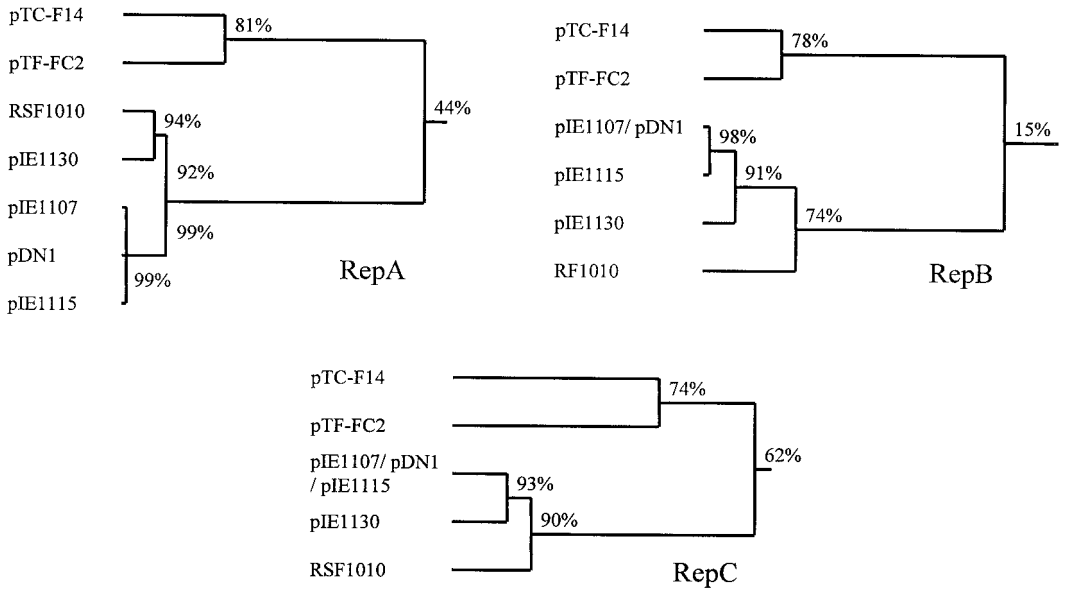


FIG. 2. Phylogenetic relationships between Rep proteins of the IncQ plasmid family. Percentages represent percent amino acid sequence identities.

thologs of the IncQ RepA helicase, the RepB′ primase, and the RepC iteron-specific DNA-binding protein (Fig. 1).

Comparison of replication proteins. Amino acid sequence alignments of the three Rep proteins have indicated that based on protein sequence comparisons, the IncQ plasmid family may be divided into two major groups (Fig. 2). Plasmids pTF-FC2 and pTC-F14 comprise one group, and plasmids RSF1010, pIE1107, pIE1115, pIE1130, and pDN1 make up the other. The RepC DNA-binding proteins are the most highly conserved of the Rep proteins, with 62% amino acid sequence identity between the different groups and >74% identity between members of the same group. Based on the amount of sequence variation between groups, the RepA helicases are intermediately conserved, with 44% amino acid identity between groups and >81% identity within groups. At only 15% amino acid sequence identity, the RepB′ primases are poorly conserved between groups, but at >74% amino acid identity, they are fairly well conserved within groups.

Given the sequence similarity between Rep protein orthologs, it is of interest to know whether any of the Rep proteins can functionally complement mutants in any of the others. Experiments of this type have been carried out between the IncQ replicons of plasmid R300B and pTF-FC2. The *oriV* region of pTF-FC2 could be complemented in *trans* by its own replication proteins but not by those of R300B. Frameshift point mutations within the *repA* and *repC* genes of pTF-FC2 were constructed, and although these mutants could be complemented by homologous unmutated *repA* and *repC* genes they could not be complemented by the *repA* and *repC* genes present on R300B (21). By cloning the *oriV* region of pTF-FC2 into a priming-deficient M13 phage and testing for an increase in plaque size, it was shown that RepB′ of pTF-FC2 acted as a primase on its own *oriV* (22) while the R300B *repB* gene was unable to prime the *oriV* of pTF-FC2 (Rawlings, unpublished). Together, these studies indicate that the RepA, RepB′, and RepC proteins of the R300B were unable, individually or in combination, to substitute for the orthologous proteins of plasmid pTF-FC2. Plasmids pTF-FC2 and R300B belong to different IncQ-like family groups, and it is possible that complementation between the more similar Rep proteins within a plasmid group might occur. However, initial studies using the *oriV* regions of the fairly closely related plasmids pTF-FC2 and pTC-F14 showed that either set of replication proteins was able to complement only its own *oriV* (27).

Origins of replication. The location of *oriV* regions relative to the replication proteins differs between the IncQ and the IncQ-like plasmids. In the IncQ-like plasmids, pDN1, pIE1107, pIE1115, pIE1130, pTF-FC2, and pTC-F14, the functional *oriV* is situated immediately downstream of the *repC* gene (Fig. 1). In plasmids pTF-FC2 and pTC-F14, this association is so close that the *oriV* region begins within the open reading frame of the COOH terminus of the RepC protein. In contrast, in the IncQ plasmid RSF1010 there is a DNA fragment of about 3.65 kp containing the *sulII*, *strA*, and *strB* genes, which separates the *oriV* region of the IncQ plasmids from the *repC* gene. The physical linkage of *repC* and *oriV* is therefore not a requirement and does not appear to confer a competitive disadvantage since the IncQ plasmids are widely distributed, especially in medical environments. Plasmid pIE1107 is unusual in that it has two *oriV*-like regions. *oriVa* is almost identical in location and sequence to the *oriV* of the IncQ plasmids. There is, however, a 19-bp deletion in the 8-bp stem-loop region which follows the AT-rich region, and *oriVa* is nonfunctional for plasmid replication (93). Plasmids pIE1115 and pIE1130 also have duplicate *oriVa* regions similar to pIE1107 but without the 19-bp deletion.

One of the most distinguishing characteristics of the *oriV* regions of members of the IncQ plasmid family is the presence of iterons (directly repeated sequences) with a 22-bp unit length. In plasmids RSF1010, pDN1, pIE1107, pIE1115, and

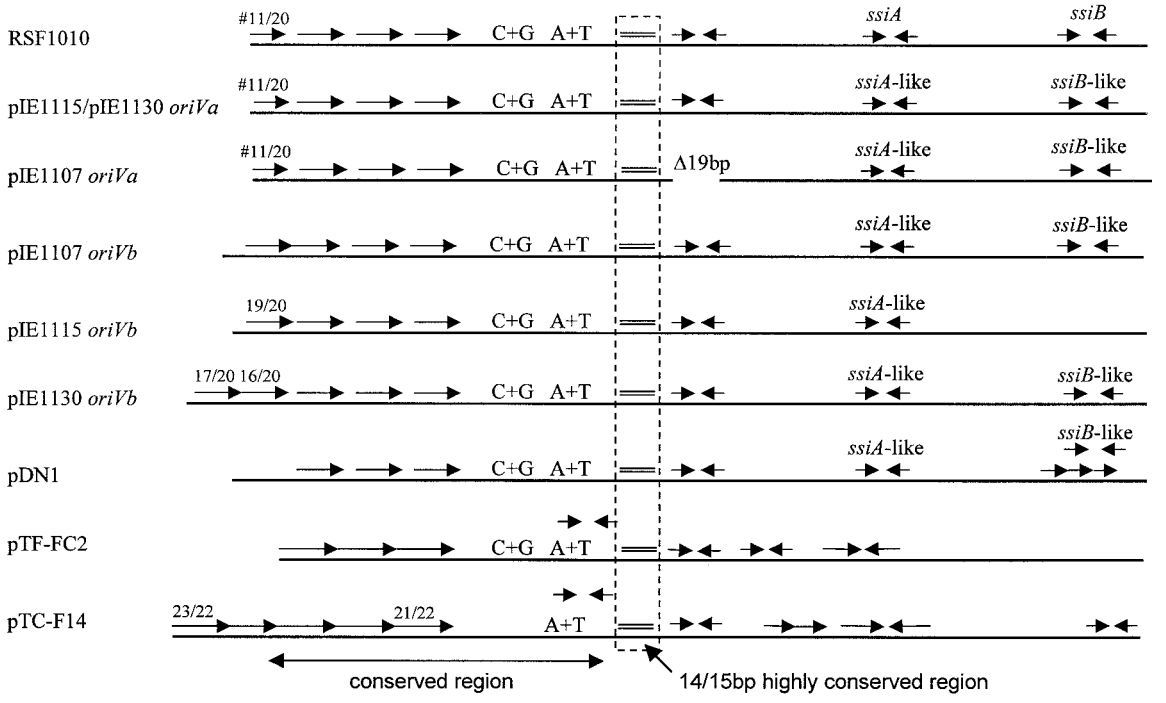


FIG. 3. Schematic diagram of the structural features within the active *oriV* and nonactive *oriV*-like regions of IncQ-like plasmids. *oriVa* and *oriVb* indicates duplicate *oriV* regions in the same plasmid. *oriVa* indicates the nonfunctional but incompatibility active *oriV*-like region. Arrows pointing in the same direction represent directly repeated sequences and arrows pointing toward each other inverted repeat sequences capable of forming stem-loop structures. Numbers above the arrows indicate the number of bases conserved in imperfectly conserved iterons, and #11/20 indicates a truncated 11bp iteron. A+T, on A+T-rich region; C+G, a G+C-rich region; *ssiA* and *ssiB*; single strand initiation sites. DNA synthesis proceeds in a single direction toward the right-hand side from *ssiA* and toward the left from *ssiB*. The sequence to the left of the highly conserved 14/15-bp region is more highly conserved than that to to the right.

pIE1130, the iterons are 20 bp long with 2-bp spacers, whereas in pTF-FC2 and pTC-F14 the iterons are 22-bp long without spacers. The number of iterons varies from three to five (Fig. 3), with 10 of the nucleotides within the direct repeats being conserved among all members of the family. A short distance from the direct repeats is a GC-rich region of 28 bp followed by an AT-rich region of 31 bp. It has been proposed that RepC-mediated DNA melting occurs at this AT-rich region (mentioned earlier), which allows access to the plasmid-encoded RepA helicase. Plasmid pTC-F14 is an exception in that no clear GC-rich region is apparent. Figure 4 shows an alignment of the direct-repeat GC-rich and AT-rich regions of five members of the IncQ plasmid family. The respective *oriVa*-like regions of pIE1107, pIE1115, and pIE1130 have been omitted because they have identical nucleotide sequences to RSF1010 within the region shown. There is 55% nucleotide sequence identity among all IncQ-type *oriV* regions within this region, with a 14- of -15-bp consensus sequence identity in the region immediately downstream of the AT-rich region. There is an abrupt decrease in the consensus nucleotide sequence identity in the *repC*-distal region adjacent to that shown in Fig. 4. The region where the *oriV* nucleotide sequence alignment is poor includes the region containing the IncQ plasmid *ssiA* and *ssiB* sites. None of the other members of the IncQ-like plasmid family have an extended inverted-repeat region (40-bp stem and 40-bp loop) identical to that present in the IncQ plasmids (48). Members of the IncQ-like plasmids have other sequences capable of forming stem-loop-like structures in the AT-rich and *repC*-distal *oriV* region (Fig. 3), but the biological activity of these has not been tested. An exception is pTF-FC2, where an exonuclease III-created deletion midway through the *cirB* stem-loop resulted in poor plasmid replication and a greatly reduced copy number (20).

Incompatibility groups. Incompatibility is the inability of coresident plasmids to be stably inherited in the absence of external selection (58). Replicon-associated incompatibility is believed to arise due to the inability of the cell to correct fluctuations in copy number between plasmids that have elements of their replication machinery in common. It has been established that the iterons of the IncQ plasmid family are themselves able to exert plasmid incompatibility (48, 65). Since the sequences of the iterons of plasmids of the IncQ family are related, it has been of interest to determine which members of the family are incompatible. All incompatibility testing has been carried out with *E. coli* host strains. Plasmids pIE1107 and RSF1010 were found to be incompatible due to the IncQ-identical iterons present in the nonfunctional *oriVa*-like region (93). If this region was deleted, the resulting *oriVb*-containing plasmid, pIE1108, was fully compatible with the IncQ plasmids. The more recently discovered plasmids pIE1115 and pIE1130 also have nonfunctional IncQ-identical iterons, which make them incompatible with RSF1010 (E. Tietze, unpublished data). If these are deleted, plasmid RSF1010 belongs to one incompatibility group, plasmids pIE1108, pIE1115, and

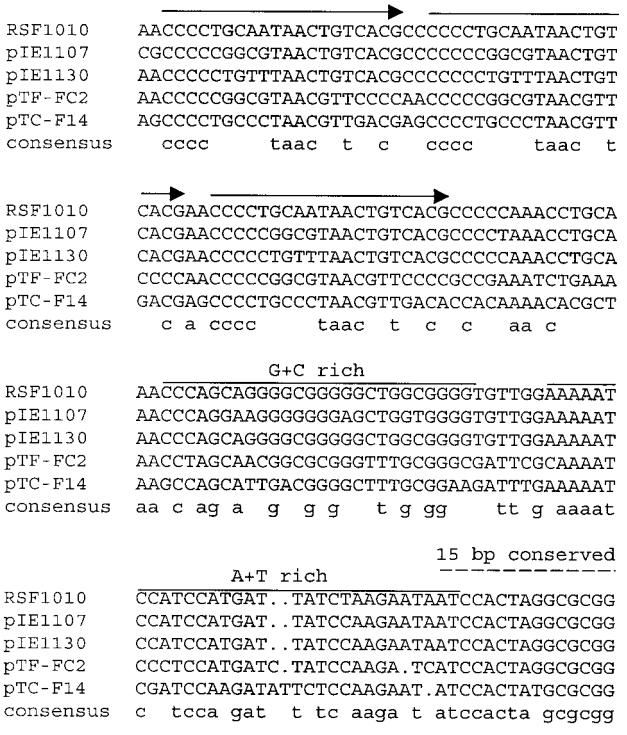


FIG. 4. Nucleotide sequence alignment of the highly conserved region within the functional *oriV* of the IncQ-like plasmids. Direct repeats are shown by arrows above the sequence, G+C and A+T regions as shown by solid lines, and the most highly conserved 15-bp region is shown by a broken line above the sequence. Sequences of plasmids pDN1 and pIE1115 have been omitted because they are almost identical to that of pIE1107 within the part of the *oriV* region shown. Plasmids pDN1 and pIE1115 have 1- and 2-bp mismatches, respectively, compared to pIE1107.

pDN1 belong to a second group, and plasmid pIE1130 belongs to a third group. Plasmids pTF-FC2 and pTC-F14 are also fully compatible with each other. However, the incompatibility relationship between the members of the IncQ plasmid family is complicated.

Since the most closely related pairs of plasmids (RSF1010/pIE1107 and pTF-FC2/pTC-F14) were fully compatible with each other, it was surprising to discover that the two pairs of the less highly conserved plasmids were asymmetrically (unidirectionally) incompatible. When the incoming selected plasmid was pIE1108, it would displace a resident pTC-F14 or a resident pTF-FC2 (27). However, if pIE1108 was the resident plasmid and pTC-F14 or pTF-FC2 was the selected incoming plasmid, these plasmids could not displace pIE1108. If plasmid RSF1010 was the incoming selected plasmid, it would displace a resident pTC-F14 but not a resident pTF-FC2. Likewise, an incoming selected pTC-F14 or pTF-FC2 would not displace a resident RSF1010. What makes the displacement of the pTC-F14 and pTF-FC2 by the IncQ plasmids more surprising is that both of these plasmids have a *pas* toxin-antitoxin, postsegregational killing system (27, 87), but this did not give them a competitive advantage over the IncQ plasmid which lacks such a system. The two plasmids isolated from heterotrophic bacteria clearly had an advantage over the plasmids isolated from the acidophilic, chemolithoautotrophic *E. coli*. One can speculate whether this is because the promoters and other functions of the plasmids isolated from bacteria which have a similar physiology to *E. coli* are more efficiently expressed in *E. coli* than are the plasmids from the chemolithoautotrophic bacteria. It would be interesting to know whether the situation is reversed in a chemolithoautotrophic host. Due to the rudimentary genetic system of *A. ferrooxidans* and *A. caldus*, incompatibility experiments are far more difficult to carry out in these hosts.

Plasmid copy number. The copy numbers of several members of the IncQ plasmid family have been reported (25) (Table 1). It has been estimated that RSF1010 has a copy number of 10 to 12 per chromosome in *E. coli* and *P. aeruginosa* (76). This is approximately the same as the 12 to 15 copies (20) or 10 to 14 copies (87) per chromosome estimated for pTF-FC2 and the 12 to 16 copies estimated for pTC-F14 in both *E. coli* and *A. caldus*. Like other iteron-containing plasmids, the copy number of IncQ plasmids is affected by the number of functional replicon-associated iterons. Becker and Meyer (7) determined the effect of inserting extra iterons on the copy number of a derivative of plasmid R1162. With two additional iterons, the number of the IncQ replicons dropped from 10–12 to only 5–7 copies per chromosome, and with three or four extra iterons it dropped to only 4–6 copies. They found that the low-copy-number derivatives were lost in the absence of selection and suggested that because R1162 does not possess a plasmid partitioning or stability system, it requires a high copy number to ensure stable inheritance by random distribution. It was proposed that this requirement for a high plasmid copy number placed a substantial metabolic burden on the cell which could enhance the selection of low-copy-number variants (7). Low-copy-number plasmids may be easily lost from the population, which favors the acquisition of accessory genes, which require a high copy number to function. This may place a restriction on the type of accessory genes which can be accommodated by the plasmid. It has been observed that several IncQ and related plasmids do have a substantial variety of accessory genes (Table 1) (Tietze, unpublished) but that many of these do not have consensus promoters or ribosome-binding sites. These genes may require a high copy number to ensure a sufficient level of gene expression. Copy numbers of plasmids of the IncQ family are remarkably consistent, at between 10 to 16 per chromosome, in spite of the number of iterons varying from three (pTF-FC2) to five (pTC-F14). In the IncQ-like plasmids with more than three iterons, the additional iterons all vary slightly from the consensus three. This variation takes the form of an extra base, a missing base, or a deletion of one of the bases of the 2-bp spacer region. It has been shown that relatively minor changes in iterons reduce or eliminate their functionality (55), and it may be that the sometimes small changes in the iterons (above the basic three) are sufficient to reduce their biological activity to maintain the copy number of IncQ and IncQ-like plasmids within the 10 to 16 copy range. Although this speculation remains to be tested, indications from standardized plasmid isolations are that all of the pIE-named plasmids have a copy number comparable to that of RSF1010 (Tietze, unpublished). It is possible that plasmids which have a toxin-antitoxin stability system (e.g., pTF-FC2 [see below]) would not have needed to depend on copy number to prevent plasmid loss and could have tolerated a lower

copy number than would plasmids where no stability system has been detected (e.g., RSF1010). However, toxin-antitoxin systems confer a relatively moderate (2.5- to 100-fold) increase in plasmid stability, and it is interesting that the "standard" copy number of plasmids of the IncQ family has been preserved irrespective of whether a stability system is present.

Mobilization Regions

Although none are self-transmissible, all plasmids of the IncQ family may be mobilized at high frequency in the presence of self-transmissible helper plasmids. Not all conjugative plasmids are equally effective at mobilizing IncQ or related plasmids, but conjugative plasmids of the *E. coli* IncPα (e.g., RK2/RP4/R68) and IncPβ (e.g., R751) groups (53, 69) are particularly efficient mobilizers of these plasmids. By using helper plasmids, the IncQ plasmids have been successfully mobilized to a large number of hosts including a wide range of gram-negative bacteria, several gram-positive bacteria including *Arthrobacter* spp. (52), *Streptomyces lividans*, and *Mycobacterium smegmatis* (29), cyanobacteria such as *Synechococcus* (43), and plant (13) and animal (99) cells. Likewise pTF-FC2 has been mobilized from *E. coli* to *Pseudomonas fluorescens* (37) and from *Agrobacterium tumefaciens* to plant cells (Rawlings, unpublished). RSF1010 can be efficiently mobilized by several other conjugation systems. For example, for mobilization into plant cells (13) and between agrobacteria (8), the T-DNA transfer apparatus of *A. tumefaciens* was used. RSF1010 plasmids can also be efficiently mobilized by chromosomally located *icm*/*dot* macrophage-killing virulence system of *Legionella pneumophila* (81, 83). Furthermore, it has been shown that some components of a type IV secretion system of *L. pneumophila* can substitute for some components of the *icm*/*dot* system that are required for RSF1010 mobilization (82).

Unlike the uniform replicons of the global IncQ plasmid family, all of which have *repA*, *repB*, and *repC* genes as well as several conserved features of the *oriV* in common, the mobilization systems are of two distinct types. The IncQ plasmid RSF1010, as well as pIE1107, pIE1115, pIE1130, and pDN1, comprise one mobilization subgroup and have a three-Mob-protein "IncQ-type" system (Fig. 1) which has substantial similarity to the two-Mob-protein system of *A. ferrooxidans* plasmid pTF1 (23). In contrast, the IncQ-like plasmids pTF-FC2 and pTC-F14 have a five-Mob-protein mobilization system (71) with a close resemblance to the Tra1 systems of the IncP plasmids and form a second subgroup.

IncQ-type systems. IncQ and other plasmids of that subgroup have a mobilization system which consists of MobA, MobB, and MobC proteins and an *oriT*-containing region. MobA is a multifunctional protein consisting of an N-terminal relaxase (78, 79) and a C-terminal DNA primase (36). The two domains appear to be able to function independently of each other. The C-terminal domain is synthesized from the same region and reading frame as the RepB protein, which is associated with vegetative plasmid replication. The relaxase activity is responsible for nicking the DNA at the *oriT* site, and the MobA primase activity is required for initiation of DNA replication that takes place during conjugal transfer. MobB and MobC serve as accessory proteins which enhance *nic* site cleavage and DNA transfer. MobB is synthesized from within the *mobA* gene but in a different reading frame, while MobC is transcribed divergently from a gene which lies on the opposite side of *oriT* (80) (Fig. 1). It has been proposed that MobB enhances relaxosome stability and *nic* site cleavage (64) while MobC helps unwind the DNA in the vicinity of the *nic* site, allowing easier access to the MobA relaxase (101). Interestingly, plasmid pTF1 appears to have only two mobilization proteins, MobL, which has an amino acid sequence related to the first 386 amino acids (aa) of the N-terminal relaxase region of MobA, and MobS, which is related to MobC (23). No equivalent of the accessory MobB protein appears to be present, and no open reading frames previously identified with DNA primase activity have been found on this 6,657-bp plasmid (47). A dendrogram showing the sequence relationship between the relaxase portion (assumed to be the N-terminal 400 aa) of the 709 aa MobA protein of RSF1010 is shown in Fig. 5. There are clearly three groups of MobA relaxases related to the IncQ plasmids. One group contains plasmids with replicons related to the IncQ plasmids as well as pAB6 and pSC101; a second group contains pTF1, pNGR23, and the Ti plasmids; and a third group contains plasmids of gram-positive bacteria. There are few plasmids in the database containing proteins related to the IncQ MobC accessory proteins; MobS of pTF1 is the most clearly related.

IncP-related systems. The alternate mobilization system of the IncQ plasmid family consists of five proteins (MobA, MobB, MobC, MobD, and MobE) and an *oriT*-containing region (71). These proteins have low but clear amino acid sequence similarity to the TraI, TraJ, TraK, TraL, and TraM proteins of the Tra1 region of the IncP plasmids, respectively (71). This relationship between the mobilization region of this group of IncQ-like plasmids and the TraI region of the IncP plasmids also applies to the order of the genes and the divergent nature of their transcription (Fig. 6). In the IncP plasmids, TraI is a relaxase of between 732 aa (RP4) (61) and 747 aa (R751) (92), and 409 aa of the N-terminal region of the MobA protein of pTF-FC2 has 26% sequence identity to the 458-aa N-terminal region of the RP4 TraI. TraJ is a DNA-binding protein that is thought to alter the local DNA structure and allow the TraI relaxase to access its binding site (60, 100). TraK is an accessory protein, also with DNA-binding activity, which is believed to wrap around a ±180-bp region of DNA near *oriT*, changing the superhelicity and further assisting TraI to access its target site (100, 102). The functions of TraL and TraM are unknown, although TraL has a Walker A/ATP/GTP-binding site (92).

Origins of transfer. Transfer origins play an essential role in the initiation of transfer-associated DNA replication and in the processing and transport of DNA from a donor to a recipient cell (46). Through comparison of a wide variety of transfer origins, five families of *oriT* sequences which show strong nucleotide conservation in the vicinity of the *nic* site have been identified (46, 100). The two- to three-Mob-protein IncQ-like plasmids belong to an *oriT* family which includes pTF1, pSC101, and the *oriT* of pTiC58. The IncQ plasmids are all highly conserved within this region, except that R1162 has a rare 1-bp mismatch compared with other members of the IncQ group (Fig. 7). The five-Mob-protein IncQ-like plasmids belong to a different *oriT* group, which includes the IncP plasmids

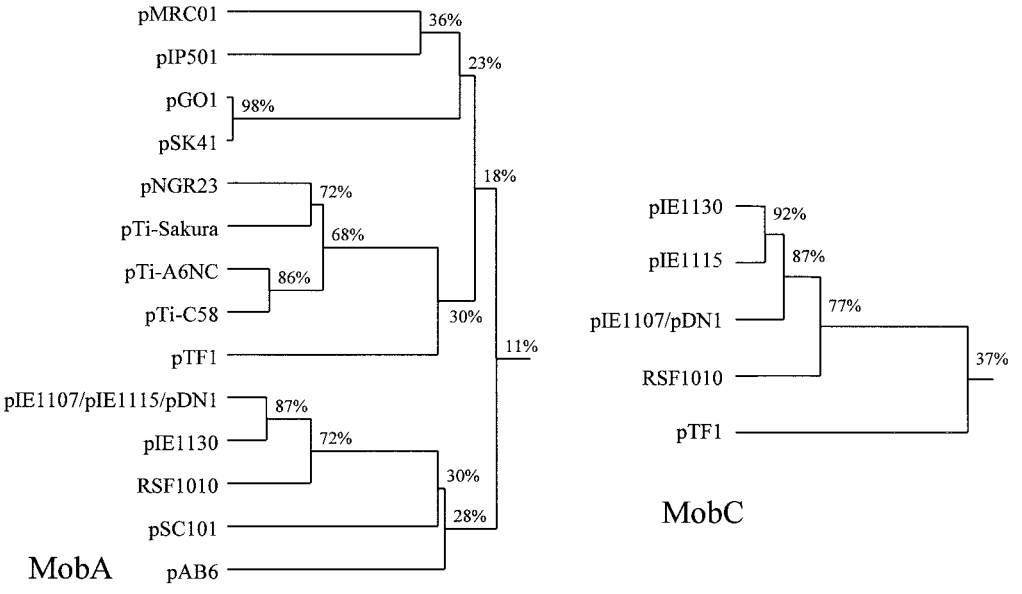


FIG. 5. Phylogenetic relationship between the MobA and MobC proteins of the IncQ-1-type plasmids and their comparison with related relaxases of mobilizable and self-transmissible plasmids. Since in the IncQ-1 plasmids the MobA proteins exist as a MobA-RepB fusion, only the N-terminal 400 aa was considered for comparison with other MobA-related proteins. The protein equivalent to MobC in pTF1 is called MobS. Percentages are percent amino acid sequence identities. GenBank accession numbers are as follows: pMRC01, g3582197; pIP501, L39769; pGO1, g1245474; pSK41, g3676420; pNGR23, P55418; pTi-Sakura, g6498282; pTi-A6NC, g2499023; pTi-C58, g2499022; pTF1, g127224; pIE1107, Z74787; pIE1115, AJ293027; pDN1, Y19120; pIE1130, AJ271879; RSF1010, M28829; pSC101, P14492; pAB6, g4884735.

RP4 and R751 and the left and right borders of the T-DNA of the Ti plasmids (Fig. 7).

ACCESSORY DNA

In addition to plasmid backbone DNA, most plasmids of the IncQ family have accessory DNA of some sort. The exception is plasmid pDN1, which appears to consist only of plasmid backbone. It is possible that plasmids without accessory genes are more common than recognized but that since these plasmids lack selectable markers they are not easily detected and therefore appear to be more rare than they actually are. As may be expected, the accessory DNA from plasmids isolated from medical or animal environments contain mainly antibiotic resistance genes, but these are also the markers for which most screening has been done. Although initial isolates of IncQ plasmids possessed mainly sulfonamide and streptomycin resistance genes, IncQ and IncQ-like plasmids may encode a wide variety of resistance genes (Table 1). Like many antibiotic resistance plasmids, acquisition of many of these genes has been as a result of transposon, insertion sequence, or integron activity. In RSF1010, the antibiotic resistance genes clearly have a different G+C content and codon usage compared with the rest of the plasmid backbone, which indicates that they have been acquired relatively recently (80). With pIE723, the *aadB* antibiotic resistance cassette has been inserted into a

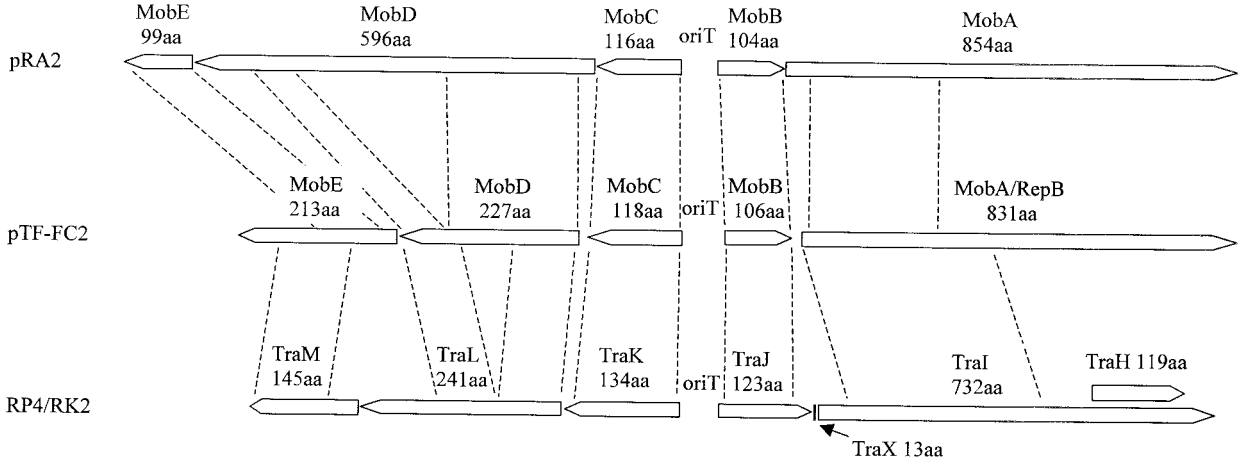


FIG. 6. Comparison of the regions involved in the mobilization of plasmids pRA2, pTF-FC2, and the IncPα plasmid RP4/RK2. Broken lines linking plasmids show the regions with amino acid sequence similarity.

FIG. 7. Two families of *oriT* nick regions to which the IncQ-family plasmids belong. Triangles show the positions of strand cleavage in plasmids for which this has been determined.

secondary site in plasmid RSF1010 as a result of integron activity in such a way that the insertion is stable (70).

It has been particularly interesting to investigate what types of genes are encoded by the accessory DNA of IncQ-like plasmids from the chemolithoautotrophic bacteria. In pTF-FC2, there is clear evidence of transposon Tn*21* activity (17). Two 38-bp terminal repeated sequences of identical nucleotide sequence to one of the ends of Tn*21* were present. Although only inactive remnants of a transposase and resolvase remained, the terminal repeats and *res* (cointegrate resolution) site were functional if Tn*21* transposase and resolvase was provided in *trans*. In addition to the above features, the 3.5-kb region between the Tn*21* ends contained a *glx*-like (glutaredoxin) gene, which was functional in *E. coli*, as well as a *merR*-like gene and a 406-aa-protein-encoding open reading frame resembling a 12-transmembrane segment multidrug transport protein (62), for which no expression could be detected in *E. coli*. The *glx* gene could complement *E. coli trx* (thioredoxin) mutants for a number of thioredoxin-dependent functions (17). It was able to substitute as electron donor in place of thioredoxin for the reduction of As^{V} to As^{III} by ArsC, the product of chromosomally located arsenate reductase gene of *A. ferrooxidans* (14; B. G. Butcher and D. E. Rawlings unpublished data). Since *A. ferrooxidans* strains are exposed to high levels of arsenic in arsenopyrite and other arsenic-containing ores, this may be the purpose for the *grx* gene being on pTF-FC2. The nucleotide sequence of the region of pTC-F14 most likely to contain accessory DNA has not yet been completed.

EVOLUTIONARY ASPECTS

Two IncQ-Type Plasmid Groups Identified

Based on a comparison of features such as the amino acid sequence similarities of the RepA, RepB, and RepC proteins, as well as the relatedness of the operon structure, Mob proteins, and *oriT* areas of the mobilization regions, there are at least two major groups within the IncQ plasmid family. We propose to call these plasmids IncQ groups 1 and 2. If additional groups are discovered, these could be named groups 3, 4, and so on. How many of these major groups may exist is uncertain and possibly awaits the investigation of plasmids in bacteria from environments other than those so far examined. Based on iteron sequence similarity and incompatibility, the two major groups can be subdivided into subgroups termed IncQ-1α for plasmids which have a functional *oriV* incompatible with RSF1010, IncQ-1β for those with an *oriV* incompatible with pIE1107, pIE1115, or pDN1; and IncQ-1γ for those with an *oriV* incompatible with pIE1130. We propose that the subgroups of the second group of IncQ-like plasmids should be named IncQ-2α for pTF-FC2 and IncQ-2β for pTC-F14. This scheme is consistent with that currently in use for the IncPα and IncPβ subgroups of IncP plasmids.

Sizes of Plasmids of the IncQ-Type Family

The single-strand displacement mechanism of replication, together with the plasmid-associated replication proteins, appears to have conferred exceptionally broad-host-range properties to plasmids of this type. In addition, an advantage may be derived from the plasmid being independent of the host priming system for lagging-strand DNA synthesis. A disadvantage of this mechanism of replication is that during replication, up to half of the plasmid will be present as a single-stranded intermediate, even if only for short periods. Single-stranded DNA is more reactive and much less physically stable than double-stranded DNA. One may speculate about whether the strand displacement replication mechanism places a limit on the size that may be attained by plasmids that replicate by this process. It is interesting that the natural plasmids which belong to the IncQ family are all fairly small, ranging in size from 5.1 kb (pDN1) to 14.0 kb (pTC-F14). None of these plasmids are self-transmissible, possibly because the coding capacity required for the synthesis of the conjugation apparatus is too large to be stably accommodated in a replicon of this type. It would be interesting to determine whether there is a difference in the limit to the size of plasmid that can be efficiently supported by a replicon of the strand displacement type compared with mechanisms in which leading- and lagging-DNA-strand synthesis takes place simultaneously.

Relaxase-Primase Fusion

As discussed above, the mobilization regions of the IncQ family plasmid backbone are of two different types (an IncQ-type and an IncP-type); however, in both types the relaxase, which is an essential activity for mobilization, has been fused to a primase, which is required for plasmid replication. A mobilization-replication fusion of this type does not appear to have happened between the IncQ-like *mob* region and non-IncQ-like replicon of pTF1 or between the IncP-type *mob* region and the unique replicon (unrelated to either IncP or IncQ plasmid families) of the non-self-transmissible plasmid pRA2 (45). Why a *mob-rep* fusion should have occurred in all plasmids of the IncQ family plasmids is unclear. The fusion in plasmid R1162 occurs because the MobA-linked primase is essential for plasmid mobilization (36). However, the *mob-rep* fusion does not appear to be required for the mobilization of all IncQ-type plasmids. In plasmid pTF-FC2, a *Cla*I site is naturally located within the coding region of this relaxase-primase

TABLE 3. Comparison of G+C contents of whole plasmid, backbones, and accessory DNA of sequenced plasmids of the IncQ family

Plasmid	G+C content (mol%)			
	Whole plasmid	Plasmid backbone	Accessory DNA	Difference
RSF1010	60.9	63.9	56.2	7.7
pIE1107	59.1	62.0	54.1	7.9
pIE1115	56.2	62.1	51.0	9.1
pIE1130	57.0	62.2	50.8	11.4
pDN1	62.3	62.3	NA[b]	NA
pTF-FC2	59.8	60.0	59.2	0.8
pTC-F14	ND[c]	59.3[a]	ND	ND

[a] Replicon only.
[b] NA, not applicable.
[c] ND, not determined.

fusion in such a way that the two activities of this multifunctional protein can be separated. By removal of DNA from either side of the *Cla*I site, it has been shown that each part can be expressed independently of the other and that only the relaxase portion is required for efficient mobilization of pTF-FC2 and only the primase portion is required for replication (22, 71). This indicates that the two parts of the large fusion protein can function independently of each other. The reason why both parts of the relaxase-primase protein have remained fused in the form of a large open reading frame so as to link the replicon and mobilization properties of the plasmid is still a mystery.

Nucleotide Sequence Composition

The nucleotide composition of the members of the IncQ plasmid family investigated to date is approximately 60% G+C (Table 3). Scholtz et al. (80) showed that the codon bias of the genes comprising the plasmid backbone (replication/maintenance and mobilization functions) was markedly different from that of the genes encoding antibiotic resistance. This was interpreted as indicating that the antibiotic resistance genes had been incorporated more recently in the evolution of RSF1010. The separation of the *repC* genes from the *oriV* region by antibiotic resistance genes in RSF1010 but not the other members of the IncQ plasmid family is consistent with the view that the antibiotic resistance genes were inserted after a functional replicon had evolved. In Table 3 we present calculations of the moles percent G+C ratios for the plasmid sequences as a whole as well as separate moles percent G+C ratios for the plasmid backbones and accessory DNA (frequently antibiotic or transposon-like regions). The difference in G+C content for the plasmid backbones compared with the accessory DNA for the IncQ-1α, IncQ-1β, and IncQ-1γ plasmids ranges from 7.7% for RSF1010 to 11.4% for pIE1130. For pTF-FC2, the difference between the accessory DNA (within the 38-bp Tn*21*-like transposon ends) is only 0.8%. This suggests that insertion of a Tn*21*-like transposon into pTF-FC2 is an evolutionarily old event or that the source of the transposon was an organism with a G+C ratio close to that of the pTF-FC2 backbone. This second possibility is likely since the G+C content of *A. ferrooxidans* genome is 59%, which is very close to the average content for pTF-FC2.

Evolution of New Incompatibility Groups

The finding of several IncQ-like plasmids with more than one *oriV* region and the unidirectional (asymmetrical) incompatibility of these plasmids toward RSF1010 is intriguing. A model for the diversification of plasmid incompatibility has been proposed by Sykora (91), and its relevance to the IncQ plasmid family has been discussed by Tietze (93) and Osborn et al. (59). According to this model, plasmid cointegrate formation can serve as mechanism for the evolution of new incompatibility groups. Cointegrate plasmids with two compatible origins of replication have an advantage over the double-plasmid state, where the two plasmids exist as separate compatible units. Each of the separate plasmids may be eliminated by invasion of an incompatible plasmid, whereas with a cointegrate, the incompatible replicon is saved from elimination by the other replicon. Cointegrate plasmids may allow for the evolution of new *rep* genes and incompatibility groups because conservative selection of one of the replicons is relaxed. Applying the model to the IncQ plasmid family, the presence of duplicate replicons would allow for the *rep* genes and the target iterons of one of the replicons to accumulate mutations, resulting in pseudogenes and altered iterons. In an environment where the functional replicon could be displaced, there will be selective pressure to restore the cointegrate state in which both replicons are functional. However, because the genes and iterons have structurally diverged, the restored replicon may have a new incompatibility specificity. Should the old *rep* gene be inactivated or the cointegrate be resolved, the new *rep* genes and iterons would come under strong selection to function as an efficient, independent, and competitive replicon. The result of this process is that an IncQ-like plasmid of a new incompatibility type would be formed.

As pointed out by Tietze (93) plasmids which have two *oriV* regions, such as pIE1107 and pIE1130, could have been derived from cointegrate formation between two plasmids followed by partial deletion of one of the replicons. An alternate explanation is that the second *oriV* could have been acquired by recombination with the *oriVa*-containing region of a different IncQ-type plasmid. Although retention of two *oriV* regions could be a driving force for further evolution, there is another possible reason for two *oriV* regions in the case of an environment like piggery manure. This environment would appear to be populated by cells containing a large variety of plasmids, including several types of IncQ or IncQ-related plasmids. A plasmid which retained an *oriV* with incompatibility to IncQ plasmids but which replicated using an alternate compatible IncQ-like *oriV* would have a distinct selective advantage in such an environment. Cointegrate plasmids of this type would not be displaced by IncQ plasmids, but since they have an IncQ incompatibility determinant associated with the nonessential *oriV*, they would be able to reduce the copy number and ultimately displace IncQ plasmids from a host cell. For example, the nonfunctional, RSF1010-identical, *oriV*-like region of plasmid pIE1107 is able to displace the IncQ plasmid RSF1010 but is itself not displaced by RSF1010 because it uses an alternate RSF1010-compatible replicon for its own replication. This could provide a plasmid like pIE1107 (e.g., pIE1115 and pIE1130) with a distinct competitive advantage in a densely populated plasmid-rich environment like piggery manure. It is

interesting that plasmid pDN1, which has a very similar core region to pIE1107, has only a single *oriV* (Fig. 1), possibly because competition from invading plasmids is not as fierce in the soil (sheep footrot) environment in which it occurs.

There is a tension between the idea of having a second *oriV* serving as a driving force for evolution or as an additional incompatibility determinant. To serve as an element for evolution, the *oriV* would need to accumulate mutations, whereas to serve as an additional incompatibility determinant, it would need to remain relatively unaltered so as retain the same incompatibility as the cointegrate plasmids most serious competitors. Interestingly, the secondary *oriV* regions of pIE1107, pIE1115, and pIE1130 have retained identical iteron sequences and hence the same incompatibility as the IncQ plasmids. Even the iteron-flanking region which contains the *ssiA* and *ssiB* sites has remained almost free of mutations. It is difficult to know whether this apparent sequence stability is as a consequence of a recent acquisition or whether there is strong selection for an unaltered secondary *oriV* to enable the plasmid to outcompete plasmids of the same incompatibility group as the extra *oriV*. Since only the *oriV* and not a second copy of the *rep* genes of the second cointegrate has been retained, the additional *oriV* probably serves as a means of giving a plasmid like pIE1107 a competitive advantage rather than being a means for the evolution of a new replicon.

Acquisition of Regulation Genes

Regulation of the genes associated with the replication and mobilization functions of the IncQ plasmids RSF1010 and R1162 has been investigated in detail. Four promoters associated with the expression of these core functions have been identified, as well as an autoregulatory gene termed *cac* (control of *repAC*) (51). Regulation of the other members of the IncQ plasmid family is largely uninvestigated. However, from an examination of the IncQ-like plasmids, only pIE1130 has a gene or open reading frame with sequence similarity to the *cac* gene or gene product. This implies that the regulatory functions of the others must be substantially different. The 350-bp region, which is located between the *rep* and *mob* genes of pIE1107, pIE1115, and pDN1, contains a small 78-aa-protein-encoding open reading frame, which may serve as a *rep* or *mob* gene regulator. The G+C content of this region of pIE1107 is 48%, compared with 64% along the *mob* and *rep* genes (59). Similarly, it has been pointed out that the G+C content of the gene for the OrfE protein adjacent to the *cac* gene of RSF1010 differs significantly from the *rep* and *mob* genes. This observation has been used to argue that this region has been recently acquired (80). Although evidence of recent acquisition of regulatory genes is not fully convincing, the IncQ plasmid family has more variation in this region than do other regions of their backbone structure.

The IncQ-like plasmid group 2 also possess small genes within the *mob-rep* intergenic region. Surprisingly, these proteins appear to have nothing to do with replication control but constitute a toxin-antitoxin proteic plasmid stability system named *pas* (plasmid addiction system) (87). Evidence that the *pas* genes do not encode regulatory proteins was provided by the introduction of a frameshift mutation within the *pasA* antitoxin gene. This resulted in very sick *E. coli* host cells and in a strong selection for the creation of two different types of spontaneous *pas* deletions. In one of these deletions, almost the whole of the *pas* system had been lost and the plasmid was able to replicate in *E. coli* with a copy number that was unaltered from that of the original replicon (87). The source of the *pas* system is unknown, but at 60%, the G+C content of the *pas* region is no different from that of the rest of the pTF-FC2 plasmid.

In the IncQ plasmid RSF1010, the *oriT* site is a major site of plasmid regulation including plasmid replication (26). However, this may not apply to all IncQ-type plasmids because although the regulation of mobilization genes of pTF-FC2 also occurs at the *oriT* region (72), the replicon can function independently of the mobilization region at a copy number that is indistinguishable from that in the intact plasmid.

Counterselection for Restriction Enzyme Recognition Sites

DNA which enters a new host during conjugation may be subject to digestion by restriction endonucleases which cleave DNA that has not been modified by cognate methyltransferases (3, 10). Promiscuous broad-host-range plasmids are likely to encounter different restriction modification systems when entering different hosts. One of the strategies that plasmids have acquired to prevent cleavage by restriction enzymes is the use of antirestriction mechanisms (15). However, these tend to target specific restriction enzymes and do not confer universal protection. The elimination of restriction enzyme cleavage sites is a more universal protection mechanism. For example, in the broad-host-range IncP plasmids, type II restriction endonuclease sites are (with two exceptions) particularly underrepresented on the backbone of broad-host-range plasmid RP4 relative to the chromososome of *P. aeruginosa*, a natural host bacterium which has a similar G+C ratio (97). It has previously been noted that the replicon of the IncQ-like plasmid pTF-FC2 had remarkably few cleavage sites for the commonly used type II restriction enzymes (21). Rather than examine the sequences of the IncQ-like plasmids for all restriction enzymes, we have used the strategy of Wilkins et al. (97) and determined the frequency of occurrence of all 64 6-bp sequences with twofold rotational symmetry. These 6-bp palindromes are the most common class of recognition sequence for DNA cleavage by type II restriction enzymes. Each plasmid as a whole was searched for the occurrence of these palindromes, and this search was repeated separately for the backbone and accessory DNA regions. The frequency has been calculated as the average number of base pairs per palindrome occurrence and is shown in Table 4. In all four plasmids examined, there is a markedly lower frequency of occurrence of the 64 6-bp palindromes in the plasmid backbone (one site every 137 to 321 bp) compared with the accessory DNA (one site every 57 to 90 bp). This difference in frequency is most noticeable for pTF-FC2. In this plasmid, no 6-bp palindrome occurs more than once, with the exception of TTGCAA, which occurs seven times. However, no restriction enzyme has been identified which recognizes this palindrome, and it may be that there has been no selection against its presence. One interpretation of the reduced occurrence of 6-bp palindromes in plasmid backbones is that these structures are relatively ancient compared with the accessory DNA, which in most cases is a

TABLE 4. Number and frequency of occurrence of the 64 6-bp palindromes within the backbone and accessory DNA of the IncQ family of plasmids

Plasmid	Whole plasmid		Plasmid backbone[a]		Accessory DNA[a]	
	Size (bp) (no. of palindromes)	No. of bp per palindrome occurrence	Size (bp) (no. of palindromes)	No. of bp per palindrome occurrence	Size (bp) (no. of palindromes)	No. of bp per palindrome occurrence
RSF1010	8,684 (84)	103	5,365 (38)	204	3,319 (46)	72
pTF-FC2	12,180 (88)	138	8,674 (27)	321	3,506 (61)	57
pIE1107	8,520 (75)	113	5,310 (36)	153	3,210 (39)	82
pIE1115	10,687 (99)	108	5,100 (37)	138	5,587 (62)	90
pIE1130	10,687 (107)	99	5,500 (36)	147	4,187 (71)	59
pDN1	5,112 (36)	142	5,112 (36)	142	NA[b]	NA

[a] The exact position of the transition between backbone and accessory DNA is not known for some plasmids.
[b] NA, not applicable.

result of recent transposition. Selection for the elimination of restriction enzyme cleavage sites from the backbone structures of plasmids of the IncQ family may have taken place as a consequence of their promiscuous nature, whereas this selection has been absent from the accessory DNA, which has presumably been acquired from narrow-host-range plasmids or chromosomes.

SUMMARY AND FUTURE DIRECTIONS

The two major groups of IncQ-like plasmids described in this review were isolated from bacteria growing in two very different environments. It is unlikely that IncQ-like plasmids would be found in bacteria as diverse as the neutrophilic, heterotrophic bacteria associated with medical samples or animal wastes and the acidophilic, obligately autotrophic bacteria associated with inorganic mining environments, if they are not also present in other ecological niches. It may therefore be assumed that other plasmids of the IncQ family will be found in a variety of other environments once these environments are examined for their presence. The Southern hybridization and PCR-based techniques used to screen for IncQ-like plasmids were based on hybridization probes and PCR primers from IncQ-like group 1 plasmids and would almost certainly not have detected IncQ-like group 2 plasmids such as pTF-FC2 or pTC-F14. Likewise, the very successful mating experiments used to capture the IncQ-like plasmids pIE1107, pIE1115, and pIE1130 in *E. coli* were dependent on the plasmids having selectable antibiotic resistance markers and would not have isolated plasmids like pDN1. A comparison of the DNA sequences of all IncQ-like plasmids discovered so far is needed to attempt to identify regions which are sufficiently conserved to be used as hybridization probes or for the design of PCR primers which would detect members of the IncQ plasmids belonging to both known major groups. The most highly conserved regions occur within the *repC* genes, but PCR primers that would detect all the currently known types of IncQ-like plasmids still need to be designed and tested before they can be used to screen for these plasmids in new environments. Alternatively, degenerate primers, possibly used together with an appropriate PCR protocol, are required.

All of the IncQ-like plasmids discovered so far have a backbone consisting of a mobilization region and a plasmid replication maintenance region. Their wide distribution indicates that the replication and mobilization combination characteristic of the IncQ plasmid family must be particularly competitive from an ecological point of view. It is still not certain what the exact reasons are for the especially broad-host-range nature of these plasmids. As suggested above, it may be that the replicon is independent of many of the proteins required by the host replication apparatus. However, this independence from the host background would also have to extend to transcription from the promoters of essential plasmid replication and mobilization functions. Differences in the stability of IncQ and IncQ-like plasmids in the absence of selection in different hosts have been observed (86). There is much scope for future studies of the expression and control of mobilization and replicon genes as well as of plasmid copy number control in a wide range of hosts. Studies of this sort should provide additional insights into the reasons for the broad-host-range, promiscuous character of the IncQ-like plasmids. In addition, Kim and Meyer (41) reported that a 75-bp antisense RNA molecule transcribed from DNA immediately upstream of the *repA* gene of IncQ plasmid R1162 regulated the plasmid copy number. Investigations into the possibility that antisense RNA may play a role in the regulation of other members of the IncQ-family have been neglected.

The lack of *rep* gene complementation between members of the IncQ plasmid family raises questions about what determines Rep protein-*oriV*-binding specificity. These cross-complementation experiments have been reported only for the R300B/pTF-FC2 and pTC-F14/pTF-FC2 plasmid couples. Other studies of this type which include members of the IncQ-like plasmid family are required. Since the RepC protein binds specifically to the iterons, one may speculate that the RepC-*oriV*-binding specificity may affect the ability of plasmids to cross-complement each other. If this is indeed the reason, it should be possible to carry out experiments to test whether would one obtain interplasmid *repA* and *repB* complementation, as long as the specific *repC* and *oriV* combination is provided. Alternately, it may be that Rep proteins from various members of the IncQ family can be mixed only if the corresponding elements of the *oriV* region with which they naturally react are exchanged as well. Since this type of speculation is fairly easily testable, more experiments of this type are required.

The observation that the backbones of all plasmids of the IncQ family discovered so far have a G+C content of 60 to 64% is interesting. Many of the IncQ and IncQ-like plasmids were isolated from bacteria with G+C contents that are con-

sistent with this fairly high G+C content (*P. aeruginosa*, 67%; *A. ferrooxidans*, 59%; *A. caldus*, 61 to 63%). However, some of the IncQ-related plasmids were originally isolated from genera with considerably lower G+C contents, such as *Dichelobacter* (45%) or *Salmonella* and *Escherichia* (48 to 52%). In spite of this, the G+C content of the plasmid backbone is uniformly high. Whether this high G+C content has biological significance or is simply a consequence of the evolutionary history of the IncQ-like plasmid family is an unanswered question. Possibly the ancestral IncQ-like plasmid evolved in bacteria with a high G+C content and there has been insufficient selection pressure to reverse this. Since the accessory DNA of the IncQ-1 plasmids has a substantially lower G+C content than and codon usage pattern different from the rest of the backbone, these regions must have been acquired from other sources. This suggests that the plasmid must reside for unknown periods in hosts with G+C contents lower than 60 to 64%. It may be that these plasmids are now so promiscuous that the length of time they spend in a host with a low G+C content before again residing in a host with a high G+C content is too short to allow the evolution of a plasmid G+C content that matches that of the host. It will be interesting to monitor this feature in IncQ-like plasmids that may be discovered in the future, especially those isolated from bacteria with G+C contents substantially different from 60 to 64%.

The lack of restriction endonuclease cleavage sites within the plasmid backbone is presumably an adaptation to the ability of the IncQ-like plasmids to spread horizontally through a variety of hosts within a mixed microbial community. However, this lack of restriction endonuclease cleavage sites does not apply to the accessory DNA, and the acquisition of DNA with an abundance of such sites may represent an "Achilles heel" that restricts rapid horizontal plasmid spread. Plasmids like pDN1 which consist only of a plasmid backbone with few restriction endonuclease cleavage sites, may have an advantage in horizontal transfer. It is possible that plasmid pDN1, which does not appear to have accessory DNA and has a very different G+C content from the host in which it was discovered, is a "lightweight" backbone plasmid cruising between hosts. However, the presence of a plasmid with its intrinsic metabolic burden would decrease host cell fitness. Under these conditions, there would be selection pressure for backbone plasmids to acquire accessory DNA that increases host fitness and helps to ensure that plasmid-containing cells are not eliminated from the population. Since the acquisition of accessory genes with their accompanying restriction enzyme cleavage sites would in turn restrict horizontal plasmid transfer, there is likely to be a tension between ease of plasmid transfer and benefit to the host. Natural backbone plasmids like pDN1 and plasmids with accessory genes like RSF1010, pTF-FC2, and others possibly represent nature's compromise solution to this problem.

ACKNOWLEDGMENTS

Work in the laboratory of D.E.R. is supported by Billiton Process Research Laboratories (Randburg, South Africa), the University of Stellenbosch, and the National Research Foundation (Pretoria, South Africa).

REFERENCES

1. **Albritton, W. L., J. L. Brunton, L. Slaney, and I. MacLean.** 1982. Plasmid-mediated sulfonamide resistance in *Haemophilus ducreyi*. Antimicrob. Agents Chemother. **21:**159–165.
2. **Anthony, L. S., M. Z. Gu, S. C. Cowley, W. W. Leung, and F. E. Nano.** 1991. Transformation and allelic replacement in *Francisella* spp. J. Gen. Microbiol. **137:**2697–2703.
3. **Arber, W., and S. Linn.** 1969. DNA modification and restriction. Annu. Rev. Biochem. **38:**467–500.
4. **Argyle, J. L., B. J. Rapp-Giles, and J. D. Wall.** 1992. Plasmid transfer by conjugation in *Desulfovibrio desulfuricans*. FEMS Microbiol. Lett. **73:**255–262.
5. **Bagdasarian, M., R. Lurz, B. Rückert, F. C. H. Franklin, M. M. Bagdasarian, J. Frey, and K. N. Timmis.** 1981. Specific purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. Gene **16:**237–247.
6. **Barth, P. T., and N. J. Grinter.** 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. **120:**618–630.
7. **Becker, E. C., and R. J. Meyer.** 1997. Acquisition of resistance genes by the IncQ plasmid R1162 is limited by its high copy number and lack of a partitioning mechanism. J. Bacteriol. **179:**5947–5950.
8. **Beijersbergen, A., A. Dendulkras, R. A. Schilperoort, and P. J. J. Hooykaas.** 1992. Conjugative transfer by the virulence system of *Agrobacterium tumefaciens*. Science **256:**1324–1327.
9. **Bingle, W. H., and J. Smit.** 1990. High-level expression vectors for *Caulobacter crescentus* incorporating the transcription/translation initiation regions of the paracrystalline surface-layer-protein gene. Plasmid **24:**143–148.
10. **Boyer, H. W.** 1971. DNA restriction and modification mechanisms in bacteria. Annu. Rev. Microbiol. **25:**153–176.
11. **Brunton, J., D. Clare, and M. A. Meier.** 1986. Molecular epidemiology of antibiotic resistance plasmids of *Haemophilus* species and *Neisseria gonorrhoeae*. Rev. Infect. Dis. **8:**713–724.
12. **Bryan, L. E., H. M. van den Elzen, and J. T. Tseng.** 1972. Transferable drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. **1:**22–29.
13. **Buchanan-Wollaston, V., J. E. Passiatore, and F. Cannon.** 1987. The *mob* and *oriT* mobilization functions of a bacterial plasmid promote its transfer to plants. Nature **328:**172–175.
14. **Butcher, B. G., S. M. Deane, and D. E. Rawlings.** 2000. The chromosomal arsenic resistance genes of *Thiobacillus ferrooxidans* have an unusual arrangement and confer increased arsenic and antimony resistance to *Escherichia coli*. Appl. Environ. Microbiol. **66:**1826–1833.
15. **Chilley, P. M., and B. M. Wilkins.** 1995. Distribution of the *ardA* family of antirestriction genes on conjugative plasmids. Microbiology **141:**2157–2164.
16. **Cotter, T. W., and M. F. Thomashow.** 1992. A conjugation procedure for *Bdellovibrio bacteriovorus* and its use to identify DNA sequences that enhance the plaque-forming ability of a spontaneous host-independent mutant. J. Bacteriol. **174:**6011–6017.
17. **Clennel, A.-M., B. Johnston, and D. E. Rawlings.** 1995. Structure and function of Tn*5467*, a Tn*21*-like transposon located on *Thiobacillus ferrooxidans* plasmid pTF-FC2. Appl. Environ. Microbiol. **61:**223–4229.
18. **Dehio, C., and M. Meyer.** 1997. Maintenance of broad-host-range incompatibility group P and group Q plasmids and transposition of Tn*5* in *Bartonella henselae* following conjugal plasmid transfer from *Escherichia coli*. J. Bacteriol. **179:**538–540.
19. **del Solar, G., R. Giraldo, M. J. Ruiz-Echevarría, M. Espinosa, and R. Díaz-Orejas.** 1998. Replication and control of circular bacterial plasmids. Microbiol. Mol. Biol. Rev. **62:**434–464.
20. **Dorrington R. A., and D. E. Rawlings.** 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171:**2735–2739.
21. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minmum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
22. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli.* Gene **108:**7–14.
23. **Drolet, M., P. Zanga, and P. C. K. Lau.** 1990. The mobilization and origin of transfer regions of a *Thiobacillus ferroxidans* plasmid: relatedness to plasmids RSF1010 and pSC101. Mol. Microbiol. **4:**1381–1391.
24. **Fling, M. E., J. Kopf, and C. Richards.** 1988. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. Plasmid **19:**30–38.
25. **Frey, J., and M. Bagdasarian.** 1989. The biology of IncQ plasmids, p. 79–93. *In* C. M. Thomas (ed.), Promiscuous plasmids of Gram-negative bacteria. Academic Press, Ltd., London, United Kingdom.
26. **Frey, J., M. M. Bagdasarian, and M. Bagdasarian.** 1992. Replication and copy number control of the broad-host-range plasmid RSF1010. Gene **113:**101–106.
27. **Gardner M. N., S. M. Deane, and D. E. Rawlings.** 2001. Isolation of a new broad host-range plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus ferrooxidans* and analysis of the plasmid replicon. J. Bacteriol. **183:**3303–3309.
28. **Goncharoff, P., J. K. Yip, H. Wang, H. C. Schreiner, J. A. Pai, D. Furgang, R. H. Stevens, D. H. Figurski, and D. H. Fine.** 1993. Conjugal transfer of

broad-host-range incompatibility group P and Q plasmids from *Escherichia coli* to *Actinobacillus actinomycetemcomitans*. Infect. Immun. **61:**2544–3547.
29. **Gormley, E., and J. Davies.** 1991. Transfer of plasmid RSF1010 by conjugation from *Escherichia coli* to *Steptomyces lividans* and *Mycobacterium smegmatis*. J. Bacteriol. **173:**6705–6708.
30. **Götz, A., R. Pukall, E. Smit, E. Tietze, R. Prager, H. Tschäpe, J. D. van Elsas, and K. Smalla.** 1996. Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. Appl. Environ. Microbiol. **62:**2621–2628.
31. **Grinter, N. J., and P. T. Barth.** 1976. Characterization of SmSu plasmids by restriction endonuclease cleavage and compatibility testing. J. Bacteriol. **128:**394–400.
32. **Guerry, P., J. van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117:** 619–630.
33. **Hall, M. C., and S. W. Matson.** 1999. Helicase motifs: the engine that powers DNA unwinding. Mol. Microbiol. **34:**867–877.
34. **Hallberg, K. B., and E. B. Lindström.** 1994. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology **140:**3451–3456.
35. **Haring, V., and E. Scherzinger.** 1989. Replication proteins of the IncQ plasmid RSF1010, p. 95–124. *In* C. M. Thomas (ed.), Promiscuous plasmids of Gram-negative bacteria. Academic Press, Ltd., London, United Kingdom.
36. **Henderson, D., and R. J. Meyer.** 1999. The MobA-linked primase is the only replication protein of R1162 required for conjugal mobilization. J. Bacteriol. **181:**2973–2978.
37. **Herrera, G., S. J. Snyman, and J. A. Thomson.** 1994. Construction of a bioinsecticidal strain of *Pseudomonas fluorescens* active against the sugarcane borer, *Eldana saccharina*. Appl. Eviron. Microbiol. **60:**682–890.
38. **Honda, Y., H. Sakai, T. Komano, and M. Bagdasarian.** 1989. RepB′ is required *in trans* for the two single-strand DNA initiation signals in *oriV* of plasmid RSF1010. Gene **80:**155–159.
39. **Honda, Y., H. Sakai, H. Hiasa, K. Tanaka, and T. Komano.** 1991. Functional division and reconstruction of a plasmid replication origin: molecular dissection of the *oriV* of the broad-host-range plasmid RSF1010. Proc. Natl. Acad. Sci. USA **88:**179–183.
40. **Kelly, D. P., and A. P. Harrison.** 1983. Genus *Thiobacillus* Beijerinck, p. 1842–1858. *In* J. T. Stanley, M. P. Bryant, N. Pfenning, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol 3. The Williams & Wilkins Co., Baltimore, Md.
41. **Kim, Y.-J., and R. J. Meyer.** 1986. Copy-number of broad host-range plasmid R1162 is regulated by a small RNA. Nucleic Acids Res. **14:**8027–8046
42. **Kim, Y.-J., and R. J. Meyer.** 1991. An essential iteron-binding protein required for plasmid R1162 replication induces localized melting within the origin at a specific site in AT-rich DNA. J. Bacteriol. **173:**5539–5545.
43. **Kreps, S., F. Ferino, C. Mosrin, J. Gerits, M. Mergeay, and P. Thuriaux.** 1990. Conjugatve tranfer and autonomous replication of a promiscuous IncQ plasmid in the cyanobacterium *Synechocystis* PCC6803. Mol. Gen. Genet. **221:**129–133.
44. **Kusano, T., K. Sugawara, C. Inoue, T. Takeshima, M. Numata, and T. Shiratori.** 1992. Electrotransofrmation of *Thiobacillus ferrooxidans* with plasmids containing a *mer* determinant as the selective marker by electroporation. J. Bacteriol. **174:**6617–6623.
45. **Kwong, S. M., C. C. Yeo, A. Suwanto, and C. L. Poh.** 2000. Characterization of the endogenous plasmid from *Pseudomonas alcaligenes* NCIB 9867: DNA sequence and mechanism of transfer. J. Bacteriol. **182:**81–90.
46. **Lanka, E., and B. M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. Annu. Rev. Biochem. **64:**141–169.
47. **Lau, P. C. K., M. Drolet, P. Zanga, and D. S. Holmes.** 1993. Plasmid technology for biomining applications, p. 635–644. *In* A. Torma, M. Apel, and C. Brierley (ed.), Biohydrometallurgical technologies, vol. 2. TMS Press, Warrendale, Pa.
48. **Lin, L.-S., Y.-J. Kim, and R. J. Meyer.** 1987. The 20 bp. Directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility in vivo and inhibits R1162 DNA replication in vitro. Mol. Gen. Genet. **208:** 390–397.
49. **Lin, L.-S., and R. J. Meyer.** 1987. DNA synthesis is initiated at two positions within the origin of replication of plasmid RSF1010. Nucleic Acids Res. **20:**8319–8331.
50. **Mack, K., and R. W. Titball.** 1996. Transformation of *Burkholderia pseudomallei* by electroporation. Anal. Biochem. **242:**73–76.
51. **Maeser, S., P. Scholtz, S. Otto, and E. Scherzinger.** 1990. Gene F of plasmid FSR1010 codes for a low-molecular weight repressor protein that autoregulates expression of the *repAC* operon. Nucleic Acids Res. **18:**6215–6222.
52. **Margesin, R., and F. Schinner.** 1997. Heavy metal resistant *Arthrobacter* sp. — a tool for studying conjugal plasmid transfer between gram-negative and gram-positive bacteria. J. Basic Microbiol. **37:**217–227.
53. **Meyer, R., M. Hinds, and M. Brasch.** 1982. Properties of R1162, a broad host range, high copy number plasmid. J. Bacteriol. **150:**552–562.
54. **Miao, D.-M., Y. Honda, K. Tanaka, A. Higashi, T. Nakamura, Y. Taguchi, H. Sakai., T. Komano, and M. Bagdasarian.** 1993. A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad-host-range plasmid RSF1010. Nucleic Acids Res. **21:**4900–4903.
55. **Miao, D.-M., H. Sakai, S. Okamoto, K. Tanaka, M. Okuda, Y. Honda, T. Komano, and M. Bagdasarian.** 1995. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. Nucleic Acids Res. **23:**3295–3300.
56. **Nakatsu, C. H., and R. C. Wyndham.** 1993. Cloning and expression of the transposable chlorobenzoate-3,4-dioxygenase genes of *Alcaligenes* sp. strain BR60. Appl. Environ. Microbiol. **59:**3625–3633.
57. **Nesvera, J., J. Hochmannova, M. Patek, A. Sroglova, and V. Becvarova.** 1994. Transfer of the broad-host-range plasmid RSF1010 and other plasmid vectors to the gram-positive methylotroph *Brevibacterium methylicum* by electrotransformation. Appl. Microbiol. Biotechnol. **40:**864–866.
58. **Novick, R. P.** 1987. Plasmid incompatibility. Microbiol. Rev. **51:**381–395.
59. **Osborn, M., S. Bron, N. Firth, S. Holsappel, A. Huddleston, R. Kiewiet, W. Meijer, J. Seegers, R. Skurray, P. Terpstra, C. M. Thomas, P. Thorsted, E. Tietze, and S. L. Turner.** 2000. The evolution of bacterial plasmids, p. 301–361. *In* C. M. Thomas (ed.), The horizontal gene pool, bacterial plasmids and gene spread. Harwood Academic Publishers, Amsterdam, The Netherlands.
60. **Pansegrau, W., D. Balzer, V. Kruft, R. Lurz, and E. Lanka.** 1990. *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. Proc. Natl. Acad. Sci. USA **87:**6665–6669.
61. **Pansegrau, W., E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas.** 1994. Complete nucleotide sequence of Birmingham IncPα plasmids, compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
62. **Paulsen, I. T., M. H. Brown, and R. Skurray.** 1996. Proton-dependent multidrug efflux systems. Mirobiol. Rev. **60:**575–608.
63. **Peng, J. B., W. M. Yan, and X. Z. Bao.** 1994. Plasmid and transoposon transfer to *Thiobacillus ferrooxidans*. J. Bacteriol. **176:**2892–2897.
64. **Perwez, T., and R. J. Meyer.** 1996. MobB protein stimulates nicking at the R1162 origin of transfer by increasing the proportion of complexed plasmid DNA. J. Bacteriol. **178:**5762–5767.
65. **Persson, C., and K. Nordstrom.** 1986. Control of replication of the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated DNA squences. Mol. Gen. Genet. **203:**189–192.
66. **Quentmeier, A., and C. G. Friedrich.** 1994. Transfer and expression of degradative and antibiotic resistance plasmids in acidophilic bacteria. Appl. Environ. Microbiol. **60:**973–978.
67. **Rawlings, D. E., and T. Kusano.** 1994. Molecular studies of *Thiobacillus ferrooxidans*. Microbiol. Rev. **58:**39–55.
68. **Rawlings, D. E., I. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **159:**737–738.
69. **Rawlings, D. E., and D. R. Woods.** 1985. Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. Appl. Environ. Microbiol. **49:**1323–1325.
70. **Recchia, G. D., and R. M. Hall.** 1995. Plasmid evolution by acquisition of mobile gene cassettes: plasmid pIE723 contains the aadB gene cassette precisely inserted at a secondary site in the IncQ plasmid RSF1010. Mol. Microbiol. **15:**179–187.
71. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **174:**6230–6237.
72. **Rohrer, J., and D. E. Rawlings.** 1993. Regulation of mobilization of the broad-host-range plasmid pTF-FC2. Mol. Microbiol. **9:**1051–1059.
73. **Rotger, R., and C. Nombela.** 1983. Characterization of penicillin-resistant β-lactamase-producing strains of *Neisseria gonorrhaoeae* isolated in Spain (1978–1981). Microbiol. Esp. **36:**115–122.
74. **Rotger, R., F. Rubio, and C. Nombela.** 1986. A multi-resistant plasmid from commensal *Neisseria* species is closely related to the enterobacterial plasmid RSF1010. J. Gen. Microbiol. **132:**2491–2496.
75. **Saano, A. K., and V. V. Zinchenko.** 1987. A new plasmid R89S: properties and genetic organization. Plasmid **17:**191–201.
76. **Sakai, H., and T. Komano.** 1996. DNA replication of IncQ broad-host-range plasmids in Gram-negative bacteria. Biosci. Biotechnol. Biochem. **60:**377–382.
77. **Scherzinger, E., V. Haring, R. Lurz, and S. Otto.** 1991. Plasmid RSF1010 DNA replication in vitro promoted by purified RSF1010 RepA, RepB and RepC proteins. Nucleic Acids Res. **19:**1203–1211.
78. **Scherzinger, E., R. Lurz, S. Otto, and B. Dobrinski.** 1992. In vitro cleavage of double- and single-stranded DNA by plasmid RSF1010-encoded mobilization proteins. Nucleic Acids Res. **20:**41–48.
79. **Scherzinger, E., V. Kruft, and S. Otto.** 1993. Purification of the large mobilization protein of plasmid RSF1010 and characterization of its site-specific DNA cleaving/DNA joining activity. Eur. J. Biochem. **217:**929–929.
80. **Scholtz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271–288.
81. **Segal, G., M. Purcell, and H. A. Schuman.** 1998. Host cell killing and

bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proc. Natl. Acad. Sci. USA **95:**1669–1674.

82. **Segal, G., J. J. Russo, and H. A. Schuman.** 1999. Relationships between a new type IV secretion system and the icm/dot system of *Legionella pneumophila*. Mol. Microbiol. **34:**799–809.
83. **Segal, G., and H. A. Schuman.** 1998. Intracellular multiplication and human macrophage killing by are *Legionella pneumophila* inhibited by conjugal components of IncQ plasmid RSF1010. Mol. Microbiol. **30:**197–208.
84. **Siddavattam, D., A. Nickles, S. Herterich, H. D. Steibl, R. Kreutzer, and W. Klingmuller.** 1995. Site-specific mutagenesis in *Enterobacter agglomerans*: construction of *nifB* mutants and analysis of the gene's structure and function. Mol. Gen. Genet. **249:**526–532.
85. **Simon, R., U. Priefer, and A. Pühler.** 1983. Vector plasmids for in vivo and in vitro manipulations of Gram-negative bacteria, p. 98–105. *In* A. Pühler (ed.) Molecular genetics of the bacterial-plant interaction. Springer-Verlag KG, Berlin, Germany.
86. **Smalla, K., H. Heuer, A. Götz, D. Niemeyer, E. Krögerrechlenfort, and E. Tietze.** 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl. Environ. Microbiol. **66:**4854–4862.
87. **Smith, A. G. S., and D. E. Rawlings.** 1997. The poison-antidote stability system of the broad-host-range *Thiobacillus ferroxidans* plasmid pTF-FC2. Mol. Microbiol. **26:**961–970.
88. **Sobecky, P. A., T. J. Mincer, M. C. Chang, and D. R. Helinski.** 1997. Plasmids isolated from marine sediment microbial communities contain replication and incompatibility regions unrelated to those of known plasmid groups. Appl. Environ. Microbiol. **63:**888–895.
89. **Sobecky, P. A., T. J. Mincer, M. C. Chang, A. Toukdarian, and D. R. Helinski.** 1998. Isolation of broad-host-range replicons from marine sediment bacteria. Appl. Environ. Microbiol. **64:**2822–2830.
90. **Sode, K., M. Tatara, H. Takeyama, J. G. Burgess, and T. Matsunaga.** 1992. Conjugative gene transfer in marine cyanobcteria: *Synechococcus* sp., *Synechocystis* sp. and *Pseudoanabaena* sp. Appl. Microbiol. Biotechnol. **37:**369–373.
91. **Sýkora, P.** 1992. Macroevolution of plasmids: a model for plasmid speciation. J. Theor. Biol. **159:**53–65.
92. **Thorsted, P. B., D. P. Macartney, P. Aktar, A. S. Haines, N. Ali, P. Davidson, T. Stafford, M. J. Pocklington, W. Pansegrau, B. M. Wlikins, E. Lanka, and C. M. Thomas.** 1998. Complete sequence of the IncPβ plasmid R751: implications for evolution and organisation of the IncP backbone. J. Mol. Biol. **282:**969–990.
93. **Tietze, E.** 1998. Nucleotide sequence and genetic characterization of the novel IncQ-like plasmid pIE1107. Plasmid **39:**165–181.
94. **Tietze, E., H. Tschäpe, and W. Voigt.** 1989. Characterization of new resistance plasmids belonging to incompatibility group IncQ. J. Basic Microbiol. **10:**695–706.
95. **Wahlund, T. M., and M. T. Madigan.** 1995. Genetic transfer by conjugation in the thermophilic green sulfur bacterium *Chlorobium tepidum*. J. Bacteriol. **177:**2583–2588.
96. **Whittle, G., M. E. Katz, E. H. Clayton, and B. F. Cheetham.** 2000. Identification and characterization of a native *Dichelobacter nodosus* plasmid, pDN1. Plasmid **43:**230–234.
97. **Wilkins, B. M., P. M. Chilley, A. T. Thomas, and M. J. Pocklington.** 1996. Distribution of restriction enzyme recognition sequences on broad host range plasmid RP4: molecular and evolutionary implications. J. Mol. Biol. **258:**447–456.
98. **Willson, P. J., H. G. Deneer, A. Potter, and W. Albritton.** 1989. Characterization of a streptomycin-sulfonamide resistance plasmid from *Actinobacillus pleauropneumoniae*. Antimicrob. Agents Chemother. **33:**235–238.
99. **Yoshida, K., T. Takegami, A. Katoh, M. Nishikawa, and T. Nishida.** 1997. Construction of a novel conjugative plasmid harboring a GFP reporter gene and its introduction into animal cells by transfection and trans-kingdom conjugation. Nucleic Acids Symp. Ser. **37:**157–158.
100. **Zechner, E. L., F. de la Cruz, R. Eisenbrandt, A. M. Grahn, G. Koraimann, E. Lanka, G. Muth, W. Pansegrau, C. M. Thomas, B. M. Wilkins, and M. Zatyka.** 2000. Conjugative-DNA transfer processes, p. 87–174. *In* C. M. Thomas (ed.), The horizontal gene pool, bacterial plasmids and gene spread. Harwood Academic Publishers, Amsterdam, The Netherlands.
101. **Zhang, S., and R. J. Meyer.** 1997. The relaxosome protein MobC promotes conjugal plasmid mobilization by extending DNA strand separation to the nick site at the origin of transfer. Mol. Microbiol. **25:**509–516.
102. **Ziegelin, G., W. Pansegrau, R. Lurz, and E. Lanka.** 1992. TraK protein of conjugative plasmid RP4 forms a specialised nucleoprotein complex with the transfer origin. J. Biol. Chem. **267:**17279–17286.

JOURNAL OF BACTERIOLOGY, Oct. 2003, p. 6104–6111 Vol. 185, No. 20
0021-9193/03/$08.00+0 DOI: 10.1128/JB.185.20.6104–6111.2003

Analysis of the Mobilization Region of the Broad-Host-Range IncQ-Like Plasmid pTC-F14 and Its Ability To Interact with a Related Plasmid, pTF-FC2

Leonardo J. van Zyl, Shelly M. Deane, and Douglas E. Rawlings*

Department of Microbiology, University of Stellenbosch, Matieland 7600, South Africa

Received 14 May 2003/Accepted 1 August 2003

Plasmid pTC-F14 is a 14.2-kb plasmid isolated from *Acidithiobacillus caldus* that has a replicon that is closely related to the promiscuous, broad-host-range IncQ family of plasmids. The region containing the mobilization genes was sequenced and encoded five Mob proteins that were related to those of the DNA processing (Dtr or Tra1) region of IncP plasmids rather than to the three-Mob-protein system of the IncQ group 1 plasmids (e.g., plasmid RSF1010 or R1162). Plasmid pTC-F14 is the second example of an IncQ family plasmid that has five *mob* genes, the other being pTF-FC2. The minimal region that was essential for mobilization included the *mobA*, *mobB*, and *mobC* genes, as well as the *oriT* gene. The *mobD* and *mobE* genes were nonessential, but together, they enhanced the mobilization frequency by approximately 300-fold. Mobilization of pTC-F14 between *Escherichia coli* strains by a chromosomally integrated RP4 plasmid was more than 3,500-fold less efficient than the mobilization of pTF-FC2. When both plasmids were coresident in the same *E. coli* host, pTC-F14 was mobilized at almost the same frequency as pTF-FC2. This enhanced pTC-F14 mobilization frequency was due to the presence of a combination of the pTF-FC2 *mobD* and *mobE* gene products, the functions of which are still unknown. Mob protein interaction at the *oriT* regions was unidirectionally plasmid specific in that a plasmid with the *oriT* region of pTC-F14 could be mobilized by pTF-FC2 but not vice versa. No evidence for any negative effect on the transfer of one plasmid by the related, potentially competitive plasmid was obtained.

Plasmid pTC-F14 was recently isolated from the moderately thermophilic (50°C), acidophilic, sulfur-oxidizing bacterium *Acidithiobacillus caldus* (11). The strain of *A. caldus* in which the plasmid was found was one of two dominant organisms in a bacterial consortium undergoing pilot-scale testing for the commercial extraction of nickel from ores (17). The 14.2-kb plasmid pTC-F14 was shown to have an IncQ-like replicon that was closely related to, but compatible with, the broad-host-range 12.2-kb plasmid pTF-FC2 (9, 10). Plasmid pTF-FC2 had been previously isolated from a different, but related, iron- and sulfur-oxidizing bacterium, *Acidithiobacillus ferrooxidans* (6, 16, 19).

Although IncQ and IncQ-like plasmids are not self-transmissible, they are efficiently mobilized by conjugative plasmids of the *Escherichia coli* IncPα and IncPβ groups. By using IncP plasmids or IncP-based helper plasmids, the IncQ plasmids have been successfully mobilized to a large number of hosts, including a wide range of gram-negative bacteria; several gram-positive bacteria, including *Arthrobacter* spp., *Streptomyces lividans*, and *Mycobacterium smegmatis*; and cyanobacteria, such as *Synechococcus*, as well as being mobilized into plant and animal cells (reviewed in reference 18). Likewise, pTF-FC2 has been mobilized from *E. coli* to several gram-negative bacteria and from *Agrobacterium tumefaciens* to plant cells (D. E. Rawlings, unpublished observations). This, together with the broad-host-range properties of their replicons, makes these plasmids highly promiscuous and interesting to study from the fundamental biology, ecology, and applied biology points of view.

There are two major groups of IncQ and IncQ-like plasmids, the most distinguishing characteristic between the groups being whether they possess a three-gene, IncQ-like mobilization system or a five-gene, IncP-like mobilization system (18). Examples of the three-*mob*-gene plasmid family are the IncQ plasmids RSF1010, R1162, and R300B and the IncQ-like plasmids pIE1107, pIE1115, pIE1130, and pDN1 (25, 27). Plasmid pTF-FC2 was the only example of an IncQ-like plasmid with a five-*mob*-gene system (19) until the discovery of plasmid pTC-F14. Recently, the sequence of another IncQ-like plasmid, pRAS3, with a five-*mob*-gene system has been observed (14), although no biology of this system has been reported. The amino acid sequences of the Mob proteins from the two groups of plasmids belonging to the IncQ family are not related to each other.

As part of this study, we report that like pTF-FC2, the mobilization genes of pTC-F14 are of the IncP type. Because pTC-F14 and pTF-FC2 are promiscuous plasmids that were isolated from acidiphilic, iron- and/or sulfur-oxidizing, chemolithotrophic bacteria that share a similar habitat, it is not unlikely that the plasmids may come into contact with each other. Plasmids pTC-F14 and pTF-FC2 have diverged sufficiently for their replicons to be compatible, which should allow them to coexist in the same host cell (9, 10). This raised questions such as have the *mob* genes diverged sufficiently to be plasmid specific, or will they complement the mobilization activity of each other? Was there competition between plasmids at the level of mobilization? That is, had one of the plasmids evolved a mobilization system that would allow it to dominate the

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Matieland 7600, South Africa. Phone: 27 21 808 5848. Fax: 27 21 808 5846. E-mail: der@sun.ac.za.

TABLE 1. Strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Genotype or description	Source or reference
Strains		
DH5α	F′/*endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 reacA1 gyrA*(Nal^r) *relA1* Δ(*lacZYA-argF*)*U169* (ϕ80*dlac*Δ(*lacZ*)*M15*)	Promega Corp., Madison, Wis.
S17.1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	23
CSH56	F$^-$ *ara* Δ(*lac pro*) *supD nalA thi*	Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
HB101	F$^-$ Δ(*mcrC-mrr*) *hsdS20 recA13 ara-14 proA2 lacY1* λ$^-$ *galK2 rpsL20*(Sm^r) *xyl-5 mtl-1 supE44*	3
Plasmids		
pUC19	Amp^r *lacZ′*; ColE1 replicon, cloning vector	28
pACYC184	Tc^r Cm^r; p15A replicon, cloning vector	5
pBR322	Amp^r Tc^r; ColE1 replicon, cloning vector	2
pKK223-3	Amp^r; ColE1 replicon, expression vector	4
pTC-F14Cm	Cm^r; natural pTC-F14 plasmid with a chloramphenicol resistance gene inserted at the single *Bam*HI site	9
pTC-F14Km	Km^r; pTC-F14Cm with the chloramphenicol resistance gene replaced by the kanamycin resistance gene from Tn*5*	This study
pDER412	Cm^r; natural pTF-FC2 plasmid with chloramphenicol resistance gene cloned into the Tn*5467* transposon	16
pMmob	Amp^r, 5,554-bp *Bam*HI-*Xba*I fragment of pTC-F14 containing all mobilization genes and the *repB* primase, cloned into pUC19	This study
pMmob1-pMmob9	Amp^r; PCR-based deletions of the mobilization region of pTC-F14 cloned into pUC19; refer to Fig. 1	This study
pMmob1184	Cm^r; minimum mobilization region one of pTC-F14 cloned into the tetracycline resistance marker of pACYC184	This study
pMmob1322	Amp^r; minimum mobilization region one of pTC-F14 cloned into pBR322	This study
pmobE	Amp^r; PCR product of *mobE* gene of pTF-FC2 cloned into pKK223-3	This study
pmobDE	Amp^r; PCR product of *mobDE* genes of pTF-FC2 cloned into pKK223-3	This study
pmobCDE	Amp^r; PCR product of *mobCDE* genes of pTF-FC2 cloned into pKK223-3	This study
pAC105	Cm^r; exonuclease III shortening of the pTF-FC2 mobilization region containing *mobC*, *-D*, and *-E* cloned into pACYC184	20
pAC209	Cm^r; exonuclease III shortening of the pTF-FC2 mobilization region containing *mobA*, *-B*, *-C*, and *-D* and a truncated *mobE* cloned into pACYC184	20
pAC218	Cm^r; exonuclease III shortening of the pTF-FC2 mobilization region containing *mobA*, *-B*, and *-C* with *mobD* and *-E* removed also in pACYC184	20
pAC221	Cm^r; exonuclease III shortening of the pDER412 mobilization region containing *mobA* and *-B* and the *oriT* cloned into pACYC184	20
pOriTF14	Amp^r; a 203-bp *Hin*dIII-*Nco*I fragment of pTC-F14 containing the *oriT* cloned into pUC19	This study
pOriTFC2	Amp^r; the *oriT* of pTF-FC2 cloned into pUC19	This study
Primers		
mobEF2	(*Eco*RI) 5′-TACAGAATTCAGCAAGCGCATGAGC-3′	This study
mobDEF2	(*Eco*RI) 5′-TACAGAATTCCCAAAACCCGACAGC-3′	This study
mobCDEF2	(*Eco*RI) 5′-TATAGAATTCCCACGTGGCGAAGCC-3′	This study
mobER2	(*Xba*I) 5′-TACATCTAGAATGTTGAGCGCGTCG-3′	This study
mobAR14	(*Eco*RI) 5′-TACAGAATTCCGGTCCATGTCGTCG-3′	This study
repBR14	(*Eco*RI) 5′-TACAGAATTCGGGTAATCGGATGGC-3′	This study
mobC′R14	(*Pst*I) 5′-TATACTGCAGCTTTCCCGCCTTTGC-3′	This study
mobCR14	(*Pst*I) 5′-TATACTGCAGTTGCCACCACCGACG-3′	This study
mobDR14	(*Pst*I) 5′-TATACTGCAGTCGGGTGTCGGTTCC-3′	This study
mobER14	(*Pst*I) 5′-TACACTGCAGCTGTCCGAAAGTAGG-3′	This study
mobAR14#2	5′-TGGCGTCGCTTGTTTGGTTC-3′	10

horizontal transfer process, thereby giving it a selective advantage in preference to the other? Here we characterize the mobilization genes of pTC-F14 and report on the ability of the mobilization systems of pTC-F14 and pTF-FC2 to interact with each other.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. The strains, plasmids, and primers used in this study are listed in Table 1.

Media and growth conditions. *E. coli* strains were grown in either Luria-Bertani broth or on LA plates (21) at 37°C, supplemented as required with antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml; streptomycin, 50 μg/ml; kanamycin, 50 μg/ml; and nalidixic acid, 50 μg/ml.

Mating assays. Donor and recipient (CSH56) cells were cultured separately overnight with appropriate antibiotic selection. Cells were washed three times in 0.85% (wt/vol) NaCl solution and mixed in a donor/recipient ratio of 1:10. An LA plate was spotted with 100 μl of this mixture and incubated at 37°C for 1 h. The agar plug was excised, suspended in 5 ml of 0.85% NaCl solution, and vigorously shaken to dislodge mating cells. Cells were pelleted by a 2-min spin in a micro-

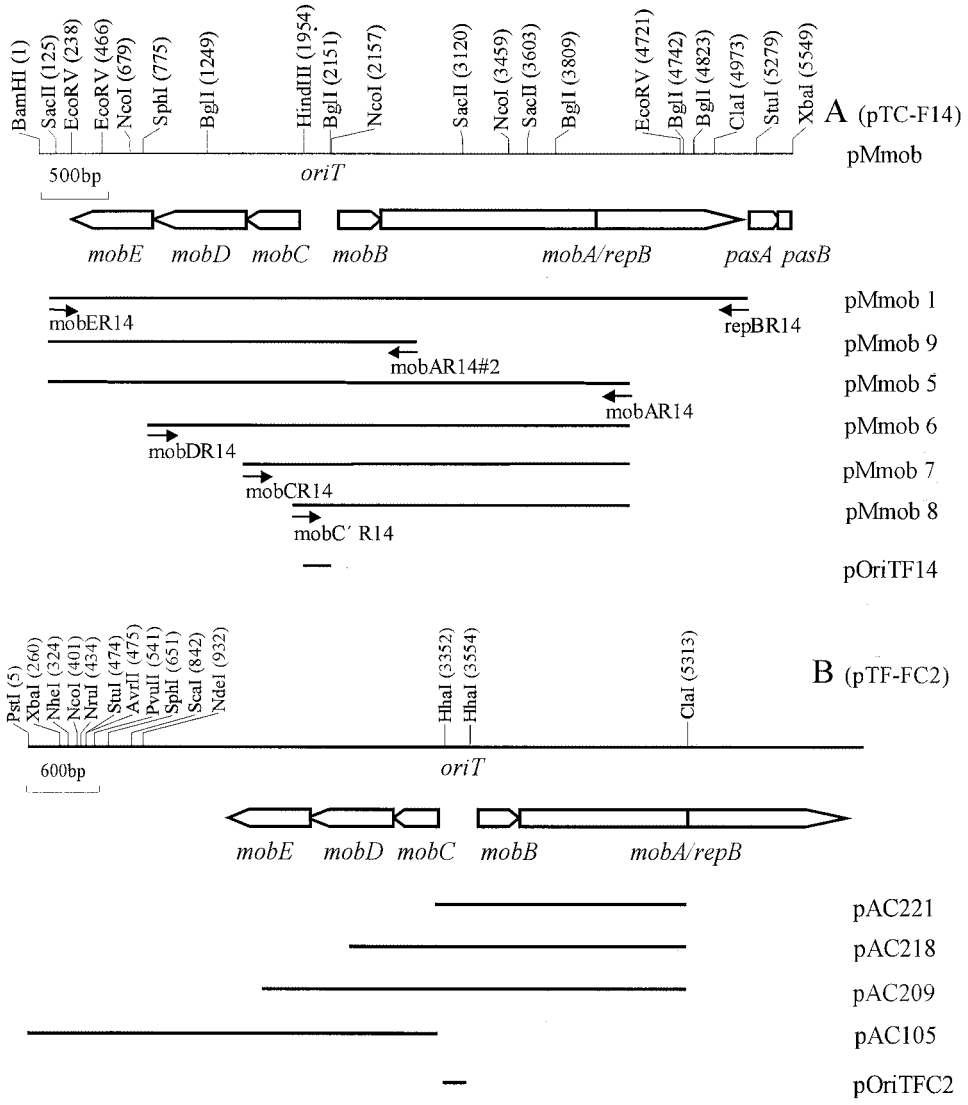


FIG. 1. Genetic and restriction endonuclease cleavage maps of the mobilization regions of plasmids pTC-F14 and pTF-FC2. (A) The 5.55-kb *Bam*HI-*Xba*I region of pTC-F14 showing the locations of the *mob*, *repB*, and *pas* genes as well as the *oriT* gene. The positions of the primers used to amplify and construct certain subclones are shown by short horizontal arrows. (B) The previously reported *mob* region of pTF-FC2 and subclones (20) used in this study.

centrifuge and resuspended in 1 ml of 0.85% NaCl solution. Serial dilutions were then plated onto media that selected for donor and transconjugant cells. The transfer frequency was calculated as the number of transconjugants per donor during the 1-h mating period.

DNA manipulations, sequencing, and bioinformatics. General techniques were performed according to standard procedures (21) or the manufacturers' recommendations. DNA was digested to give fragments with sizes of 700 bp to 1.2 kbp and cloned into the pUC19 vector. The DNA sequence was determined by a combination of sequencing from the ends of a number of subclones and synthesis of specific primers to obtain overlapping sequence from both strands. Sequencing was performed by the dideoxy chain termination method with an ABI PRISM 377 automated DNA sequencer, and the sequence was analyzed with a variety of software programs (mainly the PC-based DNAMAN [version 4.1] package from Lynnon Biosoft). Searches for sequences related to Mob proteins were performed by using the gapped-BLAST program of the National Center for Biotechnology Information at www.ncbi.nih.nlm.gov (1). Sequence alignments (based on CLUSTAL W) were carried out with the multiple alignment program, and amino acid sequence homology trees were constructed with the tree output program within the DNAMAN package.

PCRs. PCRs were performed with the Expand high-fidelity *Taq* DNA polymerase from Roche with a Hybaid PCR Sprint cycler. Plasmid pDER412 was used as template with primers mobEF2, mobDEF2, mobCDEF2, and mobER2 (listed in Table 1) to produce pmobCDE, pmobDE, and pmobE, respectively. Primers mobAR14, mobAR14#2, repBR14, mobC′R14, mobCR14, mobDR14, and mobER14 (Table 1 and Fig. 1) with pMmob as a template were used to give the mobilization region fragments (pMmob1 to pMmob9). After an initial denaturation of 60 s at 94°C, 25 cycles of 30 s at 55 to 60°C (depending on primer set) and an elongation step of up to 4 min (approximately 1 min per 1,000 bp) at 72°C were performed. A final extension step of 120 min at 72°C before cooling to 4°C completed the reaction.

Nucleotide sequence accession number. The nucleotide sequence of the 5.5-kbp region sequenced has been submitted to the EMBL-GenBank database under accession no. NC_004734/AF325537.

RESULTS

Mobilization of pTC-F14. Selectable chloramphenicol and kanamycin resistance genes were cloned into plasmid pTC-F14

TABLE 2. Mobilization frequency of plasmids and constructs

Test plasmid[a]	Plasmid present in *trans*[a]	Mobilization frequency of test plasmid[b]
pTC-F14Km		$(2.7 \pm 1.5) \times 10^{-3}$
pTC-F14Cm		$(2.8 \pm 1.8) \times 10^{-3}$
pTC-F14Cm[c]	R751	1.3×10^{-5}
pTC-F14Cm[c]	R388	$<10^{-6}$
pTF-FC2 (pDER412)		≥ 10
pTF-FC2 (pDER412)	pTC-F14Km	≥ 10
pTC-F14Km	pTF-FC2 (pDER412)	8.4 ± 0.52
pMmob (F14, *mobEDCBA repB pasA*)		≥ 10
pMmob1 (F14, *mobEDCBA repB*)		≥ 10
pMmob5 (F14, *mobEDCBA*)		$(3.3 \pm 3.1) \times 10^{-1}$
pMmob6 (F14, *mobDCBA*)		$(1.2 \pm 1.0) \times 10^{-1}$
pMmob7 (F14, *mobCBA*)		$(5.4 \pm 3.3) \times 10^{-3}$
pMmob8 (F14, *mobBA*)		$<10^{-6}$
pMmob9 (F14, *mobEDCB*)		$<10^{-6}$
pMmob8 (F14, *mobBA*)	pTC-F14Cm	2.4 ± 1.4
pMmob1184 (as for pMmob1)		$(2.1 \pm 0.8) \times 10^{-1}$
pMmob1322 (as for pMmob1)		$(2.3 \pm 1.6) \times 10^{-1}$
pTC-F14Cm	pAC105 (FC2, *mobEDC*)	$(7.9 \pm 2.1) \times 10^{-1}$
pTC-F14Cm	pAC209 (FC2, *mobDCBA*)	$(2.3 \pm 3.5) \times 10^{-3}$
pTC-F14Cm	pAC221 (FC2, *mobBA*)	$(8.9 \pm 2.4) \times 10^{-4}$
pTC-F14Cm	pmobE (FC2)	$(4.4 \pm 2.1) \times 10^{-3}$
pTC-F14Cm	pmobDE (FC2)	≥ 10
pTC-F14Cm	pmobCDE (FC2)	$(4.7 \pm 2.9) \times 10^{-1}$
pTC-F14Cm	pAC218 (FC2 *mobC*) + pmobE (FC2)	$(1.1 \pm 2.6) \times 10^{-3}$
pOriTF14		$<10^{-6}$
pOriTF14	pTC-F14Cm	1.5 ± 1.0
pOriTF14	pTF-FC2 (pDER412)	$(3.5 \pm 0.1) \times 10^{-2}$
pOriTF14	pTC-F14Km + pTF-FC2 (pDER412)	≥ 10
pOriTFC2		$<10^{-6}$
pOriTFC2	pTC-F14Cm	$<10^{-6}$
pOriTFC2	pTF-FC2 (pDER412)	≥ 10
pOriTFC2	pTC-F14Km + pAC105 (FC2, *mobEDC*)	$(3.5 \pm 4.7) \times 10^{-1}$
pOriTFC2	pTC-F14Km + pAC221 (FC2, *mobBA*)	$(1.7 \pm 0.8) \times 10^{-2}$

[a] Where relevant, genes or plasmids are indicated in parentheses.
[b] Mobilization frequency is the number of transconjugants per donor during a 60-min mating with a donor/recipient ratio of 1:10 using *E. coli* S17-1 as the donor and *E. coli* CSH56 as the recipient. A mating frequency of ≥10 is indicated when the number of transconjugants equaled the number of recipients. Mating frequencies were the average of at least three independent experiments, and standard deviations are indicated.
[c] *E. coli* HB101 was used as the donor strain.

to produce plasmids pTC-F14Cm and pTC-F14Km, respectively (Table 1). These plasmids were transformed into an *E. coli* S17-1 donor strain that has an RP4 plasmid derivative integrated into the chromosome to provide the conjugative functions required for plasmid mobilization. Both pTC-F14Cm and pTC-F14Km were mobilized to an *E. coli* CSH56 recipient strain at similar frequencies of approximately 2.8×10^{-3} transconjugants per donor. To determine whether the type of conjugative plasmid affected the mobilization frequency, we compared mobilization frequencies by using two self-transmissible plasmids different from the RP4 (IncPα) that was integrated into the chromosome of *E. coli* S17-1. Plasmid pTC-F14Cm was mobilized by R751 (IncPβ) from *E. coli* HB101 at a frequency about 100-fold lower than that by the RP4 derivative in *E. coli* S17-1, while mobilization by R388 (IncW) was not detectable (Table 2). A 5.55-kb *Bam*HI-*Xba*I fragment from pTC-F14 was subcloned into the nonmobilizable vector pUC19 and was found to be mobilized by *E. coli* S17-1 at frequencies that approached saturation. Saturation indicates that after 1 h of mating at a donor/recipient ratio of 1:10, the number of transconjugants was approximately equal to the number of recipients. This 5.55-kb fragment therefore contained all of the components needed for mobilization and was sequenced.

Analysis of the mobilization region of pTC-F14 and comparison with related plasmids. Five open reading frames were identified, arranged in a manner similar to those of pTF-FC2 (Fig. 1). However, some of the predicted amino acid sequences and characteristics of the mobilization proteins of pTC-F14 differed substantially from their counterparts in pTF-FC2. The MobA-RepB fusion and MobB proteins were the best conserved at 75.4 and 77.8% amino acid sequence identity, while the MobC, MobD, and MobE proteins were poorly conserved at 26.5, 39.8, and 21.2% amino acid sequence identity, respectively. Although all pairs of Mob proteins were of comparable sizes, the predicted pI values of the MobE proteins differed by almost 3 pH units. Surprisingly, plasmid pRAS3.1, isolated from *Aeromonas salmonicida* in Norway, has Mob proteins (GenBank accession no. AY043299.1/NC_003124.1) that are considerably more closely related to pTF-FC2 than pTC-F14 is to pTF-FC2. The sequences of the MobA, MobB, MobC, MobD, and MobE proteins of pRAS3.1 are 93.8, 88.8, 94.1, 97.4, and 88.8% identical to that of pTF-FC2, respectively, whereas they are only 75.0, 74.5, 25.8, 40.7, and 20.8% identical

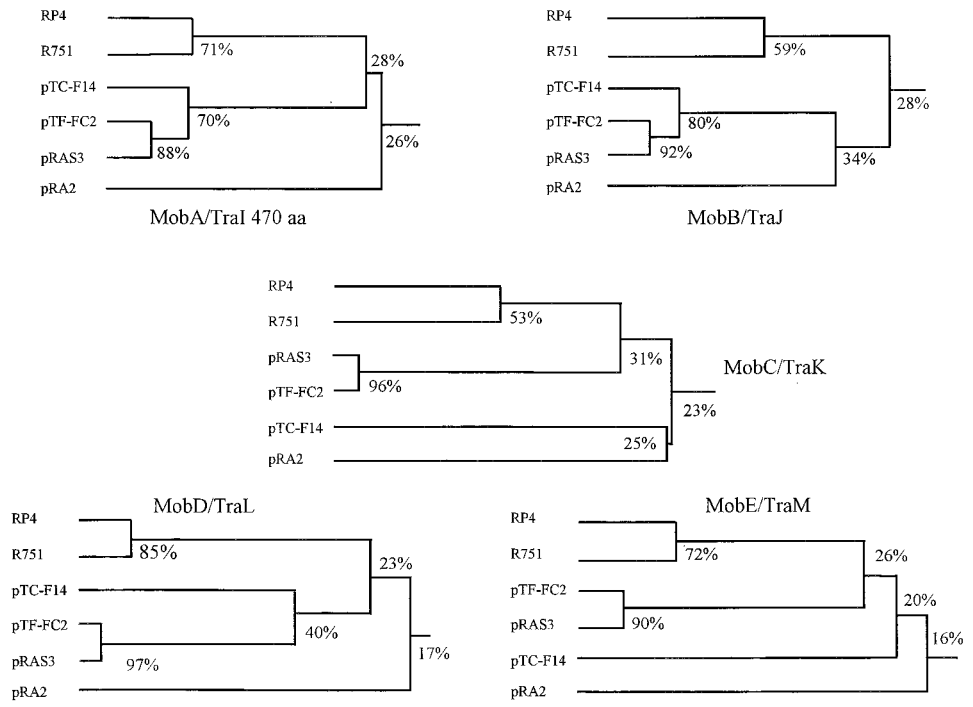


FIG. 2. Phylogenetic relationship between the MobA/TraI, MobB/TraJ, MobC/TraK, MobD/TraL, and MobE/TraM proteins of the IncP and IncQ group 2 plasmids as well as pRA2. Since in the IncQ-related plasmids the MobA plasmids exist as a MobA-RepB fusion, only the N-terminal 470 amino acids were considered for comparison. The percentages shown represent amino acid sequence identities. Accession numbers are as follows: RP4, L27758; R751, U67194; pTC-F14, AF325537; pTF-FC2, M57717; pRAS3, AY043298; and pRA2, U88088.

to that of pTC-F14. Three of the Mob proteins (MobA, MobB, and MobC) of pTC-F14, pTF-FC2, and pRAS3 had a greater than 20% amino acid sequence identity to the N-terminal 400-amino-acid portion of TraI and the complete TraJ and TraK proteins of the IncPα plasmid RP4 and the IncPβ plasmid R751, respectively. MobD and MobE had weaker but detectable sequence identity (17 to 18%) to TraL and TraM of RP4 and R751. These Mob proteins clearly belong to the IncP-like family of conjugation-associated, DNA processing proteins (Dtr), and a dendrogram showing the relationship between proteins of this family is presented in Fig. 2 (15, 24).

The *oriT* region of pTC-F14 was identified by sequence analysis and was found to be located on a 203-bp *Nco*I-*Hin*dIII fragment. This fragment was cloned into the nonmobilizable pUC19 vector (pOriT-F14) and transformed into *E. coli* S17-1, which contained a resistant pTC-F14Cm. pOriT-F14 was mobilized at a frequency that was about 500-fold greater than that of pTC-F14Cm. The *oriT* regions of IncPα and IncPβ plasmids as well as the four plasmids that have mobilization regions related to the IncP plasmids are compared in Fig. 3. The four mobilizable plasmids each contained an inverted repeat sequence that has been shown to be the site at which the relaxosome of plasmid RP4/RK2 binds prior to nicking at the *oriT* gene (26, 29). The highly conserved nucleotide hexamer that immediately precedes the *nic* site is also shown. In contrast to the mobilization proteins, for which plasmids pTF-FC2 and pRAS3 were the most closely related, the *oriT* regions of pTC-F14 and pRAS3 were considerably more closely related (matches at 42 of 50 bp) than those of pRAS3 and pTF-FC2 (32 of 50 bp) or pTC-F14 and pTF-FC2 (30 of 50 bp) (Fig. 3).

Determination of which *mob* genes are essential or nonessential for mobilization. A series of PCR-based deletions of the pTC-F14 mobilization region were made (Fig. 1). These were designed to test which genes were required for mobilization, as well as to determine the smallest region that is mobilized at the frequency of the intact *mobE-repB* region. When the entire *mobE-repB* region of pTC-F14 was cloned into the pUC19 vector (pMmob1), the mobilization frequency was at the level of saturation. This was an increase of more than 3,000-fold relative to the frequency obtained with the mobilization genes linked to its natural replicon (pTC-F14Km or pTC-F14Cm). To test whether this increase in mobilization

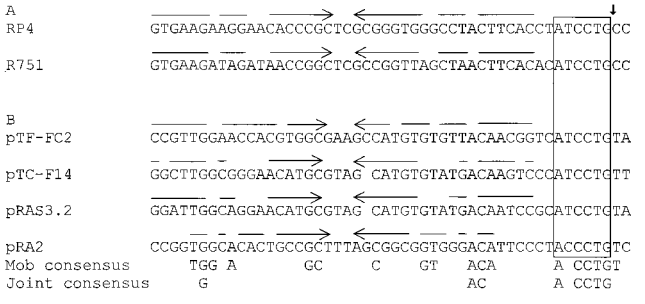


FIG. 3. Comparison of the *oriT* regions of the IncP conjugative plasmids (A) and the IncQ group 2 and pRA2 mobilizable plasmids (B). Imperfect inverted repeat sequences are shown by horizontal arrows, while the highly conserved hexamer preceding the nick site is boxed. The small vertical arrow shows the nick site as determined for RP4/RK2.

frequency was due to placement of the *mobE-repB* region in the pUC19 vector, the *mobE-repB* region of pMmob1 was cloned into vectors pACYC184 and pBR322. Both of these constructs (pMmob1184 and pMmob1322) had mobilization frequencies ~50-fold less than that of the pUC19 construct (pMmob1) but still ~100-fold higher than that of the parent plasmid (Table 2). This suggested that the increase in mobilization frequency was associated with the placement of the mobilization region within the high-copy-number vector pUC19. Deletion of most of *repB* (pMmob5) reduced the mobilization frequency by about 30-fold. The mobilization frequency was restored to saturation levels by placing a *repB*-expressing construct in *trans* with pMmob5 (data not shown). This indicated that the *repB* gene assisted but was not essential for mobilization. Using the *mobE-mobA* (pMmob5) construct as a starting point, sequential deletion of the *mobE* (pMmob6), *mobED* (pMmob7), and *mobEDC* (pMmob8) genes was carried out. Deletion of *mobE* had no discernible effect on the mobilization frequency, while deletion of both *mobE* and *mobD* (pMmob7) reduced the mobilization frequency by approximately 600-fold, whereas there was no detectable mobilization of the *mobE-mobC* deletion (pMmob8). Deletion of most of *mobA* from pMmob5 (pMmob9) resulted in a construct with a mobilization frequency below the detection limit.

Comparison of the mobilization efficiencies and interaction between the mobilization systems of pTC-F14 and pTF-FC2. The mobilization frequencies of plasmids containing the *mob* genes of pTC-F14 and the pTF-FC2 when associated with their natural replicons were compared. Plasmid pTC-F14Km was mobilized from *E. coli* S17-1 donor cells to CSH56 recipient cells at a frequency of 2.83×10^{-3} transconjugants per donor, which was more than 3,000-fold less than that of plasmid pDER412, which contained the pTF-FC2 mobilization genes (Table 2). To test whether mobilization of one plasmid was affected by coresidence of the other, both pTC-F14Km and pTF-FC2 (pDER412) were placed into *E. coli* S17-1 cells, and the frequency of transfer was measured. The frequency of mobilization of pTC-F14Km was enhanced almost to saturation in the presence of pTF-FC2, while the presence of pTC-F14 had no discernible effect on the mobilization of pTC-FC2. To determine what property of pTF-FC2 was required for this enhancement of mobilization frequency, plasmid constructs containing combinations of pTF-FC2 *mob* genes subcloned into the vector pACYC184 were introduced into *E. coli* S17-1(pTC-F14Cm) cells. Coresident plasmids pAC221 (containing pTF-FC2 *mobA* and *mobB*) and pAC209 (containing *mobA*, *mobB*, *mobC*, *mobD*, and a truncated *mobE* gene) did not increase the frequency of mobilization. In contrast, pAC105, which contained *mobC*, *mobD*, and a complete *mobE* gene, enhanced the mobilization frequency of pTC-F14 by about 100-fold, although this was about 10-fold less than when the whole of pDER412 was present. To determine whether this result was due to the *mobE* of pTF-FC2, the gene was amplified by PCR and cloned behind the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *tac* promoter in plasmid pKK223-3 (construct pmobE). This construct did not improve the mobilization frequency of pTC-F14Cm. When a combination of pTF-FC2 *mobD* and *mobE* genes (pmobDE) were placed in *trans* and induced with IPTG, mobilization of pTC-F14Cm reached saturation. IPTG induction of a combination of the *mobCDE* genes (pmobCDE) enhanced pTC-F14Cm mobilization by approximately 150-fold to about the same level as with pAC105. This indicated that it was the combination of *mobD* and *mobE* from pTF-FC2 that enhanced pTC-F14 mobilization.

Interaction at the *oriT* regions. To test whether the mobilization proteins of the two plasmids could act specifically on the *oriT* regions of each other, plasmids containing one of the cloned *oriT* fragments (pOriT-F14 and pOriT-FC2) were transformed into *E. coli* S17-1 containing either pTC-F14Km or pDER412. Both cloned *oriT* regions were functional, because they were mobilized by their respective parent plasmids at a frequency comparable to or greater than that of the parent plasmid (Table 2). The construct containing the *oriT* region of pTC-F14 (pOriT-F14) was mobilized at a frequency of 1.48 transconjugant per donor when pTC-F14 was placed in *trans*, but only at 3.48×10^{-2} with pDER412 in *trans*. With both pTC-F14 and pDER412 in *trans*, the mobilization frequency of pOriT-F14 reached saturation.

In the pTF-FC2 *oriT* complementation experiments, pDER412 was able to mobilize a construct containing its own *oriT* gene (pOriT-FC2) at a saturation frequency, while mobilization by pTC-F14 was below the limit of detection. Complementation of the *oriT* regions was therefore unidirectional, with pTF-FC2 able to mobilize the *oriT* of pTC-F14, but not vice versa. We tested whether there was any detectable interaction between the *mob* genes of pTC-F14 and the *oriT* of pTF-FC2 by providing two subsets of the *mob* genes of pTF-FC2 in *trans*. Plasmid pTC-F14Km was able to mobilize pOriT-FC2 at a frequency of 3.54×10^{-1} when in the presence of the pTF-FC2 *mobCDE* genes (pAC105) and at a frequency of 1.67×10^{-2} when the *mobAB* genes (pAC221) were present. This result was surprising and suggested that at least one of the pTF-FC2 *mobCDE* gene products, as well as one of the *mobAB* gene products, is able to independently assist in the recognition of the heterologous pTF-FC2 *oriT* by pTC-F14.

DISCUSSION

Plasmid pTC-F14 is the second member of the five-*mob*-gene, IncQ-like plasmid family to have its mobilization system investigated. Two other members of this IncQ-like plasmid family, now designated IncQ group 2, are pTF-FC2 and pRAS3, although no report on the biology of pRAS3 mobilization has been published. The observation that the amino acid sequence relationship between the mobilization proteins of plasmids pTF-FC2 and pRAS3 is much closer than that between the proteins of pTF-FC2 and pTC-F14 is remarkable (Fig. 2). The implication is that all three *mob* regions shared the same common ancestor, but pTF-FC2 and pRAS3 diverged more recently than pTF-FC2 and pTC-F14. Since divergence, pTF-FC2 and pRAS3 are now found in bacteria as different as the obligately acidophilic chemolithotrophic *A. ferrooxidans* strain FC isolated in South Africa and the neutrophilic *A. salmonocida* strain isolated in Norway. This serves to illustrate the highly promiscuous nature of the IncQ plasmid family. The 32.7-kb plasmid pRA2 is a fourth example of a plasmid containing a set of five *mob* genes that are related to the Tra1 system of the IncP plasmids (13). Plasmid pRA2 has a unique replicon with no similarity to those of the IncQ-like

plasmids, and this suggests that the five-*mob*-gene system is a mobilization module that may also be acquired by different, otherwise unrelated plasmids.

To facilitate an investigation into the minimum region required for mobilization, the *mobE-repB* region of pTC-F14 was cloned into the nonmobilizable vector pUC19. The mobilization frequency of this construct (pMmob1) was at saturation level, which was about 10^4-fold higher than when the mobilization system was linked to its natural IncQ replicon (pTC-F14Km). An increase in mobilization frequency was not likely to be due to derepression of the IncP helper plasmid, because the mobilization frequency of the pTC-F14Km plasmid was not increased (data not shown). We considered whether this increase in mobilization frequency was associated with the high copy number of the pUC19 vector (up to 500 copies) and therefore transferred the *mobE-repB* region into the lower-copy-number vectors pACYC184 (20 to 30 copies) and pBR322 (25 to 50 copies). The mobilization frequency of these constructs (pMmob1184 and pMmob1322) was reduced 20- to 30-fold but was still 100-fold higher than that of pTC-F14Km (copy number 12 to 16). The increase in mobilization frequency was consistent with an increase in vector copy number, although this observation cannot be taken as proof that the mobilization frequency was affected by copy number.

We wished to detect whether all five of the *mob* genes were required for mobilization and whether the presence of the *repB* gene affected the mobilization frequency of pTC-F14. A characteristic of all IncQ and IncQ-like plasmids is that the *mobA* and *repB* genes are fused in such a way that the MobA (nickase) and the RepB (primase) may be synthesized as separate proteins or as MobA-RepB fusion protein. All three polypeptides have been detected for plasmids RSF1010 (22) and pTF-FC2 (7; Rawlings, unpublished). A fortuitous *Cla*I site was present in plasmid pTF-FC2 that allowed deletion of the *repB* primase gene (Fig. 1). This deletion did not affect the frequency of mobilization between *E. coli* strains. In the case of pTC-F14, a PCR-generated fragment was used to delete *repB* (pMmob5), and this reduced the mobilization frequency about 30-fold compared with that of a plasmid containing an intact *repB* (pMmob1). Placement of a *repB*-expressing construct in *trans* with the RepB-truncated MobA restored mobilization frequencies to levels similar to those when MobA-RepB was present, indicating that the decrease in mobilization was not due to an increase in structural instability of the truncated MobA. The *repB* gene was therefore not essential for mobilization of pTC-F14, although unlike pTF-FC2, the presence of *repB* did enhance mobilization. This is in sharp contrast with the three-*mob*-gene IncQ plasmid R1162, in which the MobA-linked RepB primase was essential for the recovery of plasmids in recipient cells (12). These authors argue that the *mobA-repB* gene fusion of R1162 most likely occurred after the IncQ replicon acquired mobilization genes and may be unique among IncQ-like plasmids. Deletion of the pTC-F14 *mobE* gene had no noticeable effect on plasmid mobilization, while the additional deletion of *mobD* reduced mobilization (600-fold) and the further deletion of *mobC* abolished mobilization. This is in contrast to pTF-FC2, in which deletion of *mobE* reduced mobilization 150-fold, with no mobilization detected on deletion of both *mobE* and *mobD*.

The discovery that when pTF-FC2 was coresident with pTC-F14, the mobilization of the latter plasmid was increased by about 3,000-fold was unexpected. We further discovered that the presence of the combination of the pTF-FC2 *mobD* and *mobE* genes, but not the individual *mobD* and *mobE* genes, was responsible for this increase. This suggests that the apparently dispensable pTC-F14 *mobE* gene does play a role in mobilization, but the pTC-F14 *mobE* gene is not optimally functional in the mating system used (described below). The functions of MobD and MobE proteins are unknown, and the same applies to the related TraL and TraM proteins of the IncPα and IncPβ plasmids. TraL has been found to have an ATP- or GTP-binding Walker A box (24), and this box is present and highly conserved in the MobD proteins of the IncQ-like plasmids (data not shown). The role of MobD and MobE in facilitating the mobilization of one plasmid by another found in this study emphasizes the need to discover the function of the proteins.

In the present study, plasmid pTF-FC2 was clearly much more readily mobilized between *E. coli* strains than plasmid pTC-F14. Furthermore, a coresident pTF-FC2 could mobilize a plasmid containing the *oriT* of pTC-F14 (although not as efficiently as pTC-F14), while a coresident pTC-F14 could not mobilize a plasmid containing the *oriT* gene of pTF-FC2. Based on these results, plasmid pTF-FC2 might be expected to be a more promiscuous plasmid than pTC-F14. However, the fact that mobilization studies were carried out between *E. coli* strains by using the chromosomally located IncP plasmid RP4 as a conjugative helper plasmid must be taken into account. It is possible that pTF-FC2 is more suited to mobilization by this plasmid than pTC-F14, while there may be an as yet unknown helper plasmid that mobilizes pTC-F14 better than pTF-FC2. The reason for the unexpected observation that the *mobD* and *mobE* genes of pTF-FC2 were better able to assist pTC-F14 mobilization than its own genes could be because the MobD and MobE proteins are better suited to work with RP4, while the equivalent proteins of pTC-F14 may be better suited to function with a different conjugative plasmid.

The interpretation of experiments on the ability of plasmids containing the cloned *oriT* regions to be mobilized by the *mob* genes of the other plasmid is not fully clear. The *oriT* of pTC-F14 could be mobilized by its own *mob* proteins, and this mobilization frequency was enhanced in the presence of pTF-FC2. This result was consistent with the ability of pTF-FC2 to enhance the mobilization frequency of pTC-F14. Plasmid pTC-F14 was not able to mobilize a plasmid containing the *oriT* of pTF-FC2, unless some of the pTF-FC2 genes were present. What was surprising is that when we attempted to determine which of the pTF-FC2 *mobAB* or *mobCDE* genes were required, we found that either set of genes partly enhanced mobilization. A possible explanation is that more than one of the products of the pTF-FC2 *mob* genes are likely to enhance binding of the mobilization complex to the *oriT* of pTF-FC2. The nicking and processing of DNA prior to plasmid transfer by conjugation are frequently plasmid specific. For example, despite the high degree of similarity between the DNA-processing transfer proteins and the *oriT* regions of the IncP plasmids RP4/RK2 and R751, the *oriT* of RP4/RK2 cannot be transferred by R751 (8). Transfer of the RP4/RK2 *oriT* took place only when the specific *traJ* and *traK* genes of RK2/RP4 were present, with *traI* also being required, although this was

not plasmid specific. Plasmid RP4 TraJ (29) and TraK (30) proteins bind specifically to different features of the *oriT* region. In the case of pTF-FC2, it is likely that MobB (related to TraJ) and MobC (related to TraK) of pTF-FC2 could bind to its own *oriT* and thereby assist the otherwise *oriT*-specific proteins of pTC-F14 to recognize the *oriT* of pTF-FC2.

Part of the motivation for this study was to gain an understanding of the evolution of mobilization systems. The sequence similarity between the proteins associated with plasmid replication and mobilization suggests that plasmids pTC-F14 and pTF-FC2 share a common ancestor. It has been reported that plasmids pTC-F14 and pTF-FC2 are compatible in *E. coli*, and this indicates that replicons of the plasmids have diverged sufficiently for them to function as independent units. Pressure for the replicons to diverge may have arisen because the two broad-host-range, promiscuous plasmids occur in bacteria that share a similar ecological niche. This means they may have frequently encountered each other, and divergence to the point of compatiblity would mean that the plasmids will not exclude each other from the same host cell and thereby would each have an increased "replication space." It was of interest to discover whether the mobilization systems of these related plasmids would compete with each other. If one plasmid has a more dominant mobilization system, it would presumably be transferred horizontally to more host cells than the competing plasmid and thereby dominate an ecosystem. No reduction in mobilization frequency of one plasmid when coresident with the other plasmid was detected. In contrast, a coresident pTF-FC2 appeared to assist the mobilization of pTC-F14. Similarly, the cloned *oriT* region of pTF-FC2 could be mobilized by a coresident pTC-F14 when some but not all of the pTF-FC2 *mob* genes were present.

ACKNOWLEDGMENTS

This work was funded by grants from the National Research Foundation (Pretoria), The Human Resources for Industry Program (THRIP; Pretoria), the University of Stellenbosch, and the BHP-Billiton Johannesburg Technology Centre.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3308–3402.
2. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
3. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification DNA in *Escherichia coli.* J. Mol. Biol. **41:**459–472.
4. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
5. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
6. **Dorrington, R. A., and D. E. Rawlings.** 1990. Characterization of the minimum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. **172:**5697–5705.
7. **Dorrington, R. A., S. Bardien, and D. E. Rawlings.** 1991. The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli.* Gene **108:**7–14.
8. **Fürste, J. P., W. Pansegrau, G. Zieglin, M. Kröger, and E. Lanka.** 1989. Conjugative transfer of promiscuous IncP plasmids: interaction of plasmid encoded products with the transfer origin. Proc. Natl. Acad. Sci. USA **86:** 1771–1775.
9. **Gardner, M. N., S. M. Deane, and D. E. Rawlings.** 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. **183:**3303–3309.
10. **Gardner, M. N.** 2003. An investigation into the replicon of a broad host range mobilizable plasmid isolated from the moderately thermophilic bacterium *Acidithiobacillus caldus.* Ph.D. thesis. University of Stellenbosch, Stellenbosch, South Africa.
11. **Hallberg, K. B., and E. B. Lindström.** 1994. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology **140:** 3451–3456.
12. **Henderson, D., and R. J. Meyer.** 1999. The MobA-linked primase is the only replication protein of R1162 required for conjugal mobilization. J. Bacteriol. **181:**2973–2978.
13. **Kwong, S. M., C. C. Yeo, A. Suwanto, and C. L. Poh.** 2000. Characterization of the endogenous plasmid from *Pseudomonas alcaligenes* NCIB 9867: DNA sequence and mechanism of transfer. J. Bacteriol. **182:**81–90.
14. **L'Abée-Lund, T. M., and H. Sørum.** 2002. A global non-conjugative Tet C plasmid pRAS3, from *Aeromonas salmonicida.* Plasmid **47:**172–181.
15. **Pansegrau, W., E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas.** 1994. Complete nucleotide sequence of Birmingham IncPα plasmids, compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
16. **Rawlings, D. E., I. M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli.* J. Bacteriol. **158:**737–738.
17. **Rawlings, D. E., N. J. Coram, M. N. Gardner, and S. M. Deane.** 1999. *Thiobacillus caldus* and *Leptospirillum ferrooxidans* are widely distributed in continuous-flow biooxidation tanks used to treat a variety of metal-containing ores and concentrates, p. 777–786. *In* R. Amils and A. Ballester (ed.), Biohydrometallurgy and the environment toward the mining of the 21st century. Part A. Elsevier Press, Amsterdam, The Netherlands.
18. **Rawlings, D. E., and E. Tietze.** 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. **65:**481–496.
19. **Rohrer, J., and D. E. Rawlings.** 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans.* J. Bacteriol. **174:**6230–6237.
20. **Rohrer, J., and D. E. Rawlings.** 1993. Regulation of mobilization of the broad-host-range plasmid pTF-FC2. Mol. Microbiol. **9:**1051–1059.
21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Scholtz, P., V. Haring, B. Wittmann-Liebold, K. Ashman, M. Bagdasarian, and E. Scherzinger.** 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene **75:**271–288.
23. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–790.
24. **Thorsted, P. B., D. P. Macartney, P. Aktar, A. S. Haines, N. Ali, P. Davidson, T. Stafford, M. J. Pocklington, W. Pansegrau, B. M. Wilkins, E. Lanka, and C. M. Thomas.** 1998. Complete sequence of the IncPβ plasmid R751: implications for evolution and organisation of the IncP backbone. J. Mol. Biol. **282:**969–990.
25. **Tietze, E.** 1998. Nucleotide sequence and genetic characterization of the novel IncQ-like plasmid pIE1107. Plasmid **39:**165–181.
26. **Waters, V. L.** 1993. Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. Mol. Microbiol. **9:**1123–1130.
27. **Whittle, G., M. E. Katz, E. H. Clayton, and B. F. Cheetham.** 2000. Identification and characterization of a native *Dichelobacter nodosus* plasmid, pDN1. Plasmid **43:**230–234.
28. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
29. **Ziegelin, G., J. P. Fürste, and E. Lanka.** 1989. TraJ protein of plasmid RP4 binds to a 19-base pair invert sequence repetition within the transfer origin. J. Biol. Chem. **264:**11989–11994.
30. **Ziegelin, G., W. Pansegrau, R. Lurz, and E. Lanka.** 1992. TraK protein of conjugative plasmid RP4 forms a specialized nucleoprotein complex with the transfer origin. J. Biol. Chem. **267:**17279–17286.

JOURNAL OF BACTERIOLOGY, Apr. 2004, p. 2123–2133 Vol. 186, No. 7
0021-9193/04/$08.00+0 DOI: 10.1128/JB.186.7.2123–2133.2004

Plasmid Evolution and Interaction between the Plasmid Addiction Stability Systems of Two Related Broad-Host-Range IncQ-Like Plasmids

Shelly M. Deane and Douglas E. Rawlings*

Department of Microbiology, University of Stellenbosch, Matieland 7602, South Africa

Received 10 October 2003/Accepted 12 December 2003

Plasmid pTC-F14 contains a plasmid stability system called *pas* (plasmid addiction system), which consists of two proteins, a PasA antitoxin and a PasB toxin. This system is closely related to the *pas* of plasmid pTF-FC2 (81 and 72% amino acid identity for PasA and PasB, respectively) except that the *pas* of pTF-FC2 contains a third protein, PasC. As both pTC-F14 and pTF-FC2 are highly promiscuous broad-host-range plasmids isolated from bacteria that share a similar ecological niche, the plasmids are likely to encounter each other. We investigated the relative efficiencies of the two stability systems and whether they had evolved apart sufficiently for each *pas* to stabilize a plasmid in the presence of the other. The three-component pTF-FC2 *pas* was more efficient at stabilization of a heterologous tester plasmid than the two component *pas* of pTC-F14 in *Escherichia coli* host cells (±92% and ±60% after 100 generations, respectively). The PasA antidote of each *pas* was unable to neutralize the PasB toxin of the other plasmid. The *pas* proteins of each plasmid autoregulated their own expression as well as that of the *pas* of the other plasmid. The *pas* of pTF-FC2 was more effective at repressing the *pas* operon of pTC-F14 than the *pas* of pTC-F14 was able to repress itself or the *pas* of pTF-FC2. This increased efficiency was not due to the PasC of pTF-FC2. The effect of this stronger repression was that pTF-FC2 displaced pTC-F14 when the two plasmids were coresident in the same *E. coli* host cell. Plasmid curing resulted in the arrest of cell growth but did not cause cell death, and plasmid stability was not influenced by the *E. coli mazEF* genes.

Plasmid pTC-F14 is a 14-kb broad-host-range mobilizable plasmid isolated from the moderately thermophilic (optimum, 45°C), sulfur-oxidizing, acidophilic bacterium *Acidithiobacillus caldus* (previously *Thiobacillus caldus*) strain f (8). It bears a number of similarities to a 12.2-kb broad-host-range plasmid, pTF-FC2, which was isolated from a different, mesophilic (30 to 35°C), iron- and sulfur-oxidizing, chemolithotrophic, acidophilic bacterium, *Acidithiobacillus ferrooxidans* (21). Both plasmids have IncQ-like plasmid replicons consisting of an *oriV* with three or more 22-bp iterons as well as *repB* (primase-encoding), *repA* (helicase-encoding), and *repC* (DNA-binding protein-encoding) genes. A further striking similarity is that both plasmids have genes for a plasmid addiction system (*pas*) located between the *repB* and *repA* genes (8, 26).

The *pas* genes of pTF-FC2 have been reported to act as a post-segregational killing (PSK) plasmid stability system so that daughter cells that fail to inherit a plasmid are killed (26). Typical plasmid PSK systems consist of two genes, one encoding a highly expressed but short-lived antitoxin and a second gene encoding a poorly expressed but long-lived toxin (14). The *pas* of pTF-FC2 is unusual in having three genes: *pasA* which encodes an antitoxin, *pasB* which encodes a toxin, and *pasC* which was thought to enhance the ability of the antitoxin to neutralize the toxin (26). Plasmid pTC-F14 is more typical of other plasmid addiction systems in having only two genes (*pasA* and *pasB*). In all protein type toxin-antitoxin (TA) plasmid stability systems studied to date, the antitoxins have a second autoregulatory function (11). This autoregulatory role has been found to be essential for the proper functioning of the *pas* of pTF-FC2 (28). When placed under control of the *tac* promoter, abnormal expression of the *pas* genes inhibited host growth and they failed to stabilize the plasmid. Although there are clear examples of conservation between toxins and also antitoxins (e.g., RelB antitoxin, RelE toxin, and ChpAK toxin homologues) (11), in general, there is a large amount of amino acid sequence diversity between toxins and also between antitoxins which function as PSK stability systems (17, 22). Presumably, this sequence diversity allows individual PSK systems to enhance the stability of the plasmids on which they occur rather than that of coresident plasmids that may compete for limited host resources. Plasmids pTF-FC2 and pTC-F14 are unusual in that the toxins and antitoxins are highly conserved and share 81 and 72% amino acid sequence identity, respectively (8).

The discovery of closely related TA systems on two related but compatible plasmids presents an opportunity to ask interesting questions on the evolution of such systems. The bacteria from which plasmids pTC-F14 and pTF-FC2 were isolated are physiologically related and grow in a similar low-pH, inorganic mineral-rich environment. Since both plasmids are broad host range and highly mobilizable, there is a possibility that the plasmids could frequently encounter each other, as they share the same ecological replication space. If both plasmids were to occur in the same cell, then the possession of a TA system that cross-reacts with a second closely related system may not enhance the stability of each individual plasmid species in the presence of the other. We therefore wished to discover wheth-

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa. Phone: 27 21 808 5848. Fax: 27 21 808 5846. E-mail: der@sun.ac.za.

TABLE 1. Plasmid vectors, *pas*-containing constructs, and primers used in this study

Plasmid or primer	Description or sequence[a]	Source or reference
Plasmid vectors		
pBR322	Ap^r, cloning vector	3
pGEM-T	Ap^r, T-tailed PCR product cloning vector	Promega Corp.
pACYC184	Tc^r, Cm^r, p15A replicon, cloning vector	6
pKK223-3	Ap^r, ColE1 replicon, *tac* promoter	4
pMC1403	Ap^r, ColE1 replicon, *lacZYA**	5
pOU82	Ap^r, R1 replicon, *lacZYA*	10
Plasmid constructs		
pTac-F14pasA	Ap^r, pKK223-3 vector, pTC-F14 *pasA* nt 1163–1405	This study
pTac-F14pasB	Ap^r, pKK223-3 vector, pTC-F14 *pasB* nt 1364–1662	This study
pTac-F14pasAB	Ap^r, pKK223-3 vector, pTC-F14 *pasAB* nt 1163–1662	This study
pTac-F14Pr-pasAB	Ap^r, pKK223-3 vector, pTC-F14 *pasAB* own promoter nt 900–1662	This study
pGEM-F14pasA	Ap^r, pGEM-T vector, pTC-F14 *pas* region nt 900–1405	This study
pGEM-F14pasAB	Ap^r, pGEM-T vector, pTC-F14 *pas* region nt 900–1662	This study
pMC-F14Pr(pas)	Ap^r, pMC1403 vector, pTC-F14 *pas* promoter region nt 900–1207	This study
pMC-FC2Pr(pas)	Ap^r, pMC1403 vector, pTF-FC2 *pas* promoter region	This study
pOU-F14pasA	Ap^r, pOU82 replicon, pTC-F14 *pas* region nt 900–1405	This study
pOU-F14pasAB	Ap^r, pOU82 replicon, pTC-F14 *pas* region nt 900–1662	This study
pOU-FC2pasABC	Ap^r, pOU82 replicon, pTF-FC2 *pas* region, previously pOU-pasABC	26
pOU-FC2pasABC*	Ap^r, pOU82 replicon, pTF-FC2 *pas* region with frameshift mutation in *pasC*, previously pOU-pasABC	26
pTac-FC2pasA	Ap^r, pKK223-3, pTF-FC2 *pasA* with *tac* promoter, previously pTac-pasA	26
pTac-FC2pasB	Ap^r, pKK223-3, pTF-FC2 *pasB* with *tac* promoter, previously pTac-pasB	26
pTac-FC2pasA-ACYC	Ap^r, pACYC184 vector, pTF-FC2 *pasA* with *tac* promoter, previously pTac-pasA-ACYC	28
pFC2-pasABC	Km^r, pTF-FC2 minimal replicon, previously pKmM0	26
pFC2-pasA*BC	pFC2-pasABC with frameshift mutation in *pasA*, previously pKmM1	26
pFC2-pasAB*C	pFC2-pasABC with frameshift mutation in *pasB*, previously pKmM2	26
pFC2-pasABC*	pFC2-pasABC with frameshift mutation in *pasC*, previously pKmM3	26
pFC2-ΔpasABC	pFC2-pasA*BC with spontaneous deletion of *pasABC*, previously pKmM1del	26
pF14-pasAB (Km)	Km^r, pTC-F14 minimal replicon, previously pTC-F101	8
pF14-pasA*B*	pF14-pasAB (Km) with frameshift mutations in *pasA* and *pasB*	This study
pF14-pasΔAB	pF14-pasAB (Km) with StuI-XbaI deletion in *pasAB*	This study
pF14-pasAB (Cm)	Cm^r, pTC-F14 minimal replicon, previously pTC-F200	9
pKGCm	Cm^r, pACYC184 replicon	27
Primers		
Primer 1 (EcoRI)	5′-TATTGAATTCGAGCAGGAGCTAAACATGC	This study
Primer 2 (HindIII)	5′-AATGAAGCTTAACTCAATCCGCCAAGCC	This study
Primer 3 (EcoRI)	5′-ATACGAATTCCTAGAGGAAGTGGAGCGCG	This study
Primer 4 (HindIII)	5′-TCGCAAGCTTGTTTACTTTCGGTATACCTCTCG	This study
Primer 5 (EcoRI)	5′-TACTGAATTCTACCAGTGTGCCCCATCG	This study
Primer 6 (BamHI)	5′-GTAGGGATCCACTTCGGTGGGTAATCGG	This study
Primer 7 (StuI)	5′-TGAGGCCTTGGC**T**GCAGGCCACAGGACG	This study
TACPRI	5′-GACAATTAATCATCGGCTCG	25
LACZPRI	5′-CGCCAGCTGGCGAAAGGGGG	
FP2 (BamHI)	5′-AGTAGGGATCCACTTCGGCGGGCAGTCGG	28
FC2PasAPr (EcoRI)	5′-TCATGAATTCGAGGGCGCTATCCGC	This study

[a] Nucleotide (nt) numbers are based on the numbering in Fig. 1. The boldface T in primer 7 is an extra nucleotide creating a frameshift mutation in *pasA* and a new PstI site (Fig. 1).

er the two TA systems have diverged sufficiently for them to act without interference by the other. More specifically, we wished to investigate whether the antitoxin from one plasmid could neutralize the toxin of the other plasmid and whether each antitoxin could autoregulate the expression of the related system. Furthermore, we wished to determine whether a plasmid that possessed the three-component *pas* had a stability advantage over a plasmid with the related two-component *pas*, when each plasmid was on its own in a host cell or when coresident with the other.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. *Escherichia coli* strains CSH50, CSH50-I^q (27), JM105 (29), and DH5α (Promega Corp.) were grown in Luria-Bertani broth (LB) or Luria agar (LA) (23), and ampicillin (100 μg/ml), kanamycin (30 μg/ml), tetracycline (15 μg/ml), and chloramphenicol (25 μg/ml) were added as required. *E. coli* MC4100 $relA^+$ (wild type [WT]) and its derivatives, MC4100 $relA^+$ *mazEF*::*kan* (Δ*mazEF*), MC4100 *relA1* (Km^s), and MC4100 *relA1 mazEF*::*kan* were a kind gift from Hanna Engelberg-Kulka.

DNA techniques, PCR, sequencing, and analysis. Plasmids and primers are shown in Table 1. Standard methods were used for plasmid isolation, digestion, ligation, agarose gel electrophoresis, and preparation and transformation of competent *E. coli* cells (23). The PCR was carried out in a PCR Sprint temperature cycling system (Hybaid) by using the Expand high-fidelity PCR system DNA polymerase (Roche Molecular Biochemicals), and all constructs were sequenced by the dideoxy-chain termination method with an ABI PRISM 377 automated DNA sequencer to verify the integrity of PCR-amplified fragments. Sequence manipulations were performed by using DNAMAN (version 4.1; Lynnon BioSoft), and homology searches were performed by using the gapped-BLAST program of the National Center for Biotechnology Information (2).

Plasmid constructs. Primer pairs 1 and 2, 3 and 4, and 1 and 4 were used to amplify the *pasA*, *pasB*, and *pasAB* genes, respectively, with pK13 (a subclone of pTC-F14) (8) as a template for the PCR. Amplified products were cloned in *E.*

coli DH5α behind the *tac* promoter of pKK223-3 by using the EcoRI and HindIII restriction sites on the primers. Sequencing with primer TACPRI verified the integrity of the pTac-F14pasA and pTac-F14pasAB constructs. A number of attempts at the PCR, cloning, and sequencing of the *tac-pasB* construct revealed nucleotide changes in the *pasB* gene that presumably made it less lethal (and therefore cloneable). Two clones that resulted in translucent-looking *E. coli* colonies, capable of growth only at 30°C, were found to have single-base-pair deletions in the codon preceding the *pasB* stop codon. These deletions caused a frameshift, which extended PasB by 29 amino acids. To correct this, a 622-bp XbaI-SalI fragment containing the front portion of *pasB* (correctly fused to the *tac* promoter), was excised and ligated into the XbaI-SalI fragment of pTac-F14pasAB (containing the end of *pasB* plus the vector pKK223-3). This ligation mixture was transformed into *E. coli* CSH50-I^q but only gave colonies when the cells also contained pF14-pasAB (Km) (i.e., *pasA*) in *trans*. The *tac-pasB* plasmid DNA (pTac-F14pasB) was finally purified by electrophoresis of the mixed plasmid preparation (pTac-F14pasB plus pF14-pasAB [Km]) on a preparative gel, followed by excision and GFX column (Amersham) isolation of the pTac-F14pasB plasmid alone. Primer 5 (which binds 276 bp upstream of the ATG start of *pasA*) was paired with primer 2 or 4 to amplify *pasA* or *pasAB*, respectively, to include the putative promoter region known to be important for the autoregulation of the *pas* operon (28). These PCR products were cloned in pGEM-T (giving constructs pGEM-F14pasA and pGEM-F14pasAB), sequenced in both directions, and then excised with EcoRI and HindIII for cloning in pBR322. From here, the *pas* genes were excised with EcoRI and BamHI and cloned into the EcoRI-BamHI sites of pOU82 to give constructs pOU-F14pasA and pOU-F14pasAB.

Fusion of *pasA* promoter region to LacZ reporter gene and β-galactosidase assays. An in-frame translational fusion of *pasA* to a *lacZ* reporter gene was made by cloning a PCR amplification product (primers 5 and 6) into the EcoRI-BamHI sites of pMC1403 to give construct pMC-F14Pr(pas). Similarly, a *pasA-lacZ* reporter gene construct [pMC-FC2Pr(pas)] was made for pTF-FC2, containing a *pasA* upstream region of approximately 298 bp (primers FP2 and FC2PasAPr). This pMC-FC2Pr(pas) construct was regulated in the same manner as its predecessor pP2H (28), which contained a smaller upstream region of *pasA*.

The PasA-LacZ fusion constructs were transformed into *E. coli* CSH50-I^q, which was then transformed by various other constructs to be tested in *trans*. β-Galactosidase assays were performed by the method of Miller (18) by using, where possible, the following fixed parameters. The culture volume sampled was 50 μl, which was added to 950 μl of Z buffer, and the cells were made permeable by vortexing with 10 μl of toluene. A fixed assay time (usually 5 to 10 min) was chosen for each comparative experiment, after which all sample reactions were terminated. Cultures were incubated in 50 ml of LB at 37°C with the appropriate antibiotic selection.

Plasmid stability assays. Stability assays were performed by growing plasmid-containing *E. coli* cells without selection in 5 ml of LB at 37°C for 100 generations, with transfer of approximately 1,000 cells to fresh LB at 20-generation intervals. Samples taken at 20-generation intervals were diluted in saline, plated onto LA plates (to give 150 to 350 colonies per plate), and incubated at 37°C overnight in the absence of selection. Fifty colonies were patched onto LA plates with appropriate antibiotic selection, and the percentage survival was used to calculate plasmid retention. The stability assay for the pOU82 constructs was performed as described above, except that cultures were incubated at 30°C, samples taken at 20-generation time intervals were plated directly onto LA plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)/ml, and blue and white colonies were scored as plasmid-containing and plasmid-minus colonies, respectively.

Incompatibility assays. *E. coli* cells were transformed sequentially with the desired replicons, and the plasmids were maintained by selection on appropriate antibiotics. Survival of the plasmids was then tested by dropping selection by one or both antibiotics and growing the cells in 5 ml of LB for 100 generations, with transfer of approximately 1,000 cells to fresh media at 20-generation intervals. Finally, 50 colonies were replica plated to antibiotic-containing LA plates to score for plasmid retention.

Plasmid curing. Plasmid curing was based on the procedure developed in the Gerdes laboratory (15). This made use of pKG339, a plasmid that is able to stop the replication of pOU82-based constructs following induction with isopropyl-β-D-thiogalactopyranoside (IPTG). A version of pKG339, called pKGCm, was used in our laboratory (27). Prevention of replication of pOU82-based constructs results in the rapid loss of these plasmids from the cell population. An overnight culture (30°C) was inoculated into fresh medium containing antibiotic selection to ensure that plasmids were present. This 3-h preculture was diluted 1:100 into prewarmed broth minus ampicillin (but containing chloramphenicol for pKGCm selection) and grown in the absence or presence of 2 mM IPTG (added at 3 h). Culture density was monitored at a wavelength of 600 nm. Samples taken throughout the growth period were diluted in saline and plated to LA–chloramphenicol–X-Gal plates to give 100 to 500 colonies per plate. Viable cells were calculated as the total number (blue and white) of CFU per milliliter. Plasmid-containing cells were calculated as blue CFU per milliliter.

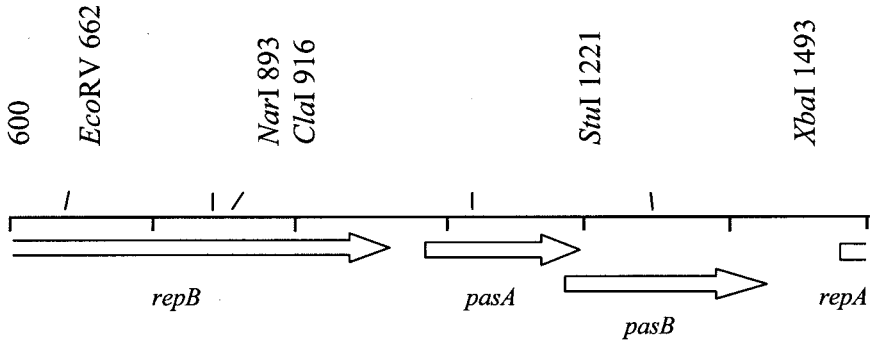
RESULTS

Comparative analysis of *pas* operons of pTC-F14 and pTF-FC2. The sequence of the pTC-F14 *pas* operon and flanking regions is shown in Fig. 1. The region between *repB* and *repA* of pTC-F14 contains two open reading frames (ORFs) with high sequence homology to *pasA* and *pasB* of pTF-FC2 at the levels of both nucleotide sequence (80 and 71%, identity, respectively) and amino acid sequence (81 and 72% identity, respectively). The most obvious difference between the two *pas* operons is that no region corresponding to the *pasC* gene of pTF-FC2 was found on pTC-F14. Nucleotide sequence similarity between the two operons terminates at the pTC-F14 *pasB* stop codon, and for the next 48 bp, there is no obvious similarity between the two plasmids. Clear nucleotide similarity (75%) resumes 68 bp immediately upstream of the *repA* gene, and there is no space to accommodate a *pasC* gene. Apart from the absence of *pasC*, the pTC-F14 *pas* operon bears striking similarity to that of pTF-FC2. Plasmid pTF-FC2 has a 7-bp duplication (5′-CAGGAGC-3′) in the promoter region of the *pas* operon, and when the PasA antitoxin was inactivated by a frameshift mutation, a spontaneous deletion occurred between the 7-bp duplications in a small percentage of the plasmids (26). This deletion inactivated the *pas* operon, and the otherwise sick host cells once again grew strongly. Plasmid pTC-F14 has a 9-bp duplication (5′-AGCAGGAGC-3′) in the same region, which includes the identical 7-bp duplication found in plasmid pTF-FC2. However, pTC-F14 *pasA* mutants have been even more difficult to construct (see below), and spontaneous deletions in the promoter region have not been detected.

Both *pasA* genes have a consensus AGGAG ribosome binding site and an AUG start typical of highly expressed genes. The start codons of both *pasB* genes (UUG in the case of pTC-F14) are situated within the 3′ end of *pasA*, and the putative ribosome binding site (GGAG) is less homologous to the consensus sequence than is the case for highly expressed genes. This arrangement of overlapping ORFs is frequently encountered in TA systems, in which the toxin is synthesized in smaller amounts relative to the antidote (e.g., *E. coli relBE*) (12).

Another *pasAB*-like operon has been discovered on pAM10.6, a plasmid isolated from *Pseudomonas fluorescens* that is unrelated to IncQ-like plasmids (20). Interestingly, the amino acid sequence identities of PasA and PasB of pAM10.6 are more closely related to those of pTC-F14 (84 and 74%, respectively) than PasA and PasB of pTF-FC2 are to those of pTC-F14 (81 and 72%, respectively). Like the other *pas* genes, the genes of the pAM10.6 *pas* also have overlapping ORFs, but unlike pTF-FC2, no *pasC* gene is present.

The *pas* genes of pTC-F14 function as a stability system. In spite of the high amino acid sequence similarity between PasA and PasB of pTF-FC2 and pTC-F14, it was necessary to demonstrate that the two-component *pas*-like system of pTC-F14 was able to function as a plasmid stability system. To deter-




```
      NarI      (primer#5)      ClaI
TGAGGGCGCCATCTACCAGTGTGCCCCATCGATCCGGGAGCAGCAGGAAAGCCGGGACTGGACCGACTACGC  960

CCGCCGCACGGCCCGCTATGCCTACAGCGCCGCAGGTGACCGGCAGGCCGCCGACCTGGGTAAGTACCGCCA 1032

GCAGTGGGAGAAGCTGGAGGGGCGCGAGCGGCAGCAGGAGCAGGCCAAGGCGCGGGAGATTGAGCGCAACGG 1104

        end of repB
GCCGAGTATGAGTCGTTAGTATTCTTCACAGCGGCATGATATACTTGTATATCATTTTGAGCAGGAGCTAAA 1176

 pasA start        (primer#6)                  StuI     ↓T
CATGCTTGCCATCCGATTACCCACCGAAGTGGAAAATCGCCTTGAGGCCTTGGCGCAGGCCACAGGACGCAC 1248
  M  L  A  I  R  L  P  T  E  V  E  N  R  L  E  A  L  A  Q  A  T  G  R  T

GAAAACCTTTTACGCCCGCGAAGCGATCCTTGAGCACCTGGACGACCTCGAAGATTTGTACCTTGCAGAGCA 1320
  K  T  F  Y  A  R  E  A  I  L  E  H  L  D  D  L  E  D  L  Y  L  A  E  Q

                                                              start pasB
ACGCCTGATCGACATTCGCGCAGGCCGAAGCCGTACCTACACGCTAGAGGAAGTGGAGCGCGATCTTGGCTT 1392
  R  L  I  D  I  R  A  G  R  S  R  T  Y  T  L  E  E  V  E  R  D  L  G  L
                                                                    L  A

GGCGGATTGAGTTTGATGACAAGGCCAAGAAAGACTTGGCTGCGCTGGATAAGAGCGTTGCCAAACGCATCA 1464
 A  D  *
W  R  I  E  F  D  D  K  A  K  K  D  L  A  A  L  D  K  S  V  A  K  R  I
                                XbaI
CGGCTTTTCTGCGAGAGCGCGTCGCCCATCTAGACGACCCGCGCAGCATTGGCGAAGCCCTCAAAGGCTCCA 1536
T  A  F  L  R  E  R  V  A  H  L  D  D  P  R  S  I  G  E  A  L  K  G  S

AGCTGGGAGACTTCTGGAAGTACCGCGTAGGGGATTGGCGAATCATTGCCAGCATTGAGGATGAAGCCTTGC 1608
K  L  G  D  F  W  K  Y  R  V  G  D  W  R  I  I  A  S  I  E  D  E  A  L

GTATTCTTGTCGTGCGTATTGGTAACCGCCGAGAGGTATACCGAAAGTAAACAACCCCGGCATGAAGGGCCG 1680
R  I  L  V  V  R  I  G  N  R  R  E  V  Y  R  K  *

GGGTTTCCCGCGCAACCGGATGAAAGGACGGCGCGAGCCGGAGTGTCAGTCCCCGGCAGCGCCTAACCACGC 1752

          start of repA
CAGTTCTGGAAAGGAGAACAGCATG                                                  1777
```

FIG. 1. Nucleotide sequence of the region of pTC-F14 carrying *pasA*, *pasB*, the 3′ end of *repB*, and the 5′end of *repA* (accession number AF325537). The amino acid sequences of PasA and PasB are indicated below the nucleotide sequences. Primers 5 and 6 (underlined) were used to make a translational fusion of the 307 bp upstream and the start region of *pasA* to a *lacZ* reporter gene. The site of insertion of a T nucleotide into the *pasA* sequence (↓) to create a frameshift and a PstI site (boldface type) are shown. Repeated sequences similar to those that flank a spontaneous deletion in pTF-FC2 are boxed, and potential ribosome binding sites are in boldface italic type. Two 9-bp inverted repeats immediately upstream of *pasA* and downstream of *pasB* are shown by opposing arrows. Restriction enzyme recognition sites are in boldface type.

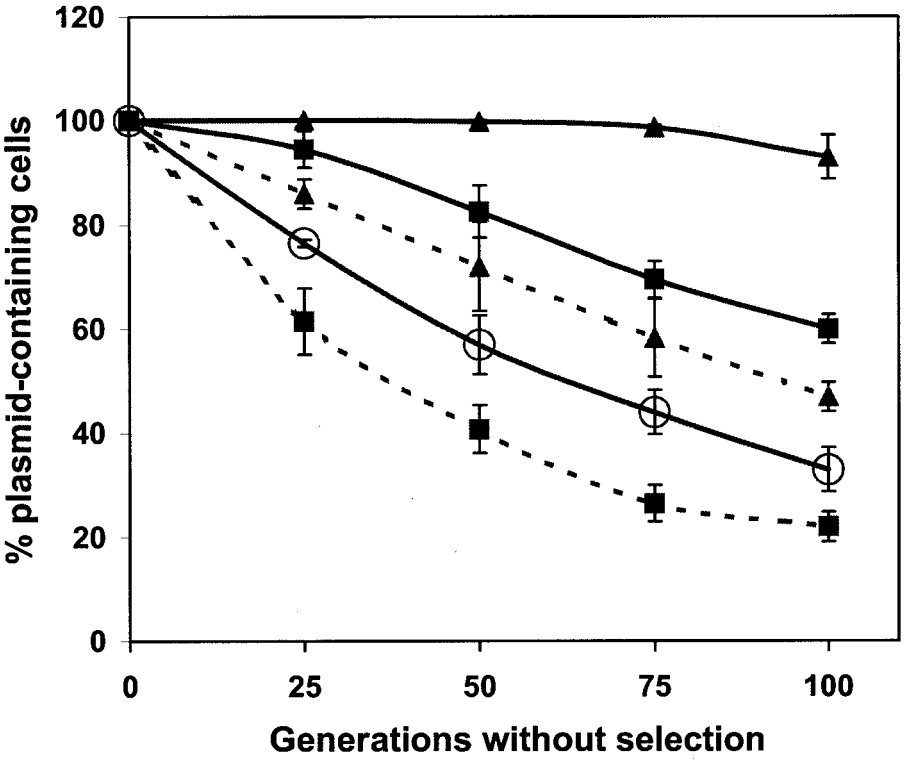


FIG. 2. Stabilization of heterologous R1 replicon by the *pasAB*(*C*) genes of pTC-F14 and pTF-FC2 in *E. coli* CSH50 at 30°C. Plasmid stability curves of the tester plasmid pOU82 (○) alone and containing the *pasABC* (▲, solid line) and *pasAB*(*C*) (▲, broken line) genes from pTF-FC2 (constructs pOU-FC2pasABC and pOU-FC2pasABC*, respectively) (Table 1) and the *pasAB* (■, solid line) and *pasA* (■, broken line) genes from pTC-F14 (constructs pOU-F14pasAB and pOU-F14pasA, respectively) (Table 1). The datum points are averages of the results from three independent determinations with the standard deviations, and values are normalized to 100% at 0 generations.

mine this, the *pasAB* operon of pTC-F14 was cloned into the unstable, low-copy-number test plasmid pOU82 (10). After 100 generations of growth without selection, the pOU82 control was retained in approximately 33% of the population while plasmids pOU-F14pasAB (plasmid pOU82 containing the pTC-F14 *pasAB* genes) and pOU-F14pasA (containing the *pasA* antidote-encoding gene only) were stabilized 60 and 22%, respectively (Fig. 2). By comparison, the three-component pTF-FC2 *pas* was more effective at plasmid stabilization, and plasmids pOU-FC2pasABC and pOU-FC2pasABC* (*pasC* inactivated) were retained in 93 and 47% of the population, respectively. Therefore, although the pTC-F14 2-component *pas* was able to stabilize the pOU82 test plasmid in *E. coli*, it was less effective than the three-component pTF-FC2 *pas*.

Difficulty in constructing a pTC-F14 *pasA* knockout. We wished to generate a *pasA* mutant of pTC-F14 to investigate whether this plasmid's PasB toxin could be neutralized by the PasA antitoxin from pTF-FC2. A T was inserted within codon 18 of *pasA*, creating a frameshift mutation and introducing a PstI site (primer 7) (Fig. 1). The frameshift mutant constructs were transformed into *E. coli* cells, and transformants were obtained which contained plasmids with the newly introduced PstI site. However, unlike *pasA* mutants of pTF-FC2, the cells containing this construct were not noticeably sick. On sequencing the construct, it was discovered that a second mutation (G→T) had occurred 5 bp from the stop codon of *pasB*, resulting in the alteration of a conserved R to an L, which presumably inactivated PasB. To overcome this, the region containing the *pasA* T-insertion frameshift was recovered by excising an 830-bp EcoRV-XbaI fragment, and an attempt was made to insert (exchange) this into the minimal replicon of pTC-F14. This exchange consistently failed, and when the frameshift-containing insertion was eventually obtained, it was found that a C had been spontaneously deleted, which resulted in the C-terminal 27 amino acids of PasB being out of frame. It was concluded that in the absence of a functional PasA antitoxin, PasB was so toxic to the host cells that a *pasA* mutant was not viable.

Autoregulation and balanced expression of *pasA* and *pasB* is required to control toxicity of the *pasAB* operon. Induced expression of the pTC-F14 *pasAB* genes from the *tac* promoter was studied in *E. coli* JM105, as had been done for the *pasABC* genes of pTF-FC2 (28). Two promoter fusion constructs were used: pTac-F14pasAB, in which the *tac* promoter was fused to the ribosome binding site and ATG start region of *pasAB* so as to exclude the natural *pas* promoter, and pTac-F14Pr-pasAB, in which the *tac* promoter was fused 276 bp upstream of *pasAB* and which includes the natural *pas* promoter. Following induction with IPTG, strong inhibition of growth was caused by expression of *pasAB* from the *tac* promoter only when the natural *pasAB* promoter was not present (results not shown). This confirmed the importance of autoregulatory feedback by PasA-PasB for the balanced production of antitoxin and toxin, as was found in the case of the pTF-FC2 *pasABC* system (28).

Cross talk between antidote and toxin of pTC-F14 and pTF-FC2. Unregulated expression caused further problems when

TABLE 2. Complementation of lethal expression of PasB toxin by antidote PasA carried in *trans*[a]

Resident plasmid in *E. coli* CSH50-I^q	No. of transformants per ng upon transformation with:					
	pKK223-3 (control)		pTac-FC2pasB		pTac-F14pasB	
	−IPTG	+IPTG	−IPTG	+IPTG	−IPTG	+IPTG
pF14-pasAB (Km)	~1,000	~1,000	~1,000	NG	~700	~700
pF14-pasΔAB	~1,000	~1,000	~1,000 (sick)	NG	NG	NG
pFC2-pasABC	~1,000	~1,000	~1,000	~1,000	~500	NG
pFC2-pasABC*	~1,000	~1,000	~1,000	~1,000	~500	NG
pFC2-ΔpasABC	~1,000	~1,000	~1,000 (sick)	NG	NG	NG

[a] pTC-F14 *pasAB* and the deletion thereof were carried on plasmids pF14-pasAB (Km) and pF14-pasΔAB, respectively. pTF-FC2 *pasABC* and *pasAB* and deletions thereof were carried on plasmids pFC2-pasABC, pFC2-pasABC*, and pFC2-ΔpasABC, respectively. pKK223-3 is the *tac* promoter fusion vector containing no insert. pTac-FC2pasB contains the *pasB* of pTF-FC2 under control of the *tac* promoter. pTac-F14pasB contains the *pasB* of pTC-F14 under control of the *tac* promoter. IPTG (1 mM) was used to induce the *tac* promoter (−, without; +, with). Colony numbers on plates after 24 h of incubation at 30°C are given. NG, no growth.

we attempted to test cross-complementation of *tac*-expressed *pas* genes from pTC-F14 and pTF-FC2. In previous work, site-directed mutagenesis was used to construct a *pasA* mutant of pTF-FC2. This *pasA* mutation (pFC2-pasA*BC) resulted in very slow growing host cells, with a small proportion forming large colonies as a result of one of two types of spontaneous *pas* deletion (26). We wished to test whether the PasB toxin of the pTF-FC2 *pasA* mutant could be neutralized by the pTC-F14 PasA antitoxin, thereby restoring rapid growth to the host cells. Cells containing the pTC-F14 and pTF-FC2 *pasA* genes under control of a *tac* promoter (pTac-F14pasA and pTac-FC2pasA, respectively) as well as cells containing the pKK223-3 vector control were transformed with the pTF-FC2 *pasA* mutant DNA (pFC2-pasA*BC). Cells were plated on kanamycin and ampicillin, to select for both plasmids, and IPTG to induce *pasA* expression. However, no transformants were obtained. This lead to the unexpected discovery that the *pasA* (antitoxin) genes of pTF-FC2 and pTC-F14, when expressed from an IPTG-induced *tac* promoter, were themselves toxic to host cells. The toxicity of pTac-F14pasA and pTac-FC2pasA alone was evident even in the absence of any other coresident constructs. The degree of toxicity varied slightly from bacterial strain to strain and was more noticeable at 37°C than at 30°C. Attempts to make cells containing the pFC2-pasA*BC construct competent and complement with pTac-F14pasA and pTac-FC2pasA in *trans* were unsuccessful, as pFC2-pasA*BC made host cells too sick to transform, even when grown at 30°C.

Furthermore, neutralization of pFC2-pasA*BC was not direct evidence of protein-protein interaction, since we could not rule out the influence of PasA binding to the promoter of pFC2-pasA*BC that would also result in a decrease in toxicity. To prove TA interaction at a purely protein level, we attempted to express the toxin from the inducible *tac* promoter (rather than the natural *pas* promoter) and then to neutralize the effect of the toxin by placing the antidote in *trans*. The pTF-FC2 toxin fusion (pTac-FC2pasB) was already available and was not fully lethal in cultures grown at 30°C when uninduced by IPTG. However, the pTC-F14 *tac* toxin gene fusion (pTac-F14pasB) was much harder to construct, as it appeared to be more toxic than the pTF-FC2 equivalent. pTac-F14pasB was extremely lethal and could not be maintained in cultures, even at 30°C, unless its antidote (on pF14-pasAB [Km]) was supplied in *trans.* Competent *E. coli* cells that already contained various plasmids expressing the pTC-F14 or pTF-FC2 antidote were then transformed with these lethal *tac-pasB* constructs to test which antidote would neutralize which toxin (Table 2).

Without induction by IPTG, it was evident that slight cross-neutralization occurred between the resident plasmid carrying the antidote and the incoming plasmid carrying the toxin in the case of both pTF-FC2 and pTC-F14. The presence of pTac-FC2pasB resulted in healthy colonies in the presence of its own TA system (pFC2-pasABC and pFC2-pasABC*) as well as in the foreign TA system (pF14-pasAB [Km]). However, deletions of either of these TA systems (pF14-pasΔAB or pFC2-ΔpasABC) resulted in sick colonies. Similarly, when not induced by IPTG, pTac-F14pasB gave healthy colonies when in the presence of its own TA system (pF14-pasAB [Km]) as well as in the foreign TA system (pFC2-pasABC and pFC2-pasABC*). In this case, however, deletion of either of these TA systems (pF14-pasΔAB or pFC2-ΔpasABC) resulted in no colonies, illustrating how much more lethal the pTC-F14 *tac-pasB* fusion was than the pTF-FC2 fusion.

When induction was initiated by IPTG, full neutralization was required between the resident plasmid carrying the antidote and the incoming plasmid carrying the toxin. In this instance, only the related antidote-toxin pairs (pF14-pasAB [Km] + pTac-F14pasB and pFC2-pasABC + pTac-FC2pasB) showed neutralization, whereas the foreign pairs (pF14-pasAB [Km] + pTac-FC2pasB and pFC2-pasABC + pTac-F14pasB) do not cross-react sufficiently to allow cell growth (Table 2). The presence of the extra gene, *pasC*, on pFC2-pasABC did not appear to affect the neutralization of the toxin by the antidote.

Bearing in mind the lethality of unregulated overexpression of the *pas* genes, further studies with *tac* promoter fusions to individual *pas* genes were dropped in favor of constructs such as pF14-pasAB (Km) (minimal replicon of pTC-F14) and pFC2-pasABC (minimal replicon of pTF-FC2) containing the entire natural promoter region and *pasAB*(*C*) operon (Table 1).

Cross-regulation of *pas* genes. The *pasABC* genes of pTF-FC2 were previously shown to be autoregulated, with the PasA antitoxin functioning as the primary regulator (28). We wished to test whether the PasA antitoxins of plasmids pTF-FC2 and pTC-F14 could regulate the *pas* genes of each other. Translational fusion *pasA-lacZ* reporter gene constructs were made for both pTF-FC2 [pMC-FC2Pr(pas)] and pTC-F14 [pMC-

TABLE 3. Regulation of *pas* operons of pTC-F14 and pTF-FC2[a]

pas-lacZ reporter construct	Coresident plasmid	β-Galactosidase activity (Miller units) ± SD	% Activity
pMC-F14Pr(pas)	pF14-pasΔAB	5,149 ± 727	100
	pF14-pasAB (Km)	1,179 ± 213	24.55 ± 1.9
	pFC2-pasABC	551 ± 188	12 ± 2.9
	pFC2-ΔpasABC	4,763 ± 709	93.5 ± 11.6
pMC-FC2Pr(pas)	pFC2-ΔpasABC	4,779 ± 1,339	100
	pFC2-pasABC	196 ± 10	4.25 ± 0.9
	pF14-pasAB (Km)	510 ± 98	10.8 ± 0.98
	pF14-pasΔAB	4,512 ± 1,048	95.05 ± 4.7

[a] β-Galactosidase activity was measured in log-phase *E. coli* strain CSH50-I^q cells containing the *pas-lacZ* reporter constructs pMC-F14Pr(pas) (pTC-F14 *pas* promoter fused to *lacZ*) and pMC-FC2Pr(pas) (pTF-FC2 *pas* promoter fused to *lacZ*). The percentage of expression of the promoter regions is calculated as the proportion of β-galactosidase activity measured relative to that in which the inactive *pas* operons (pF14-pasΔAB and pFC2-ΔpasABC, respectively) are in *trans*, and it is based on the relative values within the results from each of three experiments. Values represent the averages of the results from three independent experiments with standard deviations.

F14Pr(pas)], containing upstream regions of 298 and 276 bp for the *pasA* genes of pTF-FC2 and pTC-F14, respectively.

Although β-galactosidase activity varied from experiment to experiment, relative values within individual experiments were highly reproducible. With no other constructs in *trans*, the pTF-FC2 and pTC-F14 *pasA-lacZ* reporter genes expressed *lacZ* at comparable levels (approximately 5,200 Miller units). When the pTF-FC2 and pTC-F14 replicons from which the *pas* genes had been deleted (pFC2-ΔpasABC and pF14-pasΔAB) were placed in *trans*, levels of reporter gene expression were reduced slightly and these values were taken as the unregulated level (100% expression) (Table 3). With the natural *pasAB* genes of pTF-FC2 (pFC2-pasABC) or pTC-F14 (pF14-pasAB [Km]) placed in *trans* to the pTF-FC2 *pasA-lacZ* reporter construct, β-galactosidase activity was reduced to 4 and 10% of the unregulated levels of expression, respectively. The pTC-F14 *pasA-lacZ* fusion was expressed at about 25% of its unregulated level when its own *pas* genes were placed in *trans* (pF14-pasAB [Km]), and this level of expression was reduced to 12% when the *pas* genes of pTF-FC2 were placed in *trans* (pFC2-pasABC). This indicated that PasABC of pTF-FC2 was twice as efficient at repressing the *pas* promoter of pTC-F14 than was its own PasAB. Furthermore, pFC2-pasABC was twice as efficient at repressing its own *pas* promoter [pMC-FC2Pr(pas)] than was pF14-pasAB (Km) (Table 3).

Therefore, both *pas* operons could regulate one another, with the three-component *pasABC* system of pTF-FC2 being the stronger repressor of both promoters. PasC is not responsible for this improved repression, since pFC2-pasABC* (frameshift mutation in *pasC*) was equally effective as pFC2-pasABC in repressing both its own and pTC-F14's *pas* operon (results not shown).

It was previously shown that PasA of pTF-FC2 acted as the primary repressor of the *pasABC* operon and that PasB and PasC played minor and negligible coregulatory roles, respectively. To test whether the PasA of pTF-FC2 could regulate the pTC-F14 *pasA-lacZ* fusion on its own, a plasmid (pTac-FC2pasA-ACYC) containing the *pasA* gene of pTF-FC2 under control of the *tac* promoter was placed in *trans* to the reporter construct. After 3 h of growth of the host culture, the *pasA* gene was induced with 2 mM IPTG, and after a further 3 h, the level of reporter gene expression was found to be 13% of the unregulated expression (results not shown). Therefore, PasA is also the major regulator of the pTC-F14 *pas* operon.

***pas*-mediated plasmid stability is not dependent on *E. coli mazEF*.** Aizenman and coworkers (1) presented evidence to suggest that certain PSK systems do not kill the host cells directly but are protein synthesis inhibitors that work through the *E. coli mazEF* chromosomal TA system. MazEF-mediated cell death can be triggered by several protein synthesis inhibitors, including antibiotics (that are general inhibitors of transcription and/or translation) (24), as well as by the *phd-doc* TA module from the P1 prophage (13). This prompted us to test the stability of the *pas* module in the *E. coli* MC4100 and MC4100 *relA*/*mazEF* strains, with the pOU82 assay system. The stability of pOU-FC2pasABC was the same in both MC4100 WT and the *relA*/*mazEF* deletion strains over 100 generations without antibiotic selection (results not shown). This indicated that pOU82 stability mediated by the pTF-FC2 *pasABC* genes was not influenced by the *E. coli relA*/*mazEF* operon. Similarly, the stability conferred by the *pasAB* genes of pTC-F14 on pOU82 was not influenced by the *E. coli relA*/*mazEF* operon.

Plasmid curing does not cause cell death. Since the pOU82-based method of assaying stability is quite different from the methods used by Hazan and colleagues (13) in their *E. coli mazEF* plasmid stability experiments, a forced plasmid-curing method based on the procedure developed in the Gerdes laboratory (15) was tested. Prevention of replication results in rapid loss of the pOU82-based constructs from a cell population, and if the *pas* mediated stability was of the PSK type, this would be expected to result in the death of plasmid-minus cells. To test the ability of the pTF-FC2 PasABC to induce cell death, the pOU82-based plasmid constructs were prevented from replicating following induction of CopA inhibitor by plating cells onto LA containing 2 mM IPTG (Table 4). The procedure was effective in curing the cells of plasmids, but no cell death in either the *mazEF*-positive or -negative hosts was

TABLE 4. Ability of pTF-FC2 *pasABC* system to induce cell death upon curing[a]

Strain and plasmid construct	No. of host cells from noncured culture	% Containing plasmid	No. of host cells from cured culture	% Containing plasmid
A^+M^+(pKGcm)(pOU-FC2pasABC)	255	94	262	0
A^+M^-(pKGcm)(pOU-FC2pasABC)	328	100	326	0

[a] Colony counts (CFU) of two bacterial cultures diluted and plated onto LA containing X-Gal (blue colonies contained plasmid, white colonies did not contain plasmid) with (cured) and without (noncured) 2 mM IPTG. The *E. coli* strains used were MC4100 *relA*$^+$ and *mazEF*$^+$ (A^+M^+) and *relA*$^+$ *mazEF*-negative (A^+M^-). The results are representative of those from three separate experiments.

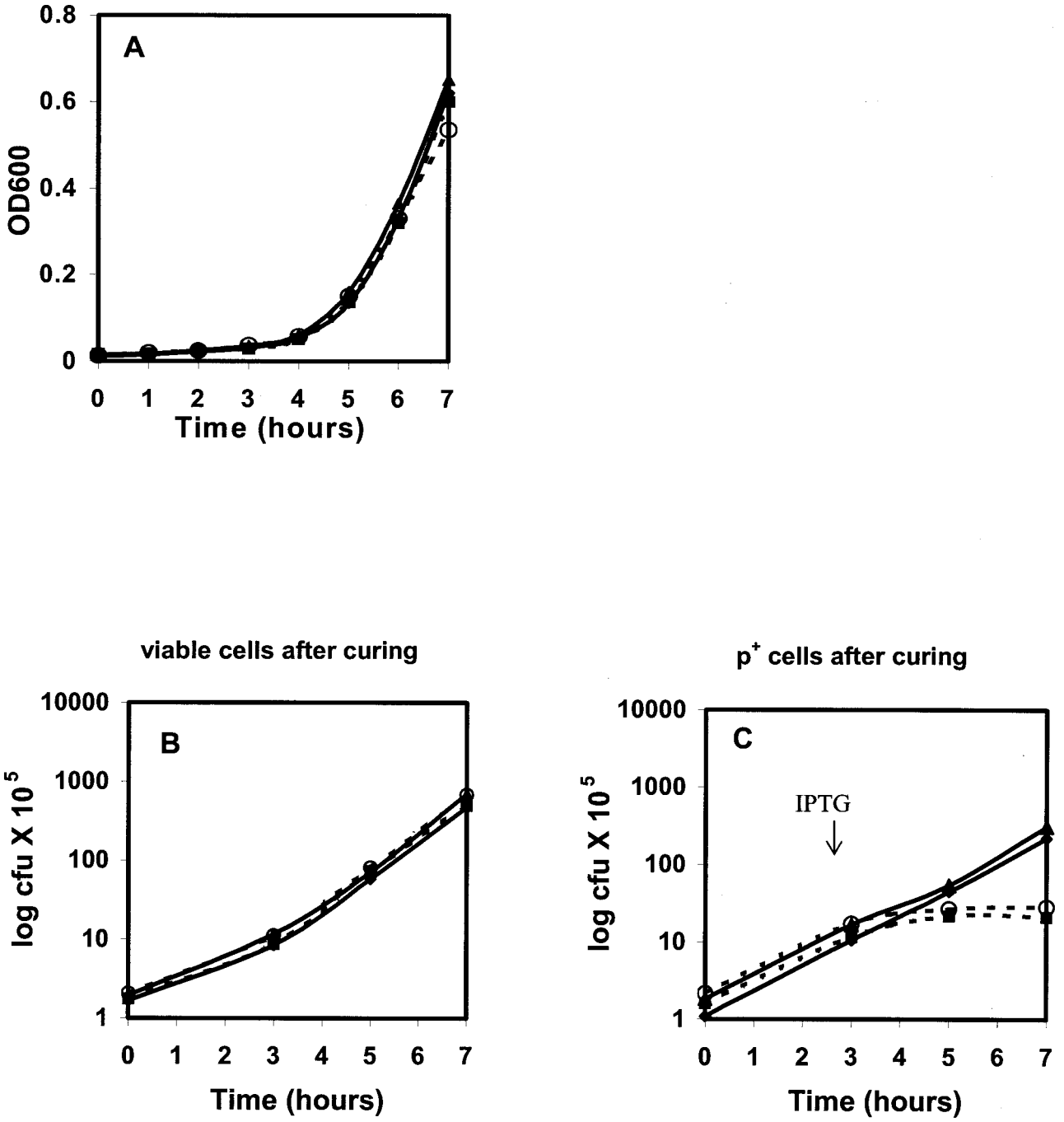


FIG. 3. Effect on host cell viability of curing of R1 replicon carrying pTF-FC2 *pasABC* genes. Turbidity readings (A), total CFU per milliliter (B), and plasmid-containing CFU per milliliter (C) of *E. coli* MC4100 (*relA*$^+$ *mazEF*$^+$) carrying pOU82 (◆, ■) and pOU-FC2pasABC (▲, ○) with (broken line) and without (solid line) the addition of IPTG at 3 h. The graphs are representative of the results from three independent experiments.

detected, as the number of CFU remained constant for cured and noncured cultures.

To confirm the result, a similar experiment was set up in which the growth of cells was monitored in liquid medium rather than on plates. A 3-h preculture of *E. coli* MC4100 *relA*$^+$ *mazEF*$^+$ containing pKGCm together with pOU82 or pOU-FC2pasABC was diluted into prewarmed broth without ampicillin and grown for a further 7 h with or without 2 mM IPTG (added at 3 h). At time zero, the inoculum contained approximately 10^5 cells per ml, of which 94% contained the pOU82-based constructs (Fig. 3).

After 7 h, turbidity readings showed that both cultures had increased in cell mass, with those containing pOU-FC2pasABC (plus IPTG) lagging slightly behind those with pOU82. Viable cell counts likewise increased over 7 h to 4×10^7 to 5×10^7 CFU/ml (for pOU82 plus or minus IPTG) and 5×10^7 to 6×10^7 CFU/ml (for pOU-FC2pasABC plus IPTG). At 0, 3, 5, and 7 h, both cultures were tested for the

TABLE 5. Competitive and noncompetitive plasmid stability in the absence of antibiotic selection[a]

Host cell	pTC-F14-based plasmid and antibiotic marker	pTF-FC2-based plasmid and antibiotic marker	Plasmid retention (%) within cells after 100 generations minus antibiotic selection for:	
			pTC-F14-based plasmids	pTF-FC2-based plasmids
E. coli CSH50	pF14-pasAB (Cm)		99.3 (1.2)	
	pF14-pasAB (Km)		99.7 (0.6)	
	pF14-pasΔAB (Km)		26.6 (29)	
	pF14-pasA*B* (Km)		5.3 (9.2)	
		pFC2-pasABC (Km)		99.0 (1.7)
		pFC2-pasAB*C (Km)		60.3 (15.4)
		pFC2-pasABC* (Km)		99.7 (0.6)
		pFC2-ΔpasABC (Km)		80.7 (14)
	pF14-pasAB (Cm)	pFC2-pasABC (Km)	1.6 (2.8)	98.0 (3.5)
	pF14-pasAB (Cm)	pFC2-pasAB*C (Km)	17.0 (5.6)	91.0 (5.6)
	pF14-pasAB (Cm)	pFC2-pasABC* (Km)	0.6 (1.2)	89.6 (5.8)
	pF14-pasAB (Cm)	pFC2-ΔpasABC (Km)	75.3 (18)	89.3 (13.6)

[a] All values are normalized to 100% at 0 generations. The percentage of plasmid retention is shown as the average of the results from at least three experiments. Standard deviations are shown in parentheses.

presence of plasmids by plating onto media containing X-Gal. At the end of 7 h, less than 10% of both types of cells contained plasmids, showing that plasmid curing was successful. Induction of cultures containing pOU-FC2pasABC with IPTG consistently caused a slight lag in the growth rate, as determined by turbidity readings. However, these cultures continued to grow at a constant, if slower, rate and produced high levels of viable plasmid-free cells. This indicated that loss of the *pas* may have temporarily slowed the growth of plasmid-minus cells but did not cause cell death. Similarly, loss of a plasmid containing a pTF-FC2 *pasC* mutant (pOU-FC2pasABC*) or a pTC-F14 *pasAB*-containing plasmid (pOU-F14pasAB) did not cause cell death on curing or the slight lag in growth described for pOU-FC2pasABC (results not shown).

Role of *pas* genes in competition between replicons of pTF-FC2 and pTC-F14 for maintenance within a bacterial population. We tested whether the presence or absence of a *pas* gene conferred a selective advantage to pTF-FC2 or pTC-F14 when both plasmids were coresident in an *E. coli* cell. *E. coli* CSH50 cells containing derivatives of plasmid pTF-FC2 or pTC-F14 were grown for approximately 100 generations without antibiotic selection, and the presence of the plasmids was determined by testing 50 colonies for kanamycin or chloramphenicol resistance. Before carrying out these competition experiments, the stability of the replicons with and without functional *pas* genes was determined. pTF-FC2 replicon-based plasmids pFC2-pasABC (intact *pasABC*) and pFC2-pasABC* (*pasAB* with inactive *pasC*) were stable for 100 generations without antibiotic selection when present on their own in host cells as were pTC-F14 replicon-based plasmids pF14-pasAB (Km) and pF14-pasAB (Cm) (intact *pasAB*) (Table 5). When the *pas* genes were deleted, plasmids containing either the pTF-FC2 replicon (pFC2-ΔpasABC) or the pTC-F14 replicon (pF14-pasΔAB) were not as stable (81 and 27% retention, respectively). Frameshift mutations within the *pasB* gene of the minimal replicon of pTF-FC2 (pFC2-pasAB*C) and *pasAB* genes of pTC-F14 (pF14-pasA*B*) were also less stable, 60 and 5%, respectively (Table 5).

To test whether the *pas* genes within the minimal replicons of one plasmid affected the stability of the other plasmid, derivatives of plasmids pTF-FC2 and pTC-F14 were cotransformed into *E. coli* CSH50, which was then subcultured for approximately 100 generations in the absence of selection for either plasmid, and the presence of the individual plasmids within single colonies was determined. When together in a cell, pTF-FC2 (pFC2-pasABC) displaced pTC-F14 (pF14-pasAB [Cm]) and less than 5% of cells retained pF14-pasAB (Cm) (Table 5). A deletion mutant of pTF-FC2 (pFC2-ΔpasABC) that completely lacks the *pasABC* region was less able to displace pF14-pasAB (Cm) (75% retention). This indicated that the displacement was not due to incompatibility of the replicons and is consistent with previous research in which the iterons of each plasmid cloned on a high-copy-number plasmid would displace the replicon from which the iterons were derived but not the other replicon (8). Plasmids with frameshifts in TF-FC2 *pasB* (pFC2-pasAB*C) and *pasC* (pFC2-pasABC*) were able to displace pF14-pasAB (Cm) (17 and 1% retention of pF14-pasAB [Cm], respectively). This indicated that only the *pasA* region of the *A. ferrooxidans* plasmid pTF-FC2 was required for the displacement of pF14-pasAB (Cm) from the bacterial population. In contrast, pTC-F14 containing an intact *pasAB* was not able to displace the pTF-FC2 replicon, even when the *pas* genes of pTF-FC2 were deleted (pFC2-ΔpasABC) or inactivated (pFC2-pasAB*C).

Noncompetitive and competitive stability of the pTC-F14 and pTF-FC2 derivatives was also studied at 25-generation intervals to determine how rapidly the plasmids were lost from host cells (results not shown). pF14-pasAB (Cm), though highly stable when alone in the host cell, becomes extremely unstable when in the presence of either pFC2-pasABC or pFC2-pasAB*C and begins to be lost from the host population within the first 25 generations.

DISCUSSION

In this work we showed that *pasAB* TA genes of pTC-F14 were able to improve the stability of an unstable, low-copy-number, heterologous replicon, pOU82. The *pasAB* of pTC-F14 or *pasABC* genes of pTF-FC2 were less effective at stabilizing pOU82 than their own replicons (pF14-pasAB [Cm]/pF14-pasAB [Km] or pFC2-pasABC, respectively). The *pasABC*

operon of pTF-FC2 consistently conferred better stability on the pOU82 replicon than the *pasAB* of pTC-F14. The *pasABC* operon in which *pasC* has been inactivated (pOU-FC2pasABC*) conferred plasmid stability to approximately the same level as the pTC-F14 *pasAB* genes (pOU-F14pasAB). This implied that *pasC* was responsible for the difference in stability conferred upon pOU82 by the two- and three-component *pas*, respectively. The role of PasC in the *pasABC* system is still unclear. If *pasC* were simply involved in improving the binding of the antidote to the toxin (i.e., more effective neutralization of toxin in cells that inherit the plasmid), one would not expect a decrease in stability when *pasC* was inactivated. In the absence of PasC, the toxin should be more effective in cells that failed to inherit the plasmid, and this could be expected to increase plasmid stability. The decrease in stability is more consistent with a role for PasC in facilitating the reaction between the PasB toxin and its cellular target. If this were true, an inactive PasC would result in a less effective toxin, which would allow more plasmid-minus cells to escape the effects of the TA module. Although the *pas* TA is related to the *E. coli relBE* family (11), the target of the *pas* TA has yet to be identified.

Using a plasmid replication inhibition system, we showed that the TA systems of both pTF-FC2 and pTC-F14 did not cause cell death upon plasmid loss. Previously, Smith and Rawlings (26) had reported that induction of *pasB* caused cell death, but we attribute this bactericidal effect to expression of the *pasB* gene from a heterologous *tac* promoter. We confirm in this study that autoregulation of the *pas* operon is essential in both systems for the balanced functioning of the TA locus. Both the pTC-F14 and pTF-FC2 antitoxins (when the *pasA* genes, alone, were cloned behind the *tac* promoter) and the toxins (when the *pasA* antitoxin genes were inactivated) appeared to inhibit *E. coli* host cells. The toxicity of the antitoxin was less severe in some *E. coli* strains (such as JM105 and XL1-Blue) but nevertheless restricted our ability to carry out experiments in which either PasA or PasB was to be expressed in the absence of each other or from an IPTG-regulated *tac* promoter. In spite of the toxicity of the individual products, when both the *pasA* and *pasB* genes of pTC-F14 or pTF-FC2 were present and autoregulated, the *pas* genes did not kill *E. coli* host cells. Examination of the cultures subjected to plasmid curing revealed a slight lag in growth that was consistent with bacteriostasis rather than cell death.

Because of the report that certain TA PSK systems do not kill host cells directly but are protein synthesis inhibitors that work through the *E. coli mazEF* chromosomal TA system (13), we tested the ability of the *pas* genes to stabilize pOU82-based plasmids in *mazEF* mutants. There was no difference in stability between *E. coli mazEF* mutant and WT strains using either the pTF-FC2 *pasABC* or the pTC-F14 *pasAB* genes. Similarly, the ability of the plasmid addiction systems to cause cell death was not affected by the *mazEF* status of the host cells.

The effectiveness of plasmid stability conferred by a TA system is correlated to the mode of action of the toxin, i.e., stasis versus death. For example, the *parD-pemK* locus is responsible for modest stability (approximately 10-fold) and acts by arresting cell division or growth (15). The *parDE* locus, on the other hand, confers a high degree of stability (approximately 500-fold) by causing cell death of plasmid-free cells. The fairly low level of plasmid stability conferred by the *pas* genes together with the observation that their function is not affected by the *mazEF* genes and that the *pas* genes do not appear to kill host cells, suggests that *pas* action is most likely the arrest of cell growth or division.

The interpretation of competition experiments between plasmids containing the two TA systems is challenging. The plasmid pTF-FC2 containing the *pasABC* system clearly displaced the plasmid pTC-F14 containing the *pasAB* system. This displacement was a function of the *pasABC* system, as when these genes were deleted, the stability of the *pasAB*-containing plasmid almost fully recovered. However, the *pasAB* genes of plasmid pTC-F14 were not able to displace pTF-FC2, even when its *pasABC* system was deleted. This raises the question of what the mechanism of *pas*-mediated plasmid displacement is and why it is unidirectional. One possibility is that the *pas* genes are involved in the regulation of plasmid replication. The *pas* genes are situated between the *repB* and *repAC* genes in a position similar to that of the *cac* genes of the IncQ plasmid RSF1010, and the *cac* genes have been shown to be involved in plasmid replication (16). An argument against *pas* involvement in replication is that when the *pasAB* genes of pTC-F14 are deleted or the *pasABC* genes of pTF-FC2 were spontaneously deleted, plasmid replication continued and no change in copy number was detected (26; this study, results not shown). Furthermore, the PasA- and PasB-like proteins of plasmid pAM10.6 are closely related to PasA and PasB of pTF-FC2 and pTC-F14, but pAM10.6 does not have an IncQ-like replicon and the *pasAB* genes of pAM10.6 do not appear to be part of the pAM10.6 replicon (20).

It is unlikely that the plasmids compete solely via the mechanism of PSK. This would require that the two replicons segregate away from one another at cell division (creating daughter cells lacking one or the other TA system and thus inducing growth inhibition or death). Since the replicons are compatible, this would be unlikely, and if it did occur, one would expect it to give rise to approximately equal numbers of daughter cells containing either pTC-F14 or pTF-FC2. Since we have shown that the non-self toxin-antidote proteins do not completely cross-neutralize one another or are equally effective at partial cross-neutralization, we would expect loss of either plasmid to invoke a PSK effect that would disadvantage or kill the host and, in doing so, eliminate the remaining plasmid. In effect, the two plasmids would be addicted to one another, and the majority of cells would be expected to carry both plasmids. We do not observe this. Instead, pTF-FC2 clearly out-competes pTC-F14, in spite of the observation that the latter seems to have the more virulent toxin.

Using *pasA-lacZ* reporter gene fusions, it was shown that the proteins of both *pas* operons could regulate each other, with the three-component *pasABC* system of pTF-FC2 being the stronger repressor of both, although this stronger repression was not attributable to the presence of *pasC*. This presents a possible mechanism for how pFC2-pasABC (pTF-FC2) outcompetes pF14-pasAB (Cm) (pTC-F14) when together in a cell: the *pas* promoter of pF14-pasAB (Cm) is twice as effectively repressed by the competitor PasA than it is by its own PasA. Conversely, the pFC2-pasABC *pas* promoter is repressed less by the competitor PasA than by its native PasA. Presumably, when both plasmid species are present in the

same host cell, pF14-pasAB (Cm) is disadvantaged through the action of PasA of pTF-FC2.

The evidence in support of PasA being responsible is that pFC2-pasAB*C (the pTF-FC2 derivative lacking a functional toxin gene) is able to out-compete pTC-F14, presumably through the action of its remaining PasA. Furthermore, pTC-F14 is unable to displace pTF-FC2 lacking its entire *pas* region (pFC2-ΔpasABC), suggesting that the survival of pFC2-ΔpasABC could be ascribed to its lack of the *pas* promoter-operator region, thus rendering it immune to any form of interference from the PasA of pTC-F14. It is possible that PasA serves multiple roles. We have shown that it is the primary regulator of the *pas* operon, causing strong repression of the promoter. Furthermore, when overexpressed, PasA can retard growth or possibly kill the bacterial host cell even in the absence of the PasB toxin. It acts as an antitoxin to the toxin. The role of PasAB(C) in plasmid biology is puzzling, especially as the whole *pas* of pTF-FC2 can be deleted without an apparent change in plasmid copy number, although there is a fairly modest but clearly detectable decrease in plasmid stability (26). It is possible that *pas* plays a role only when the copy number of the plasmid falls, whereas the upper limit for plasmid copy number is likely to be controlled by handcuffing in much the same way that it is for other iteron-containing replicons (7). In the same way that *relBE* (or *mazEF*) expression is linked to cellular stress (19), the primary role of the *pasAB*(*C*) operon may be to integrate plasmid replication into the overall metabolism of the host in which it resides. The *pas* could function under conditions in which a plasmid that was previously at a normal copy number falls, and hence, the overall expression of the *pas* operon decreases. By analogy to the *mazEF* system, failure to express the *pas* operon sufficiently would result in lower levels of PasA (since it has a shorter half-life) relative to PasB, and this would inhibit cell growth until the *pas* was again expressed at sufficiently high levels. This inhibition of cell growth would allow the plasmid copy number to catch up and would be expected to confer the relatively low levels of increased plasmid stability observed in the presence of *pas* without affecting the apparent plasmid copy number under optimal conditions.

Experiments are currently under way to determine whether readthrough from *pas* results in increased transcription of the downstream *repAC* genes, thereby ensuring that the plasmid is replicated when the copy number falls. This may not affect the maximum plasmid copy number that is likely to be set by iteron-mediated handcuffing.

ACKNOWLEDGMENTS

We thank Kenn Gerdes for the gift of plasmids pOU82 and pKG399, from which pKGCm was constructed, and Hanna Engelberg-Kulka for the *E. coli mazEF* mutants.

This work was funded by grants provided by BHP-Billiton Johannesburg Technology Centre, the University of Stellenbosch, the National Research Foundation (Pretoria, South Africa), and the Training of Human Resources for Industry Program (THRIP; Pretoria, South Africa).

REFERENCES

1. **Aizenman, E., H. Engelberg-Kulka, and G. Glaser.** 1996. An *Escherichia coli* chromosomal addiction module regulated by ppGpp: a model for programmed cell death. Proc. Natl. Acad. Sci. USA **93:**6059–6063.
2. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: A new generation of protein data base search programs. Nucleic Acids Res. **25:**3389–3402.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81:**6929–6933.
5. **Casadaban, M. J., J. Chou, and S. N. Cohen.** 1980. *In vitro* gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational initiation signals. J. Bacteriol. **142:**971–980.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifyable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
7. **Chattoraj, D. K.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. **37:**467–476.
8. **Gardner, M. N., S. M. Deane, and D. E. Rawlings.** 2001. Isolation of a new broad host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. **183:**3303–3309.
9. **Gardner, M. N.** 2003. Ph.D. thesis. University of Stellenbosch, Stellenbosch, South Africa.
10. **Gerdes, K., J. E. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
11. **Gerdes, K.** 2000. Toxin-antitoxin molecules may regulate synthesis of macromolecules during nutritional stress. J. Bacteriol. **182:**561–572.
12. **Grønlund, H., and K. Gerdes.** 1998. Toxin-antitoxin systems homologous with *relBE* of *Escherichia coli* plasmid P307 are ubiquitous in prokaryotes. J. Mol. Biol. **285:**1401–1405.
13. **Hazan, R., B. Sat, M. Reches, and H. Engelberg-Kulka.** 2001. Postsegregational killing mediated by the P1 phage "addiction module" *phd-doc* requires the *Escherichia coli* programmed cell death system *mazEF.* J. Bacteriol. **183:**2046–2050.
14. **Jensen, R. B., and K. Gerdes.** 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. **17:**205–210.
15. **Jensen, R. B., E. Grohmann, H. Schwab, R. Dìaz-Orejas, and K. Gerdes.** 1995. Comparison of *ccd* of *F*, *parDE* of RP4, and *parD* of R1 using a novel conditional replication control system of plasmid R1. Mol. Microbiol. **17:** 211–220.
16. **Maeser, S., P. Scholz, S. Otto, and E. Scherzinger.** 1990. Gene F of plasmid RSF1010 codes for a low molecular weight repressor protein that autoregulates expression of the *repAC* operon. Nucleic Acids Res. **18:**6215–6222.
17. **Magnuson, R., and M. B. Yarmolinsky.** 1998. Corepression of the P1 addiction operon by Phd and Doc. J. Bacteriol. **180:**6342–6351.
18. **Miller, J. H.** 1983. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
19. **Pedersen, K., S. K. Christensen, and K. Gerdes.** 2002. Rapid induction and reversal of a bacteriostatic condition by controlled expression of toxins and antitoxins. Mol. Microbiol. **45:**501–510.
20. **Peters, M., E. Jogi, I. Suitso, T. Punnisk, and A. Nurk.** 2001. Features of the replicon of plasmid pAM10.6 of *Pseudomonas fluorescens.* Plasmid **46:**25–36.
21. **Rawlings, D. E., I.-M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli.* J. Bacteriol. **158:**737–738.
22. **Rawlings, D. E.** 1999. Proteic toxin-antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2. FEMS Microbiol. Lett. **176:**269–277.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Sat, B., R. Hazan, T. Fisher, H. Khaner, G. Glaser, and H. Engelberg-Kulka.** 2001. Programmed cell death in *Escherichia coli*: some antibiotics can trigger *mazEF* lethality. J. Bacteriol. **183:**2041–2045.
25. **Smith, A. S. G.** 1997. Ph.D. thesis. University of Cape Town, Rondebosch, South Africa.
26. **Smith, A. S. G., and D. E. Rawlings.** 1997. The poison antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. **26:**961–970.
27. **Smith, A. S. G., and D. E. Rawlings.** 1998. The efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain and antidote degradation requires the Lon protease. J. Bacteriol. **180:**5458–5462.
28. **Smith, A. S. G., and D. E. Rawlings.** 1998. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (*pas*) is essential for plasmid stabilization. J. Bacteriol. **180:**5463–5465.
29. **Yanisch-Perron, C., J. Viera, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

Microbiology (2004), **150**, 1797–1808 DOI 10.1099/mic.0.26951-0

Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2

Murray N. Gardner and Douglas E. Rawlings

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Correspondence
Douglas E. Rawlings
der@sun.ac.za

Received 1 December 2003
Revised 9 February 2004
Accepted 11 February 2004

Two closely related but compatible plasmids of the IncQ-2α and IncQ-2β groups, pTF-FC2 and pTC-F14, were discovered in two acidiphilic chemolithotrophic bacteria. Cross-complementation and cross-regulation experiments by the replication proteins were carried out to discover what changes were necessary when the plasmids evolved to produce two incompatibility groups. The requirement of a pTC-F14 *oriV* for a RepC DNA-binding protein was plasmid specific, whereas the requirement for the RepA helicase and RepB primase was less specific and could be complemented by the IncQ-2α plasmid pTC-FC2, and the IncQ-1β plasmid pIE1108. None of the IncQ-1α plasmid replication proteins could complement the pTC-F14 *oriV*, and pTC-F14 and RSF1010 were incompatible. This incompatibility was associated with the RepC replication protein and was not due to iteron incompatibility. Replication of pTC-F14 took place from a 5·7 kb transcript that originated upstream of the *mobB* gene located within the region required for mobilization. A pTC-F14 *mobB–lacZ* fusion was regulated by the pTC-F14 *repB* gene product and was plasmid specific, as it was not regulated by the RepB proteins of pTF-FC2 or the IncQ-1α and IncQ-1β plasmids. Plasmid pTC-F14 appears to have evolved independently functioning iterons and a plasmid-specific RepC-binding protein; it also has a major replication transcript that is independently regulated from that of pTF-FC2. However, the RepA and RepB proteins have the ability to function with either replicon.

INTRODUCTION

Plasmids of the *Escherichia coli* incompatibility group Q (IncQ) are characterized by being capable of replication in a very broad host range of bacteria and by being efficiently mobilized by self-transmissible plasmids of the IncPα (RK2, RP4 and R68) and IncPβ (R751) groups (Derbyshire *et al.*, 1987; Frey & Bagdasarian, 1989). These two properties make IncQ-family plasmids highly promiscuous.

IncQ-type plasmid replicons contain three replication genes, *repA*, *repB* and *repC*, and an *oriV* region (Scholz *et al.*, 1989). In the case of the best-studied IncQ-1α plasmids, RSF1010 and R1162, the *oriV* region contains three and one half 20 bp iterons with 2 bp nucleotide spacers. The iterons exert incompatibility and serve as binding sites for the site-specific DNA-binding protein RepC (Persson & Nordström, 1986; Lin *et al.*, 1987; Haring & Scherzinger, 1989). Binding of RepC is essential for replication and is thought to introduce conformational changes leading to DNA unwinding in the adjacent A+T-rich region (Haring & Scherzinger, 1989; Kim & Meyer, 1991). This RepC-induced DNA melting serves as an entry site for RepA, a plasmid-specific helicase that unwinds the DNA in the flanking regions. One of these flanking regions contains a large inverted repeat that has two single-strand DNA initiation sites, *ssiA* and *ssiB* (Lin & Meyer, 1987; Haring & Scherzinger, 1989; Honda *et al.*, 1991). The *ssiA* and *ssiB* sites initiate priming of single-strand DNA synthesis by RepB primase on opposite strands in the leftward and rightward directions, respectively (Miao *et al.*, 1993).

We discovered two related IncQ-like plasmids in two chemolithotrophic bacteria isolated from mineral bio-oxidation processes in South Africa. Plasmid pTF-FC2 is a 12 184 bp plasmid isolated from the iron- and sulfur-oxidizing bacterium *Acidithiobacillus ferrooxidans* and plasmid pTC-F14 is a 14 159 bp plasmid from the sulfur-oxidizing bacterium *Acidithiobacillus caldus* (Rawlings *et al.*, 1984; Gardner *et al.*, 2001). Plasmids pTF-FC2 and pTC-F14 are closely related to each other and have high levels of amino acid sequence identity (72–81 %) in replication, toxin–antitoxin and two out of five mobilization proteins. In addition, the plasmids have *oriV* regions that contain 22 bp iterons with related nucleotide sequences (Gardner *et al.*, 2001). Although the two plasmids are very similar to each other, they contain origins of replication that are compatible (Gardner *et al.*, 2001). Given their close similarity, it is reasonable to assume that the two plasmids originated from a common ancestral plasmid and would have originally been incompatible. However, they have diverged sufficiently so that they are now able to coexist in the same host in the absence of selection.

The discovery of two closely related plasmids has enabled us to address some aspects of how IncQ-type plasmids have evolved to produce new compatible plasmids. Here we report on several aspects of the molecular interaction between the replicons of plasmids pTF-F14 (a member of the IncQ-2β group) and pTF-FC2 (an IncQ-2α plasmid). We have also included representatives of the IncQ-1α (RSF1010, R300B replicons) and IncQ-1β (pIE1108) groups in this plasmid-interaction study.

METHODS

Bacterial strains, bacteriophage, media and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. The *E. coli* strains were all grown at 37 °C in Luria–Bertani medium, and ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), kanamycin (30 μg ml^{-1}) and tetracycline (20 μg ml^{-1}) were added as required.

DNA techniques, sequencing and analysis. Plasmid preparation, restriction endonuclease digestions, gel electrophoresis, and cloning were carried out using standard methods (Sambrook *et al.*, 1989; Ausubel *et al.*, 1993). Where no suitable restriction sites were present, DNA fragments to be cloned for incompatibility and complementation assays were amplified by PCR. An initial denaturation step of 60 s at 94 °C was followed by 25 cycles of denaturation (30 s at 94 °C), a variable annealing step and a standard elongation step (90 s at 72 °C). Annealing temperatures were based on the mean primer annealing temperature, and extension times were altered as required (Table 1). PCR was performed in a PCR Sprint temperature cycling system (Hybaid) using the Expand High Fidelity PCR system DNA polymerase (Roche Molecular Biochemicals). The sequences of all constructs that required a PCR step were confirmed by DNA sequencing, using the dideoxy chain-termination method and an ABI PRISM 3100 genetic analyser.

Incompatibility assay. The ability of an incoming plasmid to displace a resident plasmid was used as the test for incompatibility. The incompatibility assay was performed as reported previously (Gardner *et al.*, 2001).

Complementation assay. Complementation of a plasmid *oriV* was detected by the transformation with a ColE1-*oriV*$_{incQ}$ test construct of an *E. coli* GW125a (*polA* mutant) strain containing various coresident plasmids. This was followed by selection for the presence of the *oriV*-containing construct by growth of the *E. coli* transformant on solid media containing an appropriate antibiotic.

Northern blotting. Total RNA was isolated by an adaptation of the protocol of Laing & Pretorius (1992). An overnight *E. coli* DH5α culture was diluted into pre-warmed Luria–Bertani broth, with antibiotic selection as required, and grown to an OD_{600} of 0·7–0·8 at 37 °C, with constant shaking. The cells were harvested and resuspended in 300 μl cold saline/Tris/EDTA (STE) buffer (Ausubel *et al.*, 1993). The suspension was added to 300 μl phenol (pH 6·7; Sigma), containing 0·3 g glass beads, and vortexed for 30 s before being placed on ice. SDS (4 μl of 10 % solution) was added and the solution incubated on ice for 15 min. Chloroform/isoamyl alcohol (24 : 1, v/v) was added and the solution centrifuged at high speed for 10 min. The aqueous phase was removed and extracted with 1 vol. phenol/chloroform/isoamyl alcohol (24 : 24 : 1, by vol.). To remove contaminating DNA, the precipitated nucleic acids were pelleted, resuspended in 200 μl DNase buffer (20 mM $MgCl_2$, 2 mM dithioerythritol) and incubated at 37 °C for 15 min in the presence of 5 units DNase I (RNase-free; Roche Molecular Biochemicals). Extracted RNA (30 μg) was separated in a 1 % (w/v) agarose/18 % (v/v) formaldehyde denaturing gel in 1× MOPS buffer (Ausubel *et al.*, 1993) and capillary-blotted onto a Hybond-N+ (AEC-Amersham) nylon membrane. The pTC-F14 replicon probes were labelled with [α-^{32}P]dATP (AEC-Amersham) using a Random Primed DNA labelling kit (Roche Molecular Biochemicals) and the probes were hybridized overnight to the RNA at 60 °C in hybridization buffer (7 % SDS, 1 % BSA, 1 mM EDTA, pH 8·0, 0·25 M Na_2HPO_4). After hybridization, the membrane was washed at 60 °C in 1× SSC (Ausubel *et al.*, 1993) containing 0·1 % SDS, and again in 0·1× SSC containing 0·1 % SDS. Bound probe was detected by autoradiography using MG-SR X-ray film (Konica).

RT-PCR. Total RNA was isolated using the SV Total RNA isolation system (Promega). The RNA was resuspended in 200 μl DNase buffer (18 mM Tris/HCl, pH 7·3, 9 mM $MnCl_2$, 0·9 mM NaCl) and incubated for 90 min at 28 °C in the presence of 100 units DNase I (RNase-free; Roche Molecular Biochemicals). The DNase treatment was stopped by the addition of 1 vol. phenol/chloroform/isoamyl alcohol (24 : 24 : 1, by vol.), and the RNA precipitated from the aqueous phase overnight at −20 °C by the addition of 0·1 vol. 5 M NaCl and 2 vols absolute ethanol. A two-step RT-PCR protocol was used for cDNA synthesis and cDNA product detection. The protocol of the manufacturer of the 1st Strand cDNA synthesis kit for RT-PCR (AMV; Roche Molecular Biochemicals) was used for the reverse-transcriptase reaction. The PCR was performed in a PCR Sprint temperature cycling system (Hybaid), using *Taq* DNA polymerase (Promega). For standard PCR reactions, a protocol of an initial denaturation of 60 s at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 54 °C and 90 s at 72 °C was used. A final extension step of 120 s at 72 °C, before cooling to 4 °C, completed the reaction. Extension times were altered as required for the primer pairs (Table 1), and 2 μl of the 20 μl (total volume) reverse-transcriptase reaction was used in each PCR reaction.

β-Galactosidase assays. The putative promoter regions of the pTC-F14 replicon region were PCR amplified using primer pairs LACF14MOBBF/LACF14MOBBR (*mobB* promoter), LACF14MOBAF/LACF14MOBAR (*mobA* promoter), LACF14REPBF/LACF14REPBR (*repB* promoter), primer5/primer6 (*pas* operon promoter), LACF14REPAF/LACF14REPAR (*repA* promoter) and LACF14REPCF/LACF14REPCR (*repC* promoter) (Table 1). A PCR Sprint temperature cycling system (Hybaid) and Expand High Fidelity PCR system DNA polymerase (Roche Molecular Biochemicals) were used to amplify the putative promoter regions. After an initial denaturation of 60 s at 94 °C, 25 cycles of 30 s at 94 °C, 30 s at 61 °C and 90 s at 72 °C were performed. A final extension step of 120 s at 72 °C, before cooling to 4 °C, completed the reaction. The PCR products were digested with *Eco*RI and *Bam*HI and cloned into pMC1403 (Table 1) to construct a promoter–*lacZ* reporter gene fusion. Since these recombinant plasmids were translational fusions requiring in-frame ligation of the promoter-associated ORF to the reporter gene (*lacZ*), all constructs were sequenced with primer LACZPRI (Table 1) to ensure the promoter fusions were correct. These constructs were transformed into *E. coli* CSH50I^{q}, and the β-galactosidase activity measured using the method of Miller (1972). Overnight cultures were diluted 1 : 100 into fresh pre-warmed Luria–Bertani medium containing the appropriate antibiotic selection and grown at 37 °C for 4 h. After incubation, OD_{600} was recorded, and the culture diluted 1 : 5 into Z-buffer (Miller, 1972). The exception was *E. coli* CSH50I^{q}(pMCF14PAS), which was initially diluted 1 : 500 into fresh medium with appropriate selection, grown for 3 h at 37 °C, and diluted 1 : 20 into Z-buffer. Following dilution in Z-buffer, the culture suspensions were vortexed briefly in the presence of toluene (1 %, v/v), and held at 37 °C for 45 min, before being placed at 28 °C for the remainder of the assay. The assay was started with the addition of ONPG to a final concentration of 0·67 mg ml^{-1}. After 30 min incubation [6 min for *E. coli*

Table 1. Strains, plasmids and primers used in this study

Abbreviations used in the Description column are as follows: Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; St, streptothricin; Tc, tetracycline. Descriptions of constructs into which a PCR-amplified fragment has been cloned include a reference to the position in base pairs (bp) on the GenBank published sequence (RSF1010, M28829; pTC-F14, AF325537) and the primers used. Restriction enzyme sites incorporated into primers are indicated in parentheses and underlined in the primer sequence.

Strain, plasmid or primer	Description	Reference or source
Strain		
E. coli CSH50I^{q}	*rspL* Δ(*lac–pro*) (F′ *traD36 proAB lacI*^{q} *lacZΔM15*)	Smith & Rawlings (1998)
E. coli DH5α	*ϕ*80 *lacZΔM15 endA1 recA1 gyrA96 thi*-1 *hsdR17* (r_k^-, m_k^+) *relA1 supE44 deoR* Δ(*lacZYA–argF*)*U169*	Promega
E. coli GW125a	*recA*, *polA* mutant of AB1157	Dorrington & Rawlings (1989)
Plasmid		
pTC-F14	Natural 14·2 kb plasmid from *Acidithiobacillus caldus* strain ‘f’	Gardner *et al.* (2001)
pTC-F14Cm	Cm^{r} (Cm^{r} gene cloned into pTC-F14)	Gardner *et al.* (2001)
pTF-FC2	Natural 12·2 kb plasmid from *At. ferrooxidans* FC2	Rawlings & Woods (1985)
pKE462	Tc^{r} As^{r}, R300B replicon	Dorrington & Rawlings (1989)
RSF1010K	Km^{r},1–1704 bp of RSF1010 replaced by Tn*903*	G. Ziegelin[1]
pIE1108	St^{r} Km^{r}, pIE1107 replicon with a set of non-essential IncQ iterons deleted	Tietze (1998)
pIE1108Cm	Cm^{r}, Region (St^{r}, Km^{r}) replaced with Cm^{r} gene	This study
pDER412	Cm^{r}, pTF-FC2 plasmid with pBR325 Cm^{r} gene	Rawlings *et al.* (1984)
pTV4164	Ap^{r}, pUC19 with $oriV_{pTF\text{-}FC2}$ fragment of 329 bp	Dorrington *et al.* (1991)
pTC-F101	Km^{r}, *Hin*dIII to *Sph*I pTC-F14 replicon fragment	Gardner *et al.* (2001)
pTC-F109	Ap^{r}, pGEM-T vector with PCR-amplified $oriV_{pTC\text{-}F14}$ (bp 7363–8100; SEQORI/ORIR)	Gardner *et al.* (2001)
pGEM-T®	Ap^{r}, T-tailed PCR product cloning vector	Promega
pKK223-3	Ap^{r}, P_{tac}, ColE1 replicon, expression vector	Pharmacia Biotech
pGL10	Km^{r}, RK2/RP4 replicon, cloning vector, pUC19 multiple cloning site with *E. coli lacZ* promoter	A. Toukdarian[2]
pMC1403	Ap^{r}, promoterless *lacZYA* operon, ColE1 replicon	Casadaban *et al.* (1983)
pUC19	Ap^{r}, *lacZ*′, ColE1 replicon, cloning vector	Yanisch-Perron *et al.* (1985)
pGEMRSFORIV	Ap^{r}, pGEM-T vector with PCR-amplified $oriV_{RSF1010}$ fragment (bp 2209–2772; INCQVFOR/INCQVREV)	This study
pGEMRSFORIVT	Ap^{r}, pGEM-T vector with PCR-amplified RSF1010 *oriV* to *oriT* fragment (bp 2209–3364; INCQVFOR/INCQTREV)	This study
pGEMRSFORIT	Ap^{r}, pGEM-T vector with PCR-amplified $oriT_{RSF1010}$ fragment (bp 2755–3364; INCQTFOR/INCQTREV)	This study
pGEMRSFREP	Ap^{r}, pGEM-T vector with PCR-amplified RSF1010 *mobA* to *repC* fragment (bp 3161–7725; INCQREPF/INCQREPR)	This study
pGEMRSFREP1	Ap^{r}, pGEM-T vector with PCR-amplified RSF1010 *mobA* to *cac* fragment (bp 3161–5926; INCQREPF/INCQCACR)	This study
pGEMRSFCAC	Ap^{r}, pGEM-T vector with PCR-amplified RSF1010 ORF E and *cac* fragment (bp 5271–5926; INCQCACF/INCQCACR)	This study
pGEMRSFREP2	Ap^{r}, pGEM-T vector with PCR-amplified RSF1010 ORF E to *repC* fragment (bp 5271–7725; INCQCACF/INCQREPR)	This study
pGEM-TCAC	Ap^{r}, pGEM-T vector with PCR-amplified pTC-F14 *repAC* fragment (bp 5786–7756; TACREPA/TACREPCE)	This study
pKK223-C	Ap^{r}, PCR-amplified pTC-F14 *repC* cloned behind the P_{tac} promoter (bp 6743–7756; TACREPC/TACREPCE)	This study
pKK223-AC	Ap^{r}, PCR-amplified pTC-F14 *repAC* cloned behind the P_{tac} promoter (bp 5786–7756; TACREPA/TACREPCE)	This study
pKK223-A	Ap^{r}, PCR-amplified pTC-F14 *repA* cloned behind the P_{tac} promoter (bp 5786–6727; TACREPA/TACREPAE)	This study
pGL10–C	Km^{r}, pGL10 containing PCR-amplified pTC-F14 *repC* cloned behind the P_{tac} promoter; acquired from pKK223-3	This study
pGL10–AC	Km^{r}, pGL10 containing PCR-amplified pTC-F14 *repAC* cloned behind the P_{tac} promoter; acquired from pKK223-3	This study
pGL10–A	Km^{r}, pGL10 containing PCR-amplified pTC-F14 *repA* cloned behind the P_{tac} promoter; acquired from pKK223-3	This study

Table 1. cont.

Strain, plasmid or primer	Description	Reference or source
pGL10–BA	Km^r, pGL10 containing PCR-amplified pTC-F14 *repBA* cloned behind the P_{tac} promoter; acquired from pKK223-3 (bp 4057–6727; TACREPB/TACREPAE)	This study
pGL10–B	Km^r, pGL10 containing PCR-amplified pTC-F14 *repB* cloned behind the P_{tac} promoter; acquired from pKK223-3 (bp 4057–5199; TACREPB/TACREPBE)	This study
pGL10–BAC	Km^r, pGL10 containing PCR-amplified pTC-F14 *repBAC* cloned behind the P_{tac} promoter; acquired from pKK223-3 (bp 4057–7756; TACREPB/TACREPCE)	This study
pTC-KmMO	Km^r, PCR-amplified pTC-F14 *repBAC* ligated to Km^r gene (bp 4057–7756; TACREPB/TACREPCE)	This study
pMCF14MOBB	Ap^r, PCR-amplified pTC-F14 *mobB* promoter region cloned into pMC1403 (bp 1859–2223; LACF14MOBBF/LACF14MOBBR)	This study
pMCF14MOBA	Ap^r, PCR-amplified pTC-F14 *mobA* promoter region cloned into pMC1403 (bp 2294–2689; LACF14MOBAF/LACF14MOBAR)	This study
pMCF14REPB	Ap^r, PCR-amplified pTC-F14 *repB* promoter region cloned into pMC1403 (bp 3716–4135; LACF14REPBF/LACF14REPBR)	This study
pMCF14PAS	Ap^r, PCR-amplified pTC-F14 *pas* promoter region cloned into pMC1403 (bp 4956–5263; PRIMER5/PRIMER6)	S. Deane[3]
pMCF14REPA	Ap^r, PCR-amplified pTC-F14 *repA* promoter region cloned into pMC1403 (bp 5481–5878; LACF14REPAF/LACF14REPAR)	This study
pMCF14REPC	Ap^r, PCR-amplified pTC-F14 *repC* promoter region cloned into pMC1403 (bp 6423–6823; LACF14REPCF/LACF14REPCR)	This study
pTC-F101ΔAB	Km^r, *Stu*I–*Xba*I deletion of pTC-F101 *pas* operon	S. Deane
pGL10–mob1	Km^r, PCR-amplified pTC-F14 *mobE* to *mobA*(*repB*) cloned into pGL10 (bp 70–5240)	L. van Zyl[3]
pGEM–mob5	Ap^r, PCR-amplified pTC-F14 *mobE* to *mobA* (truncated *repB*) cloned into pGEM-T (bp 70–4375)	L. van Zyl
pGL10–mob5	Km^r, *Eco*RI-*Pst*I cloning of pTC-F14 *mobE* to *mobA* from pGEM–mob5	This study
Primer		
TACREPB (*Eco*RI)	5′-TCAGGAATTCCCCGGAGCTTCAG-3′	This study
TACREPBE (*Pst*I)	5′-TTCTCTGCAGTCATGCCGCTGTG-3′	This study
TACREPA (*Eco*RI)	5′-TATTGAATTCCCCGGCAGCGCC-3′	Gardner *et al.* (2001)
TACREPAE (*Pst*I)	5′-TATTCTGCAGAGGGGGTGCGATAGC-3′	Gardner *et al.* (2001)
TACREPC (*Eco*RI)	5′-TCGCGAATTCGTGTGGCTATACCCAG-3′	This study
TACREPCE (*Hin*dIII)	5′-TCTGAAGCTTGCTGGCTTAGCGTG-3′	This study
SEQORI	5′-TATCGAGATGGCAGAGGTGCGAG-3′	Gardner *et al.* (2001)
ORIR (*Hin*dIII)	5′-TGTCAAGCTTGGCACTCTCCTG-3′	Gardner *et al.* (2001)
INCQVFOR (*Eco*RI)	5′-TATGAATTCGGCATGTTCCGCGTCC-3′	This study
INCQVREV (*Pst*I)	5′-TCACTGCAGGCTGAATGATCGACC-3′	This study
INCQTFOR (*Eco*RI)	5′-AGCGAATTCGGTCGATCATTCAGCC-3′	This study
INCQTREV (*Sal*I)	5′-TCTAGTCGACCTTCATCCATGTCGC-3′	This study
INCQREPF (*Xba*I)	5′-TGACTCTAGAAACTGGCCTAACGGC-3′	This study
INCQREPR (*Sal*I)	5′-TATAGTCGACCTATGGAGCTGTGCG-3′	This study
INCQCACF (*Xba*I)	5′-ACTGTCTAGAAGGTCATGGGTCTGC-3′	This study
INCQCACR (*Sal*I)	5′-TGCTGTCGACGCCTCCAGAATATTG-3′	This study
LACF14MOBBF (*Eco*RI)	5′-TGTCGAATTCCAGTGCAGTCCTTGC-3′	This study
LACF14MOBBR (*Bam*HI)	5′-AGGAGGATCCACGGTAAATGGCATC-3′	This study
LACF14MOBAF (*Eco*RI)	5′-TATCGAATTCAGGATGCGGACCTCG-3′	This study
LACF14MOBAR (*Bam*HI)	5′-TCTAGGATCCAGGGCTACCATTTCC-3′	This study
LACF14REPBF (*Eco*RI)	5′-TACAGAATTCCAGGCGACTAGGTGG-3′	This study
LACF14REPBR (*Bam*HI)	5′-TCAGGGATCCTGCTCCGATTTCATC-3′	This study
PRIMER5 (*Eco*RI)	5′-TACTGAATTCTACCAGTGTGCCCCATCG-3′	S. Deane
PRIMER6 (*Bam*HI)	5′-GTAGGGATCCACTTCGGTGGGTAATCGG-3′	S. Deane
LACF14REPAF (*Eco*RI)	5′-TATCGAATTCGACTTGGCTGCGCTG-3′	This study
LACF14REPAR (*Bam*HI)	5′-TTAAGGATCCAGTTCTGGCGGTTCG-3′	This study
LACF14REPCF (*Eco*RI)	5′-TAGCGAATTCCATCAGCCCTGATCG-3′	This study
LACF14REPCR (*Bam*HI)	5′-TATAGGATCCGGATCGTGTCTTGCG-3′	This study
LACZPRI	5′-CGCCAGCTGGCGAAAGGGGG-3′	This study
RTF14REPCR	5′-ATCTCGATACGGGTGTGTGG-3′	This study

Table 1. cont.

Strain, plasmid or primer	Description	Reference or source
RTF14REPCF	5′-AGAGCCGCTTGGTGTTGATG-3′	This study
RTF14REPAR	5′-ACCAACGGGCATTGTCGATC-3′	This study
RTF14REPAF	5′-CTATCTGGCCGGAGAAGATC-3′	This study
RTF14PASBR	5′-AGAAGTCTCCCAGCTTGGAG-3′	This study
RTF14PASAF	5′-AAGCGATCCTTGAGCACCTG-3′	This study
RTF14REPBJPASF	5′-TGACCAGTCGGCTATTGAGG-3′	This study
RTF14REPBR	5′-GATGACCGCCTGATAGTTGC-3′	This study
RTF14MOBAJREPBF	5′-TGACCGGGAACGACGAATAC-3′	This study
RTF14MOBAR	5′-TGGCGTCGCTTGTTTGGTTC-3′	This study
RTF14MOBBJMOBAF	5′-CTTTGAAAGCCGCCATCGAG-3′	This study
RTF14MOBBR	5′-CTCGATGGCGGCTTTCAAAG-3′	This study
RTF14MOBBF	5′-TACCGTGCAAGGACTGGAAC-3′	This study
RTF14MOBCJMOBB	5′-TGATGACATCGGCTTTCCCG-3′	This study

1, Max Planck Institute for Molecular Genetics, Berlin; 2, University of California, San Diego; 3, Stellenbosch University.

CSH50I^{q}(pMCF14PAS)] at 28 °C, the reaction was stopped with the addition of 0·42 vol. 1 M Na_2CO_3. The reaction was then centrifuged at high speed for 5 min to remove the cells, and the supernatant absorbance was measured at both 420 nm and 550 nm. The β-galactosidase activity was calculated according to the equation of Miller (1972).

RESULTS

Replication of the IncQ and IncQ-like plasmids is *polA* independent, whereas the replication of cloning vectors based on the ColE1 plasmid replicon is *polA* dependent. We had previously shown that when the *oriV* regions alone of either pTF-F14 (pTCF109) or pTF-FC2 (pTV4164) were cloned into a ColE1-based plasmid, they could not replicate in the *E. coli polA* mutant (GW125a) unless there was a coresident plasmid containing the complete replicon of the parent plasmid (Gardner *et al.*, 2001). The ability of the *oriV*-containing clones (pTCF109 and pTV4164) to replicate in the *polA* mutant was plasmid specific, i.e. a coresident plasmid containing the replicon of pTF-FC2 could

Table 2. Complementation of pTC-F14 replication proteins by IncQ and IncQ-like plasmids

The *oriV*–ColE1 construct was transformed into *E. coli* GW125a containing the coresident plasmids indicated. Results are shown as growth (+) or no growth (−) on medium selecting only for the incoming *oriV*–ColE1 construct. Plasmid isolations followed by restriction enzyme analysis to generate diagnostic banding patterns and restreaking of transformants on selective medium was used to confirm strains (not shown).

oriV–ColE1 construct transformed	Coresident pTC-F14 deletion construct	Coresident IncQ or IncQ-like plasmids				
		None	pTC-F14 (pTC-F101)	pTF-FC2 (pDER412)	pIE1108† (pIE1108Cm)	R300B (pKE462)
pTV4164	None	−	−	+	−	−
pTC-F109	None	−	+	−	−	−
pTC-F109	pGL10–BAC	+		+	+	±‡
pTC-F109	pGL10–BA	−		−	−	−
pTC-F109	pGL10–B	−		−	−	−
pTC-F109	pGL10–AC	−		+	+	−
pTC-F109	pGL10–A	−		−	−	−
pTC-F109	pGL10–C	−		+	+	−
pTC-F109	pGL10*	−		−	−	−

*Compatible PolA-independent vector.

†Transformants required 36 h of growth at 37 °C for visible colonies on solid medium, but restreaking of transformants required only overnight growth at 37 °C.

‡Few slow-growing colonies appeared after 48 h. Growth inhibition was demonstrated to be due to incompatibility between pKE462 and pGL10–BAC when selection for both was removed (not shown). The *pasAB* genes are situated between *repB* and *repA* (Fig. 2) and are present in constructs pGL10–BAC and pGL10–BA, but are absent from other pGL10 constructs.

not complement a plasmid containing the pTC-F14 *oriV* and vice versa. Similarly, the replication proteins of the IncQ-1α plasmid pKE462 (R300B replicon) and the IncQ-1β plasmid pIE1108 were unable to support the replication of the pTC-F14 *oriV* (pTF-F109) in the *E. coli* GW125a *polA* mutant (Gardner *et al.*, 2001). As these experiments had shown that the combination of all three replication proteins could not complement a heterologous pTF-F14 or pTC-FC2 *oriV*-containing construct, we wished to investigate whether all three replication proteins, RepA, RepB and RepC, were plasmid specific.

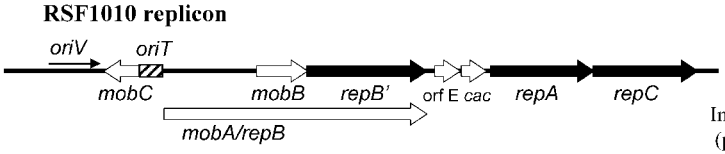
Ability of individual IncQ-plasmid replication proteins to support replication from the pTC-F14 *oriV*

The ability of pTC-F109 (ColE1-$oriV_{pTC\text{-}F14}$) to replicate in *E. coli* GW125a in the presence of the closely related pTF-FC2 as well as the less related pIE1108 and R300B replicons was retested, except that, in addition, one or more of the replication proteins of pTC-F14 was supplied *in trans* (Table 2, rows 3–9). To provide the pTC-F14 replication proteins *in trans*, fragments of pTC-F14 containing various replication genes were amplified by PCR and cloned in plasmid pGL10 (RK2/RP4 replicon vector). When pTC-F109 (ColE1-$oriV_{pTC\text{-}F14}$) was transformed into these heteroplasmid-containing recipients, *E. coli* GW125a(pDER412; pTF-FC2 replicon) and *E. coli* GW125a(pIE1108) would permit replication of the pTC-F14 *oriV* only when pTC-F14 RepC was supplied *in trans* (Table 2). This indicated that the RepC binding-protein was plasmid specific, but that the RepA and RepB of pTF-FC2 and pIE1108 were able to substitute for the equivalent replication proteins of pTC-F14.

The replication proteins of the IncQ (RSF1010/R300B) replicon were also unable to complement the replication proteins of pTC-F14. Furthermore, in the positive control, where all of the pTC-F14 replicon proteins were supplied *in trans* (pGL10–BAC), the presence of a coresident R300B plasmid resulted in a few slow-growing transformants. This suggested that replication interference between the pTC-F14 *oriV* (pTC-F109) and R300B (pKE462) had occurred (Table 2). To further investigate this interference, an *E. coli* GW125a(pKE462 + pGL10–BAC + pTC-F109) transformant was streaked onto three solid medium plates containing antibiotic selection for one of the resident plasmids. Individual colonies were restreaked twice, before being tested for the presence of the other two plasmids by plating on solid media containing the appropriate antibiotics. When pKE462 (R300B replicon) was selected, displacement of pTC-F109 (ColE1-$oriV_{pTC\text{-}F14}$) was demonstrated, but not pGL10–BAC. When pTC-F109 was selected (pGL10–BAC selected automatically, as pTC-F109 was RepBAC dependent), very weak growth was observed when testing for the presence of pKE462. This suggested that pKE462 interfered with pTC-F109 replication and that pTC-F109 or the pTC-F14 replication proteins supplied *in trans* by pGL10–BAC interfered with pKE462 replication.

Identifying the IncQ plasmid locus expressing incompatibility to pTC-F14

We previously reported that pTC-F14 was incompatible with RSF1010 (Gardner *et al.*, 2001). To determine which region of RSF1010 expressed incompatibility towards the pTC-F14 replicon (pTC-F101), a number of subclones were constructed from RSF1010 (Fig. 1). Surprisingly, plasmid incompatibility was not associated with the iteron-containing *oriV* region of RSF1010, but rather with construct pGEMRSFREP2, which contained a 2·4 kb fragment incorporating the region spanning OrfE to *repC* (Fig. 1). Since pGEMRSFCAC, containing OrfE and the *cac* gene, including the region encoding the 75 bp anti-sense



Incoming construct (plasmid selected)	% Resident (unselected) pTC-F101 plasmids remaining
pGEMRSFORIV	100
pGEMRSFORIVT	100
pGEMRSFORIT	100
pGEMRSFREP	0
pGEMRSFREP1	100
pGEMRSFCAC	100
pGEMRSFREP2	0

Fig. 1. IncQ-1α plasmid (RSF1010K) subclones used to challenge a resident pTC-F14 replicon (pTC-F101) for the identification of the IncQ-1α plasmid locus expressing incompatibility to pTC-F14. The *cac* gene is a regulator of *repA* and *repC*.

ctRNA species (Kim & Meyer, 1986), did not exert incompatibility, this suggested that the incompatibility determinant could be localized to the RSF1010 *repAC* operon (Fig. 1). Furthermore, it was found that pTC-F109 (ColE1-$oriV_{pTC\text{-}F14}$) was compatible with a resident RSF1010 replicon (RSF1010K), whereas a fragment containing the pTC-F14 *repAC* operon cloned into the ColE1-vector pGEM-T (pGEM-TCAC) displaced a resident RSF1010 replicon (data not shown). As the *repA*, *repC* and *repAC* genes of pTC-F14 had been cloned behind an IPTG-inducible *tac* promoter in vector pKK223-3, we investigated whether the overexpression of the individual *repA* and *repC* gene products affected incompatibility with RSF1010. Plasmid pKK223-3, containing either *repAC* or *repC* cloned behind a *tac* promoter, displaced approximately 90 % of coresident RSF1010K plasmids, and the level of displacement was not affected by the presence of 1 mM IPTG. In contrast, plasmid pKK223-3 containing the pTC-F14 *repA* gene was fully compatible with RSF1010K. These results suggested that the product of the *repC* gene interacts with the *oriV* region of the competing plasmid. Overexpression of the pTC-F14 *repA* and *repC* did not displace pDER412 (pTF-FC2 replicon), which suggests that only certain combinations of RepC proteins and *oriV*s are incompatible. Incompatibility could be through recognition and binding of the RepC proteins to the competing *oriV* region and subsequent inhibition of replication as a consequence of this binding. Alternatively, expression of *repC* could be affecting the regulation of the competing plasmid's *repAC* operon.

Numbers and sizes of pTC-F14 replicon transcripts

No studies on the regulation of the replication of either pTF-F14 or pTC-FC2 have been reported. We wished to investigate how many different-sized transcripts are made from the pTC-F14 replicon and whether the synthesis of these transcripts is cross-regulated by pTF-FC2 or RSF1010. Experiments to measure the size of the pTC-F14 transcripts were carried out using the Northern blot technique. Total RNA from *E. coli* DH5α(pTC-F14Cm) was isolated and probed with DNA fragments representing the pTC-F14 *repC*, *repA*, *repB*, *repAC*, *repBA*, *mobA–repB* and *mobB* gene fragments. Several signals were detected when *E. coli* DH5α(pTC-F14Cm) RNA was hybridized to the above DNA probes. A signal for a high-molecular-mass mRNA species of 5·1–5·7 kb, two smaller signals of approximately 2·4 kb and 1·6 kb, as well as a smear in the region below 1 kb were detected for each of the DNA probes tested (Fig. 2). The 5·1–5·7 kb hybridization signal is approximately the size predicted (~5·7 kb) from a large polycistronic mRNA species that was initiated upstream of *mobB* (in the $oriT_{pTC\text{-}F14}$ region) and terminated downstream of *repC* (Fig. 2).

Signals in the approximate size range of 2·4 kb and 1·6 kb, and a smear below 1 kb of varying intensity, were also detected when *E. coli* DH5α(pTC-F14Cm) RNA was hybridized to any of the above DNA probes (Fig. 2). These signals were plasmid specific, as they were absent from all pTC-F14-free control blots; however, the origin of these signals was difficult to interpret. The signals were of similar size to the predicted sizes of the *E. coli* 23S, 16S and 5S rRNA species. Therefore, the ~2·4 kb, ~1·6 kb and smaller smear signals could have been due to small intact (or degradation) products of plasmid mRNA that had been entrapped by the substantial quantity of *E. coli* rRNA present in this part of the blot. Because of these signals, it was not possible to detect whether smaller transcripts from the replication region were produced.

RT-PCR analysis of the pTC-F14 replicon

RT-PCR was used to confirm the 5·1–5·7 kb transcript detected by Northern blot analysis. The RT-PCR experimental design was such that by using the primer combinations shown in Fig. 2, the PCR products obtained would confirm which gene junctions were transcriptionally linked, allowing for the identification of polycistronic transcripts. If a large polycistronic mRNA encoding the entire region from pTC-F14 *mobB* to *repC* was indeed transcribed, this method would not allow one to detect whether additional smaller mRNA transcripts were also present.

The RT-PCR reactions (Fig. 2) were each performed on three RNA extracts to confirm the result obtained. To detect DNA contamination in the RNA extracts, PCR reactions were performed with each primer pair, using a threefold higher concentration of the *E. coli* DH5α (pTC-F14Cm) total RNA extract than that used in each RT-PCR reaction, but without AMV reverse transcriptase. No amplification products were detected. Occasionally, following PCR of the cDNA, a second, smaller PCR product was observed (e.g. Fig. 2, lane 25), but as this did not correspond to the product size expected (Fig. 2, middle lane of each set), it was deemed to be the result of mispriming.

The RT-PCR analysis (Fig. 2) confirmed the result from the Northern blot that a large transcript, which extends beyond the *repC* gene, is initiated upstream of the pTC-F14 *mobB* gene. As expected, the RT-PCR reaction did not yield a reaction product when using primers to the divergent *mobC* (RTF14MOBCJMOBB, 1795 forward) and *mobB* genes (RTF14MOBBR, 2499 reverse) (Fig. 2, lane 1 [i]). A putative promoter, 202 bp upstream of the *mobB* start codon, was identified that differs by one nucleotide from the *E. coli* σ^{70} consensus sequence in both its −35 region (TTGACT) and −10 region (TACAAT), and with a spacer of N_{16} (Harley & Reynolds, 1987). Should a transcript be initiated from this promoter that terminates downstream of *repC*, a transcript of approximately 5·7 kb is predicted.

Regulation of putative promoter regions by reporter-gene studies

Although evidence for a 5·7 kb polycistronic *mobB–repC* mRNA transcript is strong, evidence for the existence of

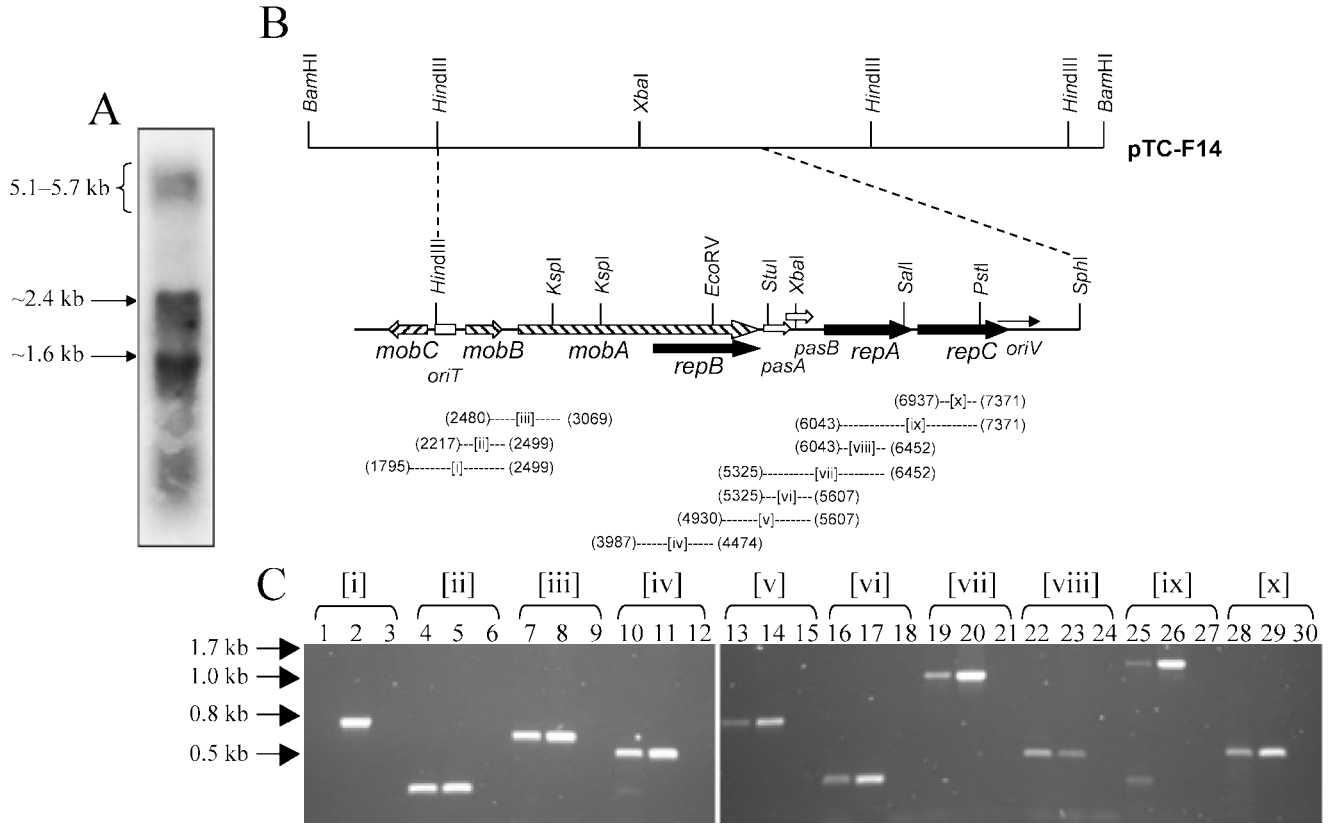


Fig. 2. Northern blot and RT-PCR analysis of the pTC-F14 replicon. (A) Example of Northern blot, showing approximate sizes of transcripts detected with a [α-^{32}P]dATP-labelled replicon probe (see text). (B) Primers used in RT-PCR and their location. Bracketed numbers represent the primers and their location (base pair position) relative to the pTC-F14 DNA sequence (GenBank accession no. AF325537). The reverse primers used in the RT reaction were as follows: (2499) RTF14MOBBR, (3069) RTF14MOBAR, (4474) RTF14REPBR, (5607) RTF14PASBR, (6452) RTF14REPAR and (7371) RTF14REPCR. Roman numerals in square brackets refer to the primer pairs used in the PCR reactions following the RT reaction. (C) RT-PCR reaction products on ethidium-bromide-stained agarose gels. The forward primers used in the PCR reaction were as follows: (1795) RTF14MOBCJMOBB, (2217) RTF14MOBBF, (2480) RTF14MOBBJMOBAF, (3987) RTF14MOBAJREPBF, (4930) RTF14REPBJPASF, (5325) RTF14PASAF, (6043) RTF14REPAF, and (6937) RTF14REPCF. The RT-PCR reaction products shown are grouped in sets of three, with the same primer pair used in each reaction of the set. Lanes 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28 are RT-PCR reactions performed on extracted *E. coli*(pTC-F14Cm) total RNA. Lanes 2, 5, 8, 11, 14, 17, 20, 23, 26 and 29 are PCR reactions performed on purified pTC-F14Cm DNA as a PCR control. Lanes 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 are exactly the same RT-PCR reactions as those performed in the first lane of each set, except that AMV reverse-transcriptase was not added to the reaction mix.

smaller constructs within this region was unclear. We therefore decided to create *lacZ*-translation reporter gene fusions to all ORFs within the *mobB–repC* region, with the exception of the gene for the PasB toxin. Reporter fusions also permitted regulation studies of the genes of this region. To allow sufficient DNA fragment length to accommodate putative promoter regions, the following PCR product size for each upstream gene region was cloned into the pMC1403 vector: 364 bp (*mobB*), 395 bp (*mobA*), 419 bp (*repB*), 307 bp (*pasA*), 397 bp (*repA*) and 400 bp (*repC*). To ensure that no host-cell background β-galactosidase activity interfered with the assays, and that any P_{tac}-controlled genes added *in trans* were repressed in the absence of IPTG, *E. coli* CSH50I^{q} was used as the host cell, as this strain contains *lacI*^{q} on an F′ plasmid (Table 2). The β-galactosidase activity for each putative promoter fusion and how this activity was affected when the parental pTC-F14 (pTC-F14Cm) plasmid and the related IncQ-type plasmids RSF1010 (RSF1010K) and pTF-FC2 (pDER412) were placed *in trans* is shown in Fig. 3.

The strongest β-galactosidase activity measured for the pTC-F14 replicon was for the plasmid addiction system (*pas*) gene translational fusion pMCF14PAS (Fig. 3). The β-galactosidase activity of 14426±2943 units obtained for *E. coli* CSH50I^{q}(pMCF14PAS) was very much greater than the substantially lower β-galactosidase activities measured for the other pTC-F14 promoter–*lacZ* fusions. The

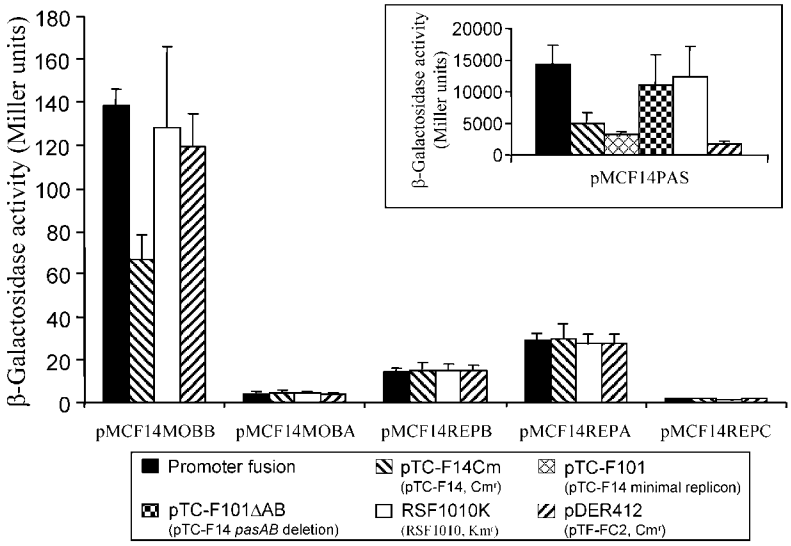


Fig. 3. Cross-regulation of putative pTC-F14 promoter regions by related IncQ plasmids present *in trans*, as determined by β-galactosidase activity. The plasmid present *in trans* to the promoter–reporter gene construct (given below each set of assays) is indicated by the key. The β-galactosidase activity for each construct was calculated from the mean of three different assays, and within each assay the β-galactosidase activity was measured from three samples Vertical error bars represent the SD of three different assays.

mobB promoter region (pMCF14MOBB) gave the second highest activity of 138 ± 8 units (Fig. 3). The negligible β-galactosidase activities (less than 5 units) measured for the putative promoter regions of pTC-F14 *mobA* and *repC* discounted these regions as containing promoter sequences active in *E. coli*, while *repB* and *repA* promoter–*lacZ* constructs had β-galactosidase activities of 14 ± 2 units and 28 ± 2 units, respectively (Fig. 3). When pTC-F14 and related IncQ-family plasmids were placed *in trans* with the *repB* and *repA* promoter–*lacZ* fusions, β-galactosidase activities were unaffected (Fig. 3), suggesting that the low level of activity was spurious.

Repression of the pTC-F14 *mobB* and *pas* operon putative promoters by pTC-F14Cm was evident from the reduction in β-galactosidase activity when this plasmid was present *in trans* (Fig. 3). The β-galactosidase activities of the pTC-F14 *mobB* (pMCF14MOBB) and *pas* operon (pMCF14PAS) promoter–*lacZ* constructs were reduced by 52 % and 65 %, respectively, when pTC-F14Cm was present *in trans* (Fig. 3). The return to 77 % of *E. coli* CSH50I^{q}(pMCF14PAS) β-galactosidase activity when pTC-F101ΔAB (*pasAB* deletion) was present *in trans* suggested that the *pas* operon putative promoter is subject to regulation by products of the *pas* operon (Fig. 3). The putative promoter region of the *pas* operon also appears to be the only promoter region which responds to cross-regulation by a related IncQ-like plasmid, as the β-galactosidase activity of the pTC-F14 *pas* operon promoter–*lacZ* fusion (pMCF14PAS) was reduced by 88 % when pTF-FC2 (pDER412) was present *in trans* (Fig. 3). Regulation of the pTC-F14 *pas* operon promoter and repression of the *pas* operon promoter by pTF-FC2 are being investigated.

The repressor of the pTC-F14 *mobB–lacZ* fusion was localized to the region of the pTC-F14 replicon upstream of the *pas* operon (Fig. 4). This was determined by β-galactosidase assays of *E. coli* CSH50I^{q}(pMCF14MOBB) into which constructs containing various regions of the pTC-F14 replicon had been transformed. Placement *in trans* of a pTC-F14 replicon containing a deletion of the *pasAB* genes (pTC-F101ΔAB) did not relieve the repression of the *mobB* promoter (41 ± 1 units; not shown), while pGL10–AC (Table 1) did not repress β-galactosidase activity (139 ± 13 units; not shown). This indicated that the repressor of this promoter was located upstream of the pTC-F14 *pas* operon. The regulation of the *mobB* promoter–*lacZ* fusion was plasmid specific, as neither RSF1010 nor pDER412 reduced expression of the fusion construct (Fig. 3). The 73 % reduction of β-galactosidase activity for the assay with pTC-F14Cm placed *in trans* (Fig. 4), compared with the 52 % reduction shown in Fig. 3, is thought to be due to variable stability of the *mobB* promoter fusion construct when pTC-F14Cm was present *in trans*. No other plasmid when placed *in trans* to the

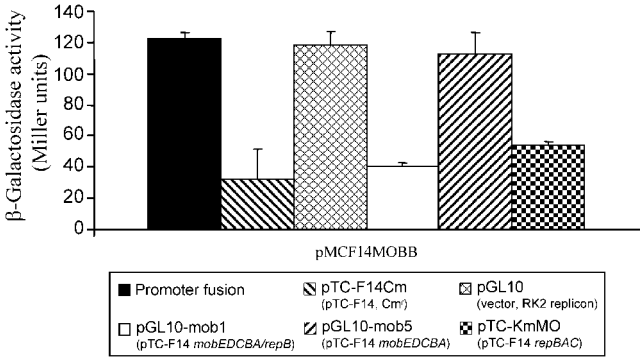


Fig. 4. Regulation of the pTC-F14 *mobB* promoter region, as determined by β-galactosidase assays. The β-galactosidase activity for each construct was calculated from the mean of three different assays, and within each assay the β-galactosidase activity was measured from three samples. Vertical error bars represent the SD of three different assays.

mobB promoter fusion construct caused promoter–reporter gene-construct instability.

The pGL10–mob1 construct, which contains the pTC-F14 mobilization region from *mobE* to *repB*, encodes a fully functional MobA–RepB fusion protein. When pGL10–mob1 was transformed into *E. coli* CSH50I^{q} (pMCF14MOBB), β-galactosidase activity was reduced to 33 % of the activity measured for *E. coli* CSH50I^{q} (pMCF14MOBB) (Fig. 4). To investigate which of the pGL10–mob1 proteins was the repressor, the pGL10–mob5 construct, which encodes a truncated MobA–RepB protein (primase domain removed), was transformed into *E. coli* CSH50I^{q}(pMCF14MOBB). With pGL10–mob5 *in trans*, the β-galactosidase activity of the *mobB* promoter–*lacZ* construct was 92 % of the activity measured for *E. coli* CSH50I^{q}(pMCF14MOBB), suggesting that the RepB protein was the repressor of the *mobB* promoter (Fig. 4).

To confirm that the RepB primase portion of pTC-F14 could repress the *mobB* promoter, the construct pTC–KmMO was transformed into *E. coli* CSH50I^{q}(pMCF14MOBB). The relaxase domain of the MobA–RepB fusion protein had been removed in the construction of pTC–KmMO by ligation of PCR-amplified pTC-F14 *repBAC*+*oriV* to a kanamycin cassette. With pTC–KmMO present *in trans*, the β-galactosidase activity was reduced to 43 % of that of *E. coli* CSH50I^{q}(pMCF14MOBB) (Fig. 4). This allowed for the identification of RepB (the primase domain of the MobA–RepB fusion protein) as the repressor of the *mobB* promoter. Regulation of the transcript beginning at the *mobB* gene was plasmid specific, as only the RepB of pTF-F14 affected expression of *mobB–lacZ*.

DISCUSSION

We are interested in the question of how two closely related replicons, such as those of the IncQ-2α and IncQ-2β plasmids pTF-FC2 and pTC-F14, which must have originated from a common ancestral plasmid, have evolved so as to become compatible. A model for the diversification of plasmid incompatibility has been proposed by Sýkora (1992) and could explain how the two replicons of pTF-FC2 and pTC-F14 became compatible. This model assumes that an ancestral IncQ plasmid dimer was formed either through recombination of plasmid progeny monomers or as a result of defective replication termination (Snyder & Champness, 2003). Pressure for at least one of the dimer replicons to acquire mutations leading to a sufficiently different replicon to the original replicon could be provided by the dimer being challenged by an identical plasmid attempting to displace the plasmid dimer from the host. Sufficient mutations would accumulate to allow for an unrelated but functional replicon to evolve, so that the second replicon of the dimer would be compatible with the ancestral plasmid. This would ensure maintenance of the dimer in the presence of the ancestral plasmid. Resolution of the plasmid dimer after mutation of one of the replicons to an unrelated, but functional, replicon would lead to two compatible sister plasmids, such as pTF-FC2 and pTC-F14. Irrespective of whether compatible plasmids can arise through the mechanism described above or as the consequence of the independent evolution of a new replicon by gradual accumulation of mutations, the driving force for the acquisition of mutations that lead to new IncQ replicon compatibility is the same, that is, the attempted displacement by a competitive plasmid.

Irrespective of the model chosen, sufficient concomitant evolution of an IncQ plasmid iteron sequence and its RepC protein would be required to produce compatible replicons. Evidence for this part of pTC-F14 and pTF-FC2 speciation has been obtained, as the two *oriV* regions are compatible (Gardner *et al.*, 2001), and in this work we showed that replication from the pTC-F14 *oriV* was RepC-specific. That is, neither the pTF-FC2 RepC protein nor any other IncQ-type plasmid which was RepC tested could substitute for the requirement of the pTC-F14 *oriV* for its own RepC. However, the RepA and RepB proteins were less plasmid specific, as the RepA and RepB proteins of pTF-FC2 and pIE1108 could substitute for the pTC-F14 proteins provided that the pTC-F14 RepC was present. This suggests that the sites in the *oriV* region to which RepA and RepB bind are less plasmid specific. As all IncQ plasmids have at least three identical 20–22 bp iterons, an as yet unsolved mystery is how mutations in one iteron are spread to all functional iteron copies. In some cases, such as pTC-F14 and pIE1130 (Smalla *et al.*, 2000), extra copies of iterons with non-identical sequences exist, but, where tested, mutated iterons have been found to be non-functional (Miao *et al.*, 1995).

It is interesting that the replicons of the closely related plasmids pTC-F14 and pTF-FC2 have become compatible, whereas the more dissimilar plasmids pTC-F14 and RSF1010 are incompatible. One can speculate that this is because pTC-F14 and pTF-FC2 are promiscuous plasmids that were isolated from acidiphilic chemolithotrophic bacteria that share the same habitat and the plasmids may encounter each other. In contrast, RSF1010 (and other IncQ-1α plasmids) are typically found in neutrophilic heterotrophic bacteria; pTF-F14 and RSF1010 are therefore unlikely to encounter each other and so have not evolved to accommodate each other. Incompatibility appears to be associated with the RepC proteins rather than with the iterons, and it is only certain combinations of RepC proteins and iterons that are incompatible. The most likely explanation is that RepC-associated incompatibility is due to non-productive binding of the heterologous RepC to the iterons of an incompatible plasmid, and that this reduces the ability of the homologous RepC to initiate replication. The replication interference was reciprocal, as RSF1010 could inhibit the replication of pTC-F14 and vice versa. No purified RepC protein was available, and as this was a fairly minor aspect of this work, DNA-binding studies were not carried out. Incompatibility

due to *repAC* is similar to that reported by Tietze (1998) for the plasmid pairs p95L28 and pDER412. The plasmid p95L28 is a ColE1-based vector (pUCBM20), into which the *oriVb* and the *repAC* operon of pIE1107 were cloned. Cloned on its own into this vector, $oriVb_{pIE1107}$ was compatible with pDER412 (pTF-FC2 replicon), but when the pIE1107 *repAC* operon was present (p95L28), the construct was incompatible with the pTF-FC2 replicon (Tietze, 1998).

An additional possibility for replication interference by two related plasmids is that the proteins involved in the control of transcription of the replication genes cross-regulate each other, and hence interfere with the replication of the heterologous plasmid. No regulation studies have been carried out on the expression of replication genes of the IncQ-2 group plasmids, and these plasmids lack the *cac* regulator (control of *repA* and *repC*), shown to be involved in RSF1010 replication (Rawlings & Tietze, 2001). Although the number of different-sized transcripts involved in the expression of the *rep* genes of pTC-F14 is uncertain, evidence from Northern hybridization and RT-PCR suggested that a large ~5·7 kb mRNA transcript is synthesized. Studies of reporter gene fusions to all of the ORFs in the region of the replicon suggested that the *mobB* and, by implication, the downstream *mobA–repB* genes are expressed at low levels relative to the *pasAB* genes. Futhermore, evidence was obtained that the pTC-F14 RepB gene product was responsible for repression of the transcript that begins at the *mobB* gene. As neither pTF-FC2 nor RSF1010, when provided *in trans*, reduced expression of the *mobB–lacZ* reporter gene, this indicated that regulation of the large pTC-F14 replication transcript was RepB specific.

The evidence obtained in this work is therefore consistent with a model in which pTC-F14 and pTF-FC2 have evolved such that the *oriV* of each plasmid is recognized only by its own RepC. However, each RepC protein is able to induce changes in the *oriV* that allows the less specific heterologous RepA helicase and RepB single-strand primase proteins to function. Besides being a primase, the RepB also appears to be a regulator of a large replication transcript. RepB, therefore, presumably has single-strand DNA-binding activity required for its primase function that is not plasmid specific and double-strand DNA-binding repressor activity that has mutated to become plasmid specific. This plasmid specificity would appear to be a requisite for pTC-F14 and pTF-FC2 to be compatible. Although some of the details of this model are speculative, it nevertheless provides a basis for future work on plasmid evolution.

Besides the large transcript, the high levels of *pasA–lacZ* expression suggested that a second transcript within the replication region is produced. A putative promoter region has been identified upstream of *pasA* that has one nucleotide difference in its −35 region (TTCACA) and a two-nucleotide difference in its −10 region (TATATC) from a consensus *E. coli* σ^{70} promoter sequence plus a consensus N_{17} spacer region (Harley & Reynolds, 1987). This transcript was not unequivocally detectable by Northern blot analysis, as the predicted size of the product fell within the region of the blot where the rRNA species were present. Internal short transcripts were also not detectable using RT-PCR, since a long transcript would serve as template for shorter transcripts. Unlike the pTC-F14 *mobB–lacZ* fusion, the *pasA–lacZ* fusion was regulated by pTF-FC2 when placed *in trans*. However, as the RepB primase is essential for IncQ-type plasmid replication, and expression of the *repB* gene is from the *mobB* promoter, pTC-F14 is likely to have retained control over its own replication. The role of the *pas* in the replication of pTC-F14 and pTC-FC2 is unknown and puzzling, as the entire *pas* of both pTF-FC2 and pTC-F14 (Smith & Rawlings 1997; Deane & Rawlings, 2004) could be deleted without a detectable effect on plasmid replication or copy number. This *pasA* cross-regulation is the subject of a recent study (Deane & Rawlings, 2004).

This study has highlighted a difficulty with what may be understood by plasmid incompatibility. A formal definition of plasmid incompatibility is that it is the failure of two coresident plasmids to be stably inherited in the absence of external selection (Novick, 1987). However, what may be frequently inferred is that two plasmids that are incompatible belong to the same incompatibility group because they have replicons or partitioning systems that are closely related. This does not hold in the case of pTC-F14 and RSF1010, which clearly belong to two very different plasmid homology groups. In addition, their iterons have substantially different sequences, and so might be expected to be compatible; however, they are incompatible. In contrast, plasmids pTF-FC2 and pTC-F14 are clearly more closely related to each other than RSF1010 is to pTC-F14, but the more closely related plasmids are compatible. This is a clear instance where replicon incompatibility does not reflect the sequence relatedness of plasmid replicons.

ACKNOWLEDGEMENTS

We thank Erhard Tietze for plamid pIE1108, Gunther Ziegelin for RSF1010K and Aresa Toukdarian for cloning vector pGL10. This work was supported by grants from the National Research Foundation, the Technology for Human Resource Development Programme (Pretoria, South Africa) and BHP Billiton Minerals Technology (Randburg, South Africa).

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1993). *Current Protocols in Molecular Biology*. New York: Wiley Interscience.

Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. & Chou, J. (1983). β-Galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. *Methods Enzymol* **100**, 293–308.

Deane, S. M. & Rawlings, D. E. (2004). Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad-host-range IncQ-like plasmids. *J Bacteriol* **186**, 2123–2133.

Derbyshire, K. M., Hatfull, G. & Willetts, N. S. (1987). Mobilization of the non-conjugative plasmid RSF1010: a genetic analysis of its origin of transfer. *Mol Gen Genet* **206**, 154–160.

Dorrington, R. A. & Rawlings, D. E. (1989). Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. *J Bacteriol* **171**, 2735–2739.

Dorrington, R. A., Bardien, S. & Rawlings, D. E. (1991). The broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. *Gene* **108**, 7–14.

Frey, J. & Bagdasarian, M. (1989). The molecular biology of IncQ plasmids. In *Promiscuous Plasmids of Gram-Negative Bacteria*, pp. 79–93. Edited by C. M. Thomas. London: Academic Press.

Gardner, M. N., Deane, S. M. & Rawlings, D. E. (2001). Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. *J Bacteriol* **183**, 3303–3309.

Haring, V. & Scherzinger, E. (1989). Replication proteins of IncQ plasmid RSF1010. In *Promiscuous Plasmids of Gram-Negative Bacteria*, pp. 95–124. Edited by C. M. Thomas. London: Academic Press.

Harley, C. B. & Reynolds, R. P. (1987). Analysis of *E. coli* promoter sequences. *Nucleic Acids Res* **15**, 2343–2361.

Honda, Y., Sakai, H., Hiasa, H., Tanaka, K., Komano, T. & Bagdasarian, M. (1991). Functional division and reconstruction of a plasmid replication origin: molecular dissection of the *oriV* of the broad-host-range plasmid RSF1010. *Proc Natl Acad Sci U S A* **88**, 179–183.

Kim, K. & Meyer, R. J. (1986). Copy-number of broad host-range plasmid R1162 is regulated by a small RNA. *Nucleic Acids Res* **14**, 8027–8046.

Kim, Y.-J. & Meyer, R. J. (1991). An essential iteron-binding protein required for plasmid R1162 replication induces localized melting within the origin at a specific site in AT-rich DNA. *J Bacteriol* **173**, 5539–5545.

Laing, E. & Pretorius, I. S. (1992). Synthesis and secretion of an *Erwinia chrysanthemi* pectate lyase in *Saccharomyces cerevisiae* regulated by different combinations of bacterial and yeast promoter and signal sequences. *Gene* **121**, 35–45.

Lin, L.-S. & Meyer, R. J. (1987). DNA synthesis is initiated at two positions within the origin of replication of plasmid R1162. *Nucleic Acids Res* **15**, 8319–8331.

Lin, L.-S., Kim, Y.-J. & Meyer, R. J. (1987). The 20 bp, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility *in vivo* and inhibits R1162 DNA replication *in vitro*. *Mol Gen Genet* **208**, 390–397.

Miao, D.-M., Honda, Y., Tanaka, K., Higashi, A., Nakamura, T., Taguchi, Y., Sakai, H., Komano, T. & Bagdasarian, M. (1993). A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. *Nucleic Acids Res* **21**, 4900–4903.

Miao, D.-M., Sakai, H., Okamoto, S., Tanaka, K., Okuda, M., Honda, Y., Komano, T. & Bagdasarian, M. (1995). The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. *Nucleic Acids Res* **23**, 3295–3300.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Novick, R. P. (1987). Plasmid incompatibility. *Microbiol Rev* **51**, 381–395.

Persson, C. & Nordström, K. (1986). Control of replication of the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated sequences. *Mol Gen Genet* **203**, 189–192.

Rawlings, D. E. & Tietze, E. (2001). Comparative biology of IncQ and IncQ-like plasmids. *Microbiol Mol Biol Rev* **65**, 481–496.

Rawlings, D. E. & Woods, D. R. (1985). Mobilization of *Thiobacillus ferrooxidans* plasmids among *Escherichia coli* strains. *Appl Environ Microbiol* **49**, 1323–1325.

Rawlings, D. E., Pretorius, I. & Woods, D. R. (1984). Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. *J Bacteriol* **158**, 737–738.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M. & Scherzinger, E. (1989). Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. *Gene* **75**, 271–288.

Smalla, K., Heuer, H., Gotz, A., Niemeyer, D., Krogerrecklenfort, E. & Tietze, E. (2000). Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. *Appl Environ Microbiol* **66**, 4854–4862.

Smith, A. S. & Rawlings, D. E. (1997). The poison-antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. *Mol Microbiol* **26**, 961–970.

Smith, A. S. & Rawlings, D. E. (1998). Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain, and antidote degradation requires the Lon protease. *J Bacteriol* **180**, 5458–5462.

Snyder, L. & Champness, W. (2003). *Molecular Genetics of Bacteria*. Washington, DC: American Society for Microbiology

Sýkora, P. (1992). Macroevolution of plasmids: a model for plasmid speciation. *J Theor Biol* **159**, 53–65.

Tietze, E. (1998). Nucleotide sequence and genetic characterization of the novel IncQ-like plasmid pIE1107. *Plasmid* **39**, 165–181.

Yanisch-Perron, C., Vieira, J. & Messing, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Plasmid 53 (2005) 137–147
PLASMID
www.elsevier.com/locate/yplas

Review

The evolution of pTF-FC2 and pTC-F14, two related plasmids of the IncQ-family

Douglas E. Rawlings*

Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, South Africa

Received 28 October 2004; revised 9 December 2004

Communicated by Kornelia Smalla

Abstract

Two plasmids, pTF-FC2 and pTC-F14, that belong to the IncQ-like plasmid family were isolated from two related bacteria, *Acidithiobacillus ferrooxidans* and *Acidithiobacillus caldus*, respectively. The backbone regions of the two plasmids share a sufficiently high amount of homology to indicate that they must have originated from the same ancestral plasmid. Although some of their replication proteins could complement each other, the plasmids have evolved sufficiently for their replicons to have become compatible. This compatibility has occurred by changes in the iteron sequence, RepC (iteron binding protein) specificity and the regulation properties of the RepB primase. Two of the five mobilization genes have remained highly conserved, whereas the other three genes appear to have evolved such that each plasmid is mobilized most efficiently by a different self-transmissible plasmid. Plasmids pTF-FC2 and pTC-F14 do not appear to compete at the level of mobilization. The antitoxins of the toxin–antitoxin (TA) plasmid stability systems were partly able to neutralize the toxins of the other plasmid and also to partly cross-regulate the TA systems of the other plasmid with the antitoxin of pTF-FC2 being the most effective cross-regulator. Other aspects of the evolution of the two plasmids are described and the danger of making the assumption that incompatibly of IncQ-like plasmids is a reflection of the degree of relatedness of two plasmids is discussed.

Keywords: Plasmid evolution; IncQ-like plasmids; Plasmid incompatibility; Plasmid .mobilization; Toxin–antitoxin systems

1. Introduction

The 12180 bp broad host-range, mobilizable, IncQ-like plasmid, pTF-FC2, was isolated from a strain of the mesophilic, acidiphilic, iron-, and sulfur-oxidizing, chemolithotrophic bacte-rium, *Acidithiobacillus ferrooxidans* in 1982 (Rawlings et al., 1984). This bacterium was a member of a consortium of bacteria that was being adapted for use as an inoculum in a biooxidation process to pretreat

* Fax: +27 21 808 5846.
E-mail address: der@sun.ac.za.

0147-619X/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2005.01.001

arsenopyrite concentrates in preparation for the extraction of gold (Dew et al., 1997; Rawlings and Silver, 1995). About 15 years later, a 14149 bp plasmid, TC-F14, was isolated from a related bacterium, *Acidithiobacillus caldus* (Gardner et al., 2001). *At. caldus*, like *At. ferrooxidans*, is also an acidiphilic, chemolithotroph, but differs in that it is moderately thermophilic (optimum 45 °C) and oxidizes sulfur but not iron (Hallberg and Lindström, 1994). *At. ferrooxidans* and *At. caldus* share overlapping ecological niches. The *At. caldus* strain from which pTC-F14 was isolated came from a pilot plant that was being used to leach nickel from ores (Rawlings et al., 1984). As the same microbial inoculum had been transferred and adapted to new minerals over many years, it could have included descendants of microorganisms that were present 15 years previously. These two IncQ-like plasmids have replicons, mobilization, and toxin–antitoxin systems that are closely related (Table 1), although the accessory genes that they carry are very different (Fig. 1). As the backbone structures of both plasmids are closely related one can assume that they must have originated from the same ancestral

Table 1
Replication, addiction, and mobilization associated proteins of pTC-F14 and comparison with pTF-FC2

Protein	Function	pTC-F14			pTF-FC2			% Amino acid identity
		Amino acids	Mol mass (Da)	p*I*	Amino acids	Mol mass (Da)	p*I*	
RepA	Replication specific helicase	291	31,289	5.92	290	31,227	6.21	81.0
RepB	Plasmid specific DNA primase	352	40,623	9.73	352	40,111	9.77	78.4
RepC	Iteron-specific binding protein	303	33,712	9.28	299	33,740	8.99	74.2
PasA	Antitoxin of plasmid addiction system	74	8523	4.46	74	8453	4.71	81.1
PasB	Toxin of plasmid addiction system	90	10,483	10.36	90	10,307	10.4	72.2
PasC	Toxin–antitoxin accessory protein	—	—	—	71	7676	3.76	—
MobA-RepB	oriT-specific relaxase	833	95,792	9.50	831	94,854	9.59	75.0
MobB	oriT-processing accessory protein	103	11,198	9.72	106	11,605	9.79	77.4
MobC	DNA-binding accessory protein	131	13,969	10.03	118	12,941	10.01	22.7
MobD	Mobilization protein of unknown function	226	24,698	6.60	227	25,274	5.25	39.4
MobE	Mobilization protein of unknown function	220	23,811	5.53	213	23,093	8.19	19.8

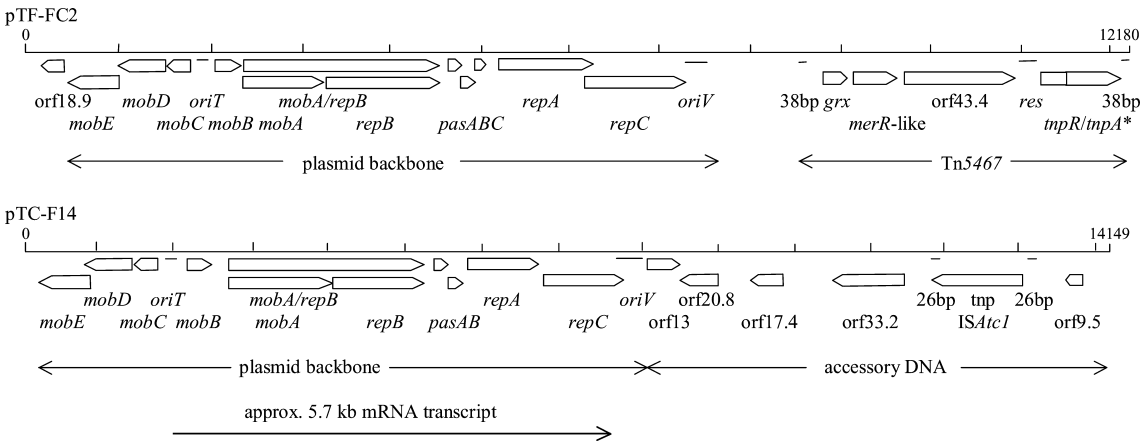


Fig. 1. A comparison of the genes, orfs (open reading frames) and other features associated with plasmids pTF-FC2 and pTC-F14. Plasmid backbones are similar while the accessory DNA is unrelated. In pTF-FC2 the accessory DNA consists mostly of Tn*5467* (Clennel et al., 1995), a defective Tn*21*-like element containing genes for a functional glutaredoxin (*grx*) and a MerR-like regulator of orf43.4. Orf43.4 is of unknown function but related to permeases of the major facilitator superfamily. The accessory DNA of pTC-F14 contains an insertion element (IS*Atc1*) related to IS*Afe1* of *At. ferrooxidans* (Holmes et al., 2001) and several other orfs of unknown function.

plasmid. At the earliest stages of their divergence, the two plasmids would have had replicons, mobilization regions, and plasmid stability toxin–antitoxin systems that would have complemented and cross-reacted with each other. Discovery of these two related plasmids has enabled us to investigate to what extent the plasmids are still able to interact with each other and thereby afforded us the opportunity to gain insights as to how the two plasmids have evolved.

2. Interaction and evolution of the plasmid replicons

Knowledge of the replication of plasmids of the IncQ family has been mostly derived from studies of the plasmids RSF1010 and R1162 (Frey and Bagdasarian, 1989; Sakai and Komano, 1996). IncQ replicons consist of three genes, *repA*, *repB*, and *repC*, and an *oriV* region (Scholz et al., 1989). The *oriV* region contains three and one-half 20 bp iterons with 2 bp nucleotide spacers. The iterons serve as binding sites for the site-specific, DNA-binding protein, RepC (Haring and Scherzinger, 1989; Lin et al., 1987; Persson and Nordstrom, 1986). They also serve as determinants of incompatibility and copy number control. In other iteron-containing plasmids, iteron-associated incompatibility (and copy number control) is believed to arise from the inhibition of replication of one plasmid by another due to reversible inter-plasmid binding at the iterons by a process known as 'handcuffing' (Chattoraj, 2000). Binding of RepC is essential for replication and is thought to introduce conformational changes leading to DNA unwinding in the adjacent A + T-rich region (Haring and Scherzinger, 1989; Kim and Meyer, 1991). This RepC-induced DNA melting serves as an entry site for RepA, a plasmid specific helicase that unwinds the DNA in the flanking regions. One of these flanking regions contains two plasmid-specific, single-strand DNA initiation sites, *ssiA* and *ssiB* (Haring and Scherzinger, 1989; Honda et al., 1991; Lin and Meyer, 1987). The plasmid-encoded RepB primase initiates the priming of single-stranded DNA synthesis on opposite strands in the leftward and rightward directions, respectively (Honda et al., 1989).

As can be seen from Fig. 1, plasmids pTF-FC2 and pTC-F14 have the backbone structure of a typical IncQ-family replicon. The amino acid sequences of the RepA, RepB, and RepC replication proteins of pTF-FC2 and pTC-F14 are highly conserved at between 74 and 81% amino acid sequence identity (Table 1). The iterons of the *oriV* regions are also related, but differ in both nucleotide sequence and in the number of copies of iterons (Gardner et al., 2001). Plasmid pTF-FC2 has three perfectly conserved 22 bp tandem repeats while plasmid pTC-F14 has five tandem repeats of which the middle three are identical while the outer two are either 23 or 21 instead of 22 bp (Fig. 2). The sequence of the 22 bp repeats of the two plasmids differs by 7 bp.

As the replicons of plasmids pTF-FC2 and pTC-F14 must have originated from the same

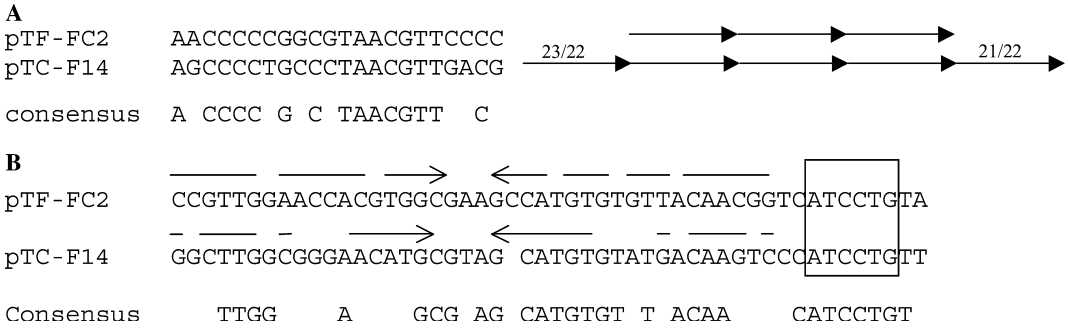


Fig. 2. (A) Sequence of the 22 bp interons within the *oriV* regions of pTF-FC2 and pTC-F14. Arrows indicate the number of iterons and variations in iteron length. (B) Sequence of the *oriT* regions. Arrows above the sequence indicate broken inverted repeat symmetry and the box indicates the highly conserved sequence around the *nic* site of IncQ-like and IncP-like plasmids.

ancestor, at the start of the process of divergence they would have been incompatible. When investigating the two replicons, a major point of interest was whether the two plasmids had evolved sufficiently for them to have become compatible and if so, what changes had taken place. In the absence of external selection, plasmids pTF-FC2 and pTC-F14 were found to be compatible which suggested that the RepC protein of one plasmid was unable to bind to the iterons of the other plasmid (Gardner and Rawlings, 2004). This conclusion was supported by the finding that replication from the *oriV* region of each plasmid could not be complemented by the related plasmid, but that heterologous complementation was possible when the plasmid's own RepC was provided in trans. The RepA helicases and the RepB primases of the two plasmids were therefore not plasmid-specific as the *repA* and *repB* genes from either plasmid could promote replication from the *oriV* of the other plasmid. The conclusion from this work was that the RepC-binding iterons in the *oriV* regions of pTF-FC2 and pTC-F14 have diverged sufficiently for the two plasmids to be compatible with each other and that the RepC proteins have coevolved with the iterons to the point where they no longer bind to the *oriV* of the other plasmid. Furthermore, there has been less pressure for the RepA helicase or RepB primase to evolve as these two proteins were capable of cross-complementing the heterologous plasmid.

Relatively little work has been carried out on the regulation of the replication of pTF-FC2 and pTC-F14. However, in the case of pTC-F14, there is at least one large 5.7 kb transcript that extends from *mobB* to the end of *repC* (see Fig. 1) (Gardner and Rawlings, 2004). Reporter gene fusions to all genes on this transcript, with the exception of *pasB*, have been made and their regulation investigated. It was found that replication was dependent on transcription from *mobB* and that the pTC-F14 RepB protein was a repressor of this transcription. When pTF-FC2 was placed in trans with the pTC-F14 *mobB-lacZ* reporter fusion, no repression of the reporter took place whereas with pTC-F14 in trans repression did occur. The conclusion was that repression of the 5.7 kb transcript by RepB was plasmid-specific. The RepB of pTC-F14 therefore has two functions, to serve as a replication primase and as a repressor of the large transcript required for replication. While RepB primase function was not plasmid-specific, the RepB repressor function was plasmid-specific. The plasmid-specific repression of the replicon transcript was consistent with the observation that the two replicons were compatible.

A surprising finding was that the RepC of plasmid RSF1010 (but not the iterons themselves) was found to exert incompatibility towards pTC-F14 (Gardner and Rawlings, 2004) even though the amino acid sequences of the pTC-F14 and RSF1010 RepC proteins are much less similar than the amino acid sequences of pTC-F14 and pTF-FC2 (Fig. 3). The RepC of RSF1010 did not show detectable incompatibility towards pTF-FC2. This incompatibility appeared to be due to nonproductive binding of the RepC of RSF1010 to the iterons of pTC-F14 in a manner that inhibited the pTC-F14 RepC from binding to its own *oriV*. However, the RepA helicase or RepB primase of RSF1010 were unable to initiate replication from the *oriV* of pTC-F14 which is consistent with the observation that the amino acid sequences of these proteins from these two plasmids are more distantly related than plasmids pTC-F14 and pTF-FC2 (Fig. 3).

The finding that distantly related replicons were incompatible while more closely related plasmids were compatible, raises a difficulty in respect to what is generally assumed by plasmid incompatibility. A formal definition of plasmid incompatibility is that it is the failure of two coresident plasmids to be stably inherited in the absence of external selection (Novick, 1987). However, what may be frequently inferred is that two plasmids that are incompatible belong to the same incompatibility group because they have replicons or partitioning systems that are closely related. This does not hold in the case of pTC-F14 and RSF1010. Plasmid pTC-F14 and RSF1010 clearly belong to two very different plasmid homology groups (see Fig. 3 for how the Rep proteins fall into two homology groups) and their iterons have substantially different sequences, nevertheless, they are incompatible. In contrast, plasmids pTF-FC2 and pTC-F14 are clearly more closely related to each other than RSF1010 is to pTC-F14, but in spite of their close relationship, they are compatible.

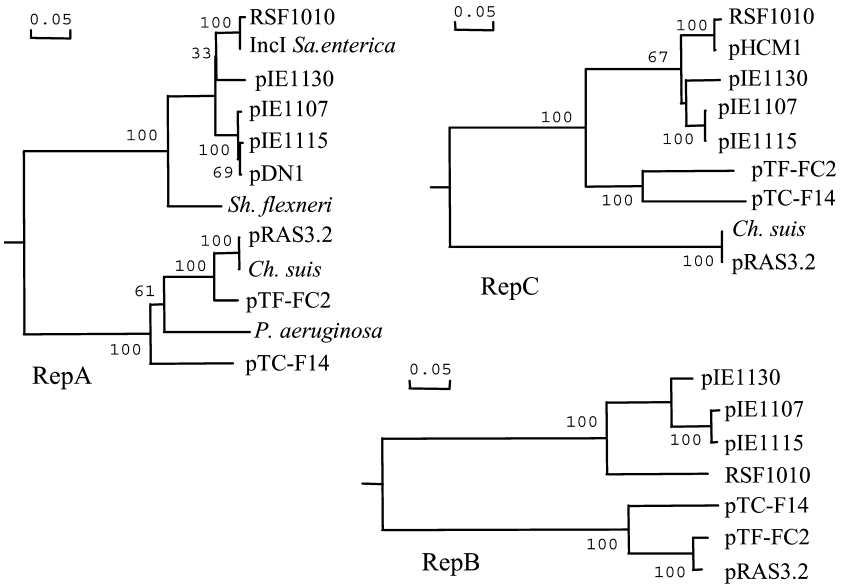


Fig. 3. Phylogenetic relationships between the RepA (helicase), RepB (primase), and RepC (iteron-binding) proteins of the IncQ family of plasmids and other related proteins. The RepA and RepB proteins fall into two distinct phylogenetic groups with pTC-F14 and RSF1010 belonging to different groups, while in the case of RepC, a third deeply branching group of proteins is apparent. Bacterial names: *Salmonella enterica*, *Shigella flexneri*, *Pseudomonas aeruginosa*, and *Chlamydia suis* represent products of chromosomally located genes associated with pathogenicity islands that appear to have been acquired as a result of transposon and plasmid activity.

This is a clear instance where replicon incompatibility does not reflect the sequence relatedness of plasmid replicons.

3. Evolution of the mobilization regions

Both plasmids pTF-FC2 and pTC-F14 belong to what is known as group 2 of the IncQ-like plasmids, all of which have a mobilization region that consists of an *oriT*-containing region and five *mob* genes (Rawlings and Tietze, 2001). The five mob gene products, MobA, MobB, MobC, MobD, and MobE have low but clear amino acid sequence similarity to the TraI, TraJ, TraK, TraL, and TraM proteins of the Tra1 region of the IncP plasmids, respectively (Rohrer and Rawlings, 1992). In the IncP plasmids TraI is a relaxase of between 732aa (RP4) (Pansegrau et al., 1994) and 747aa (R751) (Thorsted et al., 1998) and 409aa of the N-terminal region of the MobA protein of pTF-FC2 has 26% sequence identity to the 458aa N-terminal region of the RP4 TraI. TraJ is a DNA-binding protein that is thought to alter the local DNA structure and allow the TraI relaxase to access its binding site (Pansegrau et al., 1990; Zechner et al., 2000). TraK is an accessory protein, also with DNA-binding activity, which is believed to wrap around a ±180 bp region of DNA near the *oriT* changing the superhelicity and further assisting TraI to access its target site (Zechner et al., 2000; Ziegelin et al., 1992). The functions of TraL and TraM are unknown, although TraL has a Walker A/ATP/GTP-binding site (Thorsted et al., 1998).

Unlike the highly conserved *rep* gene products, the amino acid sequences of two of the *mob* gene products, MobA and MobB are highly conserved whereas the sequence relatedness of the remaining three, MobC, MobD, and MobE, is low (Table 1). The discovery of the two related mobilization systems raised the question of whether the mob genes had diverged sufficiently to have become plasmid-specific or whether they could complement the mobilization activity of each other? As can be seen in Fig. 2, the *oriT* regions of

plasmids pTF-FC2 and pTC-F14 are substantially different. Nevertheless, in cross-complementation studies, the *mob* genes of pTF-FC2 were able to mobilize a pTC-F14 *oriT*-containing test plasmid but at a frequency of approximately 50-fold less than pTC-F14 could mobilize a test plasmid containing its own *oriT* (van Zyl et al., 2003). In contrast, the *mob* genes of pTC-F14 were unable to mobilize a pTF-FC2 *oriT*-containing test plasmid. Therefore, the mobilization systems of the two plasmids have diverged to the point where there is a limited amount of unidirectional cross-complementation and a substantial amount of plasmid-specificity.

Although replicon-associated plasmid incompatibility is believed to be due to competition between related replicons, it was not known whether the mobilization systems of the related plasmids would compete. That is, whether there is competition at the level of mobilization such that when two plasmids are present in the same host, one plasmid dominates the horizontal transfer process, thereby giving it a selective advantage over the other. Under such circumstances, the more efficiently mobilized plasmid would be expected to dominate a given ecosystem.

It was found that plasmid pTF-FC2 was mobilized between strains of *Escherichia coli* by the self-transmissible IncPα plasmids (RP4 or RK2) at a frequency of approximately 3500 times greater than plasmid pTC-F14. However, when both plasmids were coresident in the same donor cell, the frequency of transfer of plasmid pTF-FC2 remained unaltered whereas the frequency of mobilization of pTC-F14 was raised to almost the same level as that of pTF-FC2 (van Zyl et al., 2003). Therefore, rather than compete with each other, plasmid pTF-FC2 increased the ability of pTC-F14 to be mated between *E. coli* hosts. An investigation into which gene products of pTF-FC2 were required for this increase in mobilization indicated that a combination of the MobD and MobE proteins was responsible. These are two of the Mob proteins that are not well-conserved between pTF-FC2 and pTC-F14 and that are weakly related to the TraL and TraM proteins of RP4 (between 20 and 23% amino acid sequence identity, respectively). We hypothesize that the MobD and MobE proteins of pTF-FC2 are better adapted to function with the conjugation system of RP4 than the MobD and MobE proteins of pTC-F14. Presumably, the MobD and MobE proteins of pTC-F14 are better adapted to the conjugation system of some other self-transmissible plasmid that has still to be discovered. It is of interest that a plasmid of 45–50 kb is present in the same *At. caldus* strain from which pTC-F14 was isolated. However, whether this plasmid is a self-transmissible plasmid that permits mobilization of pTC-F14 at high frequency awaits the isolation of this larger plasmid.

During plasmid mobilization studies, no evidence of competition or a negative effect of one plasmid on the mobilization of the other was detected. Rather, the opposite occurred, that is, when coresident in *E. coli*, the more efficiently mobilized plasmid (pTF-FC2) enhanced the mobilization of the less efficiently mobilized plasmid (pTC-F14). Although the two mobilization systems have diverged substantially, the pressure for them to diverge is less likely to have arisen from competition and more likely to be the result of the need to adapt to different self-transmissible plasmids. As discussed above, the amino acid sequences of MobB and MobA of pTF-FC2 and pTC-F14 are closely related while those of MobC, MobD, and MobE are very different (Table 1). It is unlikely that accumulation of point mutations would have generated these large amino acid sequence differences as this would imply that the mutation rate of these genes is higher than the other genes on the plasmid. The differences between MobC, MobD, and MobE of the two plasmids is more likely to be the result of gene swapping or some other mechanism by which the block of genes was acquired from another plasmid. Because of the argument presented earlier, we propose that this gene acquisition has enabled pTF-FC2 and pTC-F14 to become better suited to mobilization by different conjugative plasmids.

3.1. Evolution of the toxin–antitoxin systems

Plasmids pTF-FC2 and pTC-F14 have toxin–antitoxin (TA), post-segregational killing-like

systems that have been named *pas* (plasmid addiction system) and are situated within the replicon between the *repB* and *repA* genes (Deane and Rawlings, 2004; Smith and Rawlings, 1997). TA systems stabilize a plasmid within a population because daughter cells that fail to inherit a plasmid are either killed or their growth is inhibited (Gerdes, 2000; Hayes, 2003; Lewis, 2000). Typical TA systems consist of two genes, one encoding a highly expressed but short-lived antitoxin, and a second gene encoding a poorly expressed but long-lived toxin (Jensen and Gerdes, 1995). The *pas* of pTC-F14 consists of a PasA antitoxin and a PasB toxin, while the *pas* of pTF-FC2 is unusual in that it has a third gene, *pasC*. PasC enhanced the ability of the TA to stabilize the plasmid when cloned into a low copy number test plasmid, but this enhancement was not apparent in the natural replicon (copy number 12–15) and the function of PasC is unknown (Smith and Rawlings, 1997). In all protein type toxin–antitoxin (TA) plasmid stability systems studied to date, the antitoxins are not only able to neutralize the toxin but also have an autoregulatory function (Gerdes, 2000).

As is the case with the Rep proteins, the amino acid sequences of the proteins of pTF-FC2 and pTC-F14 TA systems are highly conserved (see Table 1). The discovery of two closely related TA systems on compatible plasmids allowed us to ask some interesting questions concerning the evolution of these systems. Since both pTF-FC2 and pTC-F14 are broad host range, mobilizable, promiscuous plasmids isolated from bacteria that can grow in a similar ecological niche, it is possible that the two plasmids could encounter each other in the same host cell. It may be argued that if the two TA systems functioned independently of each other, should either plasmid be lost, growth of the host cell would be negatively affected and the remaining plasmid would therefore also be disadvantaged. If the two TA systems could cross-react efficiently, then should either plasmid be lost, the host cell would keep growing unharmed and the TA system would fail to stabilize the individual plasmids, although it would help to ensure that at least one of the plasmids was retained. We were therefore interested to investigate whether the two TA systems were able to cross-react. More specifically whether the PasA antitoxin of one plasmid was able to neutralize the PasB toxin of the other and whether the PasA toxin of one plasmid was able to autoregulate the expression of the *pas* of the other plasmid.

Experiments to determine whether the PasB toxin of one plasmid could be neutralized by the PasA antitoxin of the other plasmid by the complementation of *pasA* mutants by the heterologous *pasA* gene were not possible because plasmids containing *pasA* mutants were lethal to the host cell (Deane and Rawlings, 2004). Attempts were made to express the PasB toxin from an IPTG inducible *tac* promoter and thereby to test for the ability of the PasA antitoxin to neutralise the heterologous PasB toxin. It was possible to make such constructs only when they were cloned into cells containing the homologous plasmid to provide sufficient antitoxin to neutralize the toxin. Without induction by IPTG, sufficient PasB was produced from the *tac* promoter for it to be lethal to the host cells unless either the homologous or heterologous plasmid was coresident in the host to produce sufficient antitoxin to neutralize the toxin. On induction by IPTG, PasB toxin neutralization by the heterologous PasA antitoxin was overwhelmed and only cells containing a plasmid capable of producing the homologous antitoxin survived. These experiments indicated that clear though limited cross-neutralization occurred between the toxin of one plasmid and the antitoxin of the other. A reporter gene (*lacZ*) translationally fused to both of the *pasA* genes indicated that either TA system was able to cross-regulate the other. Surprisingly, the heterologous pTF-FC2 *pas* was able to repress the pTC-F14 *pas* operon more efficiently than the pTC-F14 *pas* was able to repress itself. These studies showed that the antitoxins of the two TA systems could at least partially neutralize the toxin of the other system and also autoregulate the heterologous TA operon. It was also shown that PasC did not enhance the ability of the pTF-FC2 antitoxin to autoregulate the pTC-F14 *pas* or to neutralize the pTC-F14 toxin (Deane and Rawlings, 2004).

As the antitoxins of two TA systems could interact, at least partially, we wished to know how the two systems would stabilize the individual plasmids when coresident in the same host. Also

whether the three gene *pasABC* system of pTF-FC2 or the two gene *pasAB* system of pTC-F14 was more efficient. When present on their own in a host cell, each *pas* was approximately equally efficient at conferring plasmid stability to the natural replicon (Deane and Rawlings, 2004). Not all *E. coli* cells are equally sensitive to the toxin coded by *pas* (Smith and Rawlings, 1998) The reason for why *pas* toxicity and its ability to stabilize plasmids is observed in some *E. coli* strains but not in others is not yet understood. However, when a *pas*-sensitive host cell contained both plasmids and in the absence of selection for either plasmid, a replicon containing the two gene *pasAB* system was rapidly displaced by a replicon containing the three gene *pasABC* system. This displacement was not due to the presence of the *pasC* (or *pasB*) gene, but appeared to be due to the ability of the PasA antitoxin of pTF-FC2 to more effectively repress the *pas* of pTC-F14 than the PasA of pTC-F14.

The evolutionary paths of the two *pas* TA modules is unclear. It has been postulated that as the amino acid sequences of the proteins of TA modules appeared to be highly variable, the rate of evolution of TA proteins may be higher than other plasmid associated proteins (Rawlings, 1999). However, the degree of divergence of the TA proteins of pTC-F14 and pTF-FC2 was approximately the same as other ORFs within the *mobB-repC* operon. Whether the three-component *pasABC* was the forerunner of the two-component *pasAB* or vice versa is not obvious. That is, it is not possible to say whether the *pasC* gene present on pTF-FC2 was acquired by this plasmid or lost from pTC-F14. The *pas* found on the *Pseudomonas fluorescens* plasmid, pAM10.6 also has a two-component *pas* (Peters et al., 2001) and most other TA systems consist of only two components, but this observation does not indicate whether the two or three components *pas* originated first. The *pas* of pTF-FC2 and pTC-F14 have retained the ability to partly interact and the *pas* of pTF-FC2 was responsible for the rapid displacement of pTC-F14. The primary role of the *pas* in plasmid biology is uncertain as the *pas* is not very effective at plasmid stabilization. Deletion of *pas* from either pTF-FC2 or pTC-F14 resulted in a decrease in plasmid stability but there were no discernable changes in plasmid copy number or replication efficiency (Deane and Rawlings, 2004; Smith and Rawlings, 1997). It has been suggested that the *pas* may inhibit host cell division when the plasmid copy number falls below a threshold level, possibly as the result of uneven plasmid distribution after host cell division. The effect of this could be a relatively small increase in plasmid stability without an easily detectable change in copy number in most cells. However, this possibility remains to be tested.

4. Conclusions

Although plasmids pTF-FC2 and pTC-F14 are so closely related that they must have evolved from the same ancestral plasmid, the evolution of the replicons has reached the point where the two plasmids are now compatible. Compatibility has occurred due to a change in the sequence of the 22 bp iteron with a corresponding evolution in the RepC-iteron binding specificity. The RepA and RepB proteins are not plasmid-specific with respect to their helicase and primase activities, but the RepB proteins have diverged such that the repressor activity of the pTC-F14 RepB is plasmid-specific. How mutations in the iterons occur during plasmid evolution remains a puzzle. All plasmids of the IncQ-family so far discovered have at least three 22 bp iterons (or 20 bp iterons with 2 bp spacers), the sequences of which are identical within a given plasmid (Rawlings and Tietze, 2001). Furthermore, it has been shown that a single base pair change can render an iteron biologically inactive (Miao et al., 1995). The mechanism by which mutations in all three copies arise simultaneouly is not clear. However, certain members of the IncQ family (such as pTC-F14) can have more than three iteron copies and this could provide a possible means for the accumulation of mutations.

The divergently transcribed mobilization genes of the two plasmids are likely to have evolved along a different route. No obvious intercellular competition was detected between the mobilization systems and the divergence between plasmids pTF-FC2 and pTC-F14 has most likely occurred as a result of the accumulation of point mutations in

the case of *mobA* and *mobB* and gene swapping in the case of *mobC*, *mobD*, and *mobE*. We speculate that the pressure for gene swapping arose from the need for plasmids pTF-FC2 and pTC-F14 to adapt their mobilization systems to different conjugative plasmids.

The evolution of the TA systems appears to have followed a path whereby the two systems are still able to functionally interact such that partial neutralization of the toxins by the heterologous antitoxins and some cross-regulation of *pas* gene expression still occurs. This means that if both plasmids were coresident in a single cell, should either one of the plasmids be lost, the host cell should survive and the 'replication space' that it provides would be preserved. The disadvantage of this is that neither plasmid would be to ensure its own stable vertical inheritance in the presence of the other plasmid. Perhaps individual plasmid stability is less important in highly mobilizable plasmids such as pTF-FC2 and pTC-F14 because should either plasmid become lost, the host might be readily reinfected by the plasmid that has been lost. The retention of a level of TA interaction can be considered to be a more sophisticated system of plasmid stability than a highly competitive system where if either plasmid was lost the host would be rendered nonviable by the other plasmids TA system with a loss of 'replication space' for both plasmids.

The observation that the replicons of two closely related plasmids, pTF-FC2 and pTC-F14, are compatible and that two less closely related plasmids pTC-F14 and RSF1010 are incompatible (Gardner et al., 2001, 2004) is interesting. Although the reason for this unexpected finding is not known, one can speculate on possible reasons for this. Plasmids pTF-FC2 and pTC-F14 were isolated from an environment where they could possibly encounter each other, while plasmids pTC-F14 and RSF1010 were isolated from bacteria that are unlikely to encounter each other (*At. caldus* and *E. coli*, respectively). If plasmids pTF-FC2 and pTC-F14 do encounter each other, they might have been under evolutionary pressure to become compatible. Plasmids pTC-F14 and RSF1010 might not have experienced similar pressure to be co-resident within a host cell. Therefore, in spite of their greater sequence divergence, the evolutionary pressure that led to pTC-F14 and RSF1010 sequence divergence has not had to take replicon incompatibility into account and the plasmids are still incompatible. The observation that the mobilization systems of the two plasmids are substantially different can be interpreted to be in conflict with the idea that the plasmids have spent long periods of time in the same host cell. Divergence of the mobilization genes suggests that the plasmids might have spent a sufficient period of time in different hosts to allow for the adaptation of the mobilization systems to different conjugative plasmids. Similarly, the observation that pTF-FC2 displaced pTC-F14 in *E. coli* host cells in which the *pas* was toxic, suggests that during plasmid divergence, *pas*-associated 'incompatibility' has not been eliminated. This might also be considered to support the idea that the two plasmids might not have experience enough evolutionary pressure to have become fully adapted to the presence of each other. However, as pointed out previously, the *pas* was not equally effective in all *E. coli* hosts and therefore the evolutionary pressures on the *pas* might have been different in different hosts.

One should guard against an over-interpretation of the competitive pressures on the plasmids to evolve. Studies on the interaction of these broad host range plasmids were carried out in only one type of bacterium, namely *E. coli*, and not the difficult-to-work with 'natural' bacteria from which the plasmids were isolated. Furthermore, as IncQ-family plasmids are highly promiscuous and have a very wide host range, one should not think of the bacteria from which the plasmids were isolated as the only 'natural' hosts. The evolutionary pressure arising from the *pas*-associated 'incompatibility' may have varied depending on the host, whereas replicon-associated incompatibility is likely to have been experienced irrespective of the host, This may be the explanation for compatible replicons but less 'compatible' TA systems.

The contribution of the accessory DNA to plasmid evolution is not known. Most of the above studies were done with plasmids or pieces of plasmids from which the accessory DNA had been

deleted. Plasmid pTC-F14 is cryptic and although plasmid pTF-FC2 encodes for a functional glutaredoxin and MerR-like regulatory protein (Clennel et al., 1995), the contribution of these proteins to plasmid or host cell biology is unknown.

Acknowledgments

The author acknowledge the long term financial support of the National Research Foundation (Pretoria), BHP-Billiton, the University of Stellenbosch and the research of many talented post-graduate students and post-doctoral associates.

References

Chattoraj, D.K., 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. 37, 467–476.

Clennel, A.-M., Johnston, B., Rawlings, D.E., 1995. Structure and function of Tn*5467*, a Tn*21*-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid, pTFFC2. Appl. Environ. Microbiol. 61, 4223–4229.

Deane, S.M., Rawlings, D.E., 2004. Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad host range IncQ-like plasmids. J. Bacteriol. 186, 2123–2133.

Dew, D.W., Lawson, E.N., Broadhurst, J.L., 1997. The BIOX® process for biooxidation of gold-bearing ores or concentrates. In: Rawlings, D.E. (Ed.), Biomining: Theory, Microbes and Industrial Processes. Springer, Berlin, pp. 45–80.

Frey, J., Bagdasarian, M., 1989. The molecular biology of IncQ plasmids. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram-Negative Bacteria. Academic Press, London, pp. 79–93.

Gardner, M.N., Deane, S.M., Rawlings, D.E., 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. 183, 3303–3309.

Gardner, M.N., Rawlings, D.E., 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiology 150, 1797–1808.

Gerdes, K., 2000. Toxin-antitoxin molecules may regulate synthesis of macromolecules during nutritional stress. J. Bacteriol. 182, 561–572.

Hallberg, K.B., Lindström, E.B., 1994. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. Microbiology 140, 3451–3456.

Haring, V., Scherzinger, E., 1989. Replication proteins of IncQ plasmid RSF1010. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram-negative Bacteria. Academic Press, London, pp. 95–124.

Hayes, F., 2003. Toxins–antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. Science 301, 1496–1499.

Holmes, D.S., Zhao, H.-L., Levican, G., Ratouchniak, J., Bonnefoy, V., Varela, P., Jedlicki, E., 2001. ISAfe1, an ISL3 family insertion sequence from *Acidithiobacillus ferrooxidans* ATCC19859. J. Bacteriol. 183, 4323–4329.

Honda, Y., Sakai, H., Komano, T., Bagdasarian, M., 1989. RepB' is required in trans for the two single-strand DNA initiation signals in *oriV* of plasmid RSF1010. Gene 80, 155–159.

Honda, Y., Sakai, H., Hiasa, H., Tanaka, K., Komano, T., 1991. Functional division and reconstruction of a plasmid replication origin: molecular dissection of the *oriV* of the broad host-range plasmid RSF1010. Proc. Natl. Acad. Sci. USA 88, 179–183.

Jensen, R.B., Gerdes, K., 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. 17, 205–210.

Kim, Y.-J., Meyer, R.J., 1991. An essential iteron-binding protein required for plasmid R1162 replication induces localized melting within the origin at a specific site in AT-rich DNA. J. Bacteriol. 173, 5539–5545.

Lewis, K., 2000. Programmed death in bacteria. Microbiol. Mol. Biol. Rev. 64, 503–514.

Lin, L.-S., Meyer, R.J., 1987. DNA synthesis is initiated at two positions within the origin of replication of plasmid R1162. Nucleic Acids Res. 15, 8319–8331.

Lin, L.-S., Kim, Y.-J., Meyer, R.J., 1987. The 20 bp, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility in vivo and inhibits R1162 DNA replication in vitro. Mol. Gen. Genet. 208, 390–397.

Miao, D.-M., Sakai, H., Okamoto, S., Tanaka, K., Okuda, M., Honda, Y., Komano, T., Bagdasarian, M., 1995. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. Nucleic Acids Res. 23, 3295–3300.

Novick, R.P., 1987. Plasmid incompatibility. Microbiol. Rev. 51, 381–395.

Pansegrau, W., Balzer, D., Kruft, V., Lurz, R., Lanka, E., 1990. In vitro assembly of relaxosomes at the transfer origin of plasmid RP4. Proc. Natl. Acad. Sci. USA 87, 6665–6669.

Pansegrau, W., Lanka, E., Barth, P.T., Figurski, D.H., Guiney, D.G., Haas, D., Helinski, D.R., Schwab, H., Stanisich, V.A., Thomas, C.M., 1994. Complete nucleotide sequence of Birmingham IncPα plasmids, compilation and comparative analysis. J. Mol. Biol. 239, 623–663.

Persson, C., Nordstrom, K., 1986. Control of replication of the broad host range plasmid RSF1010: the incompatibility determinant consists of directly repeated DNA sequences. Mol. Gen. Genet. 203, 189–192.

Peters, M., Jogi, E., Suitso, I., Punnisk, T., Nurk, A., 2001. Features of the replicon of plasmid pAM10.6 of *Pseudomonas fluorescens*. Plasmid 46, 25–36.

Rawlings, D.E., Silver, S., 1995. Mining with microbes. Bio/Technology 13, 773–778.

Rawlings, D.E., Pretorius, I.-M., Woods, D.R., 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158, 737–738.

Rawlings, D.E., 1999. Proteic toxin–antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2. FEMS Microbiol. Lett. 176, 269–277.

Rawlings, D.E., Tietze, E., 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. 65, 481–496.

Rohrer, J., Rawlings, D.E., 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. 174, 6230–6237.

Sakai, H., Komano, T., 1996. DNA replication of IncQ broad-host-range plasmids in Gram-negative bacteria. Biosci. Biotech. Biochem. 60, 377–382.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., Scherzinger, E., 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 75, 271–288.

Smith, A.S.G., Rawlings, D.E., 1997. The poison antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. 26, 961–970.

Smith, A.S.G., Rawlings, D.E., 1998. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain and antidote degradation requires the Lon protease. J. Bacteriol. 180, 5458–5462.

Thorsted, P.B., Macartney, D.P., Aktar, P., Haines, A.S., Ali, N., Davidson, P., Stafford, T., Pocklington, M.J., Pansegrau, W., Wilkins, B.M., Lanka, E., Thomas, C.M., 1998. Complete sequence of the IncPβ plasmid R751: implications for evolution and organisation of the IncP backbone. J. Mol. Biol. 282, 969–990.

van Zyl, L.J., Deane, S.M., Rawlings, D.E., 2003. Analysis of the mobilization region of the broad host-range IncQ-like plasmid pTC-F14 and its ability to interact with a related plasmid, pTF-FC2. J. Bacteriol. 185, 6104–6111.

Zechner, E.L., de la Cruz, F., Eisenbrandt, R., Grahn, A.M., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C.M., Wilkins, B.M., Zatyka, M., 2000. Conjugative-DNA transfer processes. In: Thomas, C.M. (Ed.), The Horizontal Gene Pool, Bacterial Plasmids and Gene Spread. Harwood Academic Publishers, Amsterdam, pp. 87–174.

Ziegelin, G., Pansegrau, W., Lurz, R., Lanka, E., 1992. TraK protein of conjugative plasmid RP4 forms a specialized nucleoprotein complex with the transfer origin. J. Biol. Chem. 267, 17279–17286.

Plasmid 62 (2009) 98–107

Contents lists available at ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

The effect of the location of the proteic post-segregational stability system within the replicon of plasmid pTF-FC2 on the fine regulation of plasmid replication

Gwynneth F. Matcher, Douglas E. Rawlings *

Department of Microbiology, University of Stellenbosch, Private Bag X1, JC Smuts Building, Matieland 7602, South Africa

ARTICLE INFO

Article history:
Received 30 March 2009
Revised 11 May 2009
Available online 28 May 2009

Communicated by B. Funnell

Keywords:
Plasmid replication
Plasmid stability
Toxin–antitoxin module
Plasmid copy number

ABSTRACT

The broad host-range IncQ-2 family plasmid, pTF-FC2, is a mobilizable, medium copy number plasmid that lacks an active partitioning system. Plasmid stability is enhanced by a toxin–antitoxin (TA) system known as *pas* (plasmid addiction system) that is located within the replicon between the *repB* (primase) and the *repA* (helicase) and *repC* (DNA-binding) genes. The discovery of a closely related IncQ-2 plasmid, pRAS3, with a completely different TA system located between the *repB* and *repAC* genes raised the question of whether the location of *pas* within the replicon had an effect on the plasmid in addition to its ability to act as a TA system. In this work we demonstrate that the presence of the strongly expressed, autoregulated *pas* operon within the replicon resulted in an increase in the expression of the downstream *repAC* genes when autoregulation was relieved. While deletion of the *pas* module did not affect the average plasmid copy number, a *pas*-containing plasmid exhibited increased stability compared with a *pas* deletion plasmid even when the TA system was neutralized. It is proposed that the location of a strongly expressed, autoregulated operon within the replicon results in a rapid, but transient, expression of the *repAC* genes that enables the plasmid to rapidly restore its normal copy number should it fall below a threshold.

1. Introduction

Many plasmids possess post-segregational killing (PSK) plasmid stability systems that prevent plasmid loss by killing daughter cells that fail to inherit a plasmid or by inhibiting further cell division (Jensen and Gerdes, 1995; Rawlings, 1999; Hayes, 2003). Proteic plasmid PSK systems typically consist of two genes, one encoding a highly expressed but short-lived antitoxin, and a second gene encoding a poorly expressed but long-lived toxin. Failure to inherit the plasmid results in the destruction of the unstable antitoxin so that the stable toxin is able to prevent the growth of plasmid free progeny cells (Jensen and Gerdes, 1995). Current opinion is that plasmids can be viewed as being modular in structure and toxin–antitoxin (TA) systems are considered to be an example of a plasmid module. TA systems can function as a unit independent of a given plasmid replicon and cloning a TA system into an unstable test plasmid typically results in an increase in the stability of the test plasmid (Smith and Rawlings, 1997).

Plasmids pTF-FC2 (12.2 kb) and pTC-F14 (14.2 kb) are broad host-range, mobilizable plasmids isolated from a mesophilic (30–35 °C), iron- and sulfur-oxidizing bacterium, *Acidithiobacillus ferrooxidans* and the moderately thermophilic (optimum, 45 °C), sulfur-oxidizing bacterium, *Acidithiobacillus caldus*, respectively (Rawlings, 2005). Both plasmids have IncQ-like plasmid replicons consisting of an *oriV* with three or more 22-bp iterons as well as *repB* (primase-encoding), *repA* (helicase-encoding) and *repC* (DNA-binding protein-encoding) genes (Fig. 1). A further striking similarity is that both plasmids have genes for a plasmid

* Corresponding author. Fax: +27 21 808 5846.
E-mail address: der@sun.ac.za (D.E. Rawlings).

0147-619X/$ - see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2009.05.003

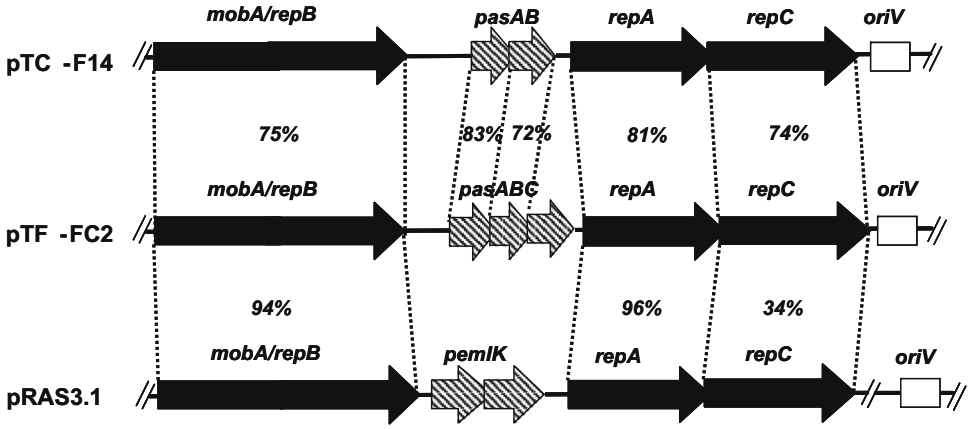


Fig. 1. Schematic map of the replication regions of IncQ-2 plasmid family illustrating the amino acid identity between the respective gene products. Sequence alignment was done with blastp hosted on the NCBI web site. No significant identity was observed for the *pemIK*-like genes with the corresponding *pas* toxin–antitoxin module.

addiction system (*pas*) located between the *repB* and *repA* genes (Smith and Rawlings, 1997; Gardner et al., 2001). The *pas* of pTF-FC2 is unusual in having three genes: *pasA* which encodes an antitoxin, *pasB* which encodes a toxin, and *pasC* which is thought to enhance the ability of the antitoxin to neutralize the toxin. Plasmid pTC-F14 is more typical of other plasmid addiction systems in having only two genes (*pasA* and *pasB*). The toxins and antitoxins of pTF-FC2 and pTC-F14 are highly conserved and share 83% and 72% amino acid sequence identity, respectively (Rawlings, 2005). An essential component of the toxin–antitoxin systems is the autoregulation of their expression. In the case of pTF-FC2, PasA represses its own operon by 25-fold, whilst PasAB together result in a 100-fold repression of expression of the *pasABC* genes (Smith and Rawlings, 1998a,b).

Plasmids pRAS3.1 and pRA3.2 are almost identical IncQ-plasmids that were isolated from the fish pathogen *Aeromonas salmonicida* in Norway (L'Abée-Lund and Sørum, 2002). The pRAS3 plasmids have mobilization and replication genes that are similar in order and sequence to both pTF-FC2 and pTC-F14, with the sequence identity to pTF-FC2 being greater than to pTC-F14 (Fig. 1). Together these three plasmids constitute the only known members of the IncQ-2 subgroup of IncQ-plasmids. A striking difference between the pRAS3 plasmids and pTF-FC2 and pTC-F14 is that the pRAS3 plasmids have a toxin–antitoxin (TA) post-segregational system located between *repB* and *repA* that is totally unrelated in sequence to the *pas* of pTF-FC2 and pTC-F14 (Loftie-Eaton and Rawlings, unpublished). The high conservation of most of the plasmid backbone of the pRAS3 plasmids and pTF-FC2 and pTC-F14 suggested that the plasmids had originated from the same ancestor plasmid but acquired their TA-modules independently. These dissimilar TA systems are located in exactly the same position within the replicon (between the *repB* and *repAC* genes) in the IncQ-2 plasmids (Fig. 1). This raised the question of whether there was a biological function for the location of the TA systems in addition to their ability to function as a plasmid stability mechanism.

In previous studies on the *pas* of pTF-FC2, it was discovered that a frame-shift mutation in the *pasA* (antitoxin) gene severely inhibited the growth of *Escherichia coli* host cells. Occasionally normal growing bacterial colonies would appear on plates and when the plasmids in these were investigated it was discovered that they had undergone spontaneous deletions of the *pas* (Smith and Rawlings, 1997). Two types of deletions were discovered: a smaller deletion in which mainly the *pas* promoter region was missing and a larger deletion in which almost the entire *pas* was absent. Surprisingly, the copy number of the plasmids appeared to remain within the 2-fold sensitivity of the assay and no adverse effects on plasmid replication was observed. Previous work on expression of the replication genes of pTC-F14 had indicted that the level of expression of the *pas* genes was approximately 100-fold greater than expression of the downstream *repA* and *repC* genes (Gardner and Rawlings, 2004). The observation that two non-sequence-related TA systems are present upstream of the *repAC* genes of IncQ-2 plasmids raised the possibility that the location of a strong, autoregulated promoter may have a previously unrecognized effect on plasmid replication. An investigation into this possibility was the basis for this study.

2. Materials and methods

2.1. Bacterial strains and plasmids

To maintain plasmid pTF-FC2 within *E. coli* DH5α (*F⁻ endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZ-ΔM15 Δ(lacZYA-argF)U169, hsdR17*(r_K^- m_K^+), λ^-), a chloramphenicol marker from pBR325 was placed within the PstI restriction endonuclease site of pTF-FC2 to form plasmid pDR412 (Rawlings et al., 1984). Plasmid constructs are detailed in Table 1 and were created using pGL10, pMC1403, pBAD28, and pGEM-T vectors as backbones with PCR products generated from pDR412 using primers listed in Table 1. Plasmid pDR412del3 contains a deletion from the stop codon of *repB* to the stop codon of *pasC* while pDR412del4 contains a deletion from the start codon of *pasA* to the stop codon of *pasC* (and has therefore retained the *pas* promoter). These deletions were generated via PCR (using primers FC2lacPvu, FC2lacPasA,

Table 1
Primers and plasmid constructs used in this study.

Plasmid/primer	Description	Reference or source
Plasmid vectors		
pMC1403	Amp^R, *lacZYA* (with the *lacZ* promoter deleted), ColE1 replicon	Casadaban et al. (1980)
pGL10	Kan^R, cloning vector with pUC19 multiple cloning site, RK2/RP4 replicon	A. Toukdarian[a]
pGEM-T	Amp^R, T-tailed PCR cloning vector, ColE1 replicon	Promega
pBAD28	Amp^R, Cm^R, arabinose-inducible expression vector , pACYC184 replicon	Guzman et al. (1995)
Plasmid constructs		
pDR412	Cm^R, pTF-FC2 with Cm^R marker from pBR325	Rawlings et al. (1984)
pDR412del1	Cm^R, pDR412; spontaneous deletion of the entire *pas* operon	Smith and Rawlings (1997)
pDR412del3	Cm^R, pDR412; designed deletion of the entire *pas* operon from the bp following the stop codon of *repB* to the stop codon of *pasC*	This study
pDR412del4	Cm^R, pDR412; designed deletion from the start codon of *pasA* to the stop codon of *pasC* so as to retain the *pas* promoter	This study
pGM-MC	Kan^R, Promoterless *lacZYA* genes from pMC1403 with pGL10 as the vector backbone	This study
pGMORep	Kan^R, pDR412 fragment from *mobD* (MunI site) to the start codon of *repA*, fused to a promoterless *lacZ* gene of pGM-MC	This study
pGMOPas	Kan^R, pDR412 fragment from *mobD* (MunI site) to the start codon of *pasA*, fused to a promoterless *lacZ* gene of pGM-MC	This study
pGM1Rep	Kan^R, pDR412del1 fragment from the *mobD* (MunI site) to the start codon of *repA*, fused to a promoterless *lacZ* gene of pGM-MC	This study
pGEMPasProm	Amp^R, PCR product from pDR412 encoding for the *pas* promoter region in the pGEM-T vector	This study
pGEMPasOp	Amp^R, PCR product from pDR412 encoding for the entire *pas* operon in the pGEM-T vector	This study
pGL10PasOp	Kan^R, PCR product from pDR412 encoding for the entire *pas* operon in the pGL10 vector	This study
pBADRepAC	Amp^R, Cm^R, *repAC* from pTF-FC2 placed in pBAD28	This study
pDR412ΔRepAC	Kan^R, pDR412 with *repAC* deleted (from the ApaI site in *repA* to the DraI site in *repC*) with R6 K ori and Kan^R replacing the Cm^R marker	This study
Primers used in plasmid constructions		
pTF-FC2AF	5′-ACGCCATAGAGAAAGATACGA-3′	This study
pTF-FC2AR	5′-GAACTTCTGCCATGTTGATG-3′	This study
RepA-BADFwd	5′-GAGCTCAAAAGGAGACAAGCATGGCTT-3′	This study
RepC-delitn Rev	5′-TTAAGCTGGCGGCTGCGGCACATTGCGGCGCGGGTTGGGCC-3′	This study
FC2lacPasA	5′-AGG*GGATCC*ACTTCGGCGGGCAGTC	This study
RepA-FC2-R	5′-TTGTTGACGTGGTGCAGGTA-3′	This study
FC2-RepB-stop	5′-*GGATCC*CTAGAGACTCATTCCCGGCGAG-3′	This study
FC2-Pas-stop	5′-*GGATCC*GGCGTGGCTGGCATC-3′	This study
FC2lacPvuI	5′-GCGGCAGCGCGATCGACGCC-3′	This study
FC2lacRepA	5′-GCT*GGATCC*TCATTGGTGAAGGCCGC-3′	This study

[a] University of California ; BamHI sites italicized and underlined.

FC2-Pas-stop, and RepA-FC2-R for pDR412del3 while pDR412del4 utilized primers FC2lacPvu, FC2-RepB-stop, FC2-Pas-stop and RepA-FC2-R) and resulted in the insertion of a BamHI site in place of the *pas* genes.

Construction of the *lacZ* reporter gene fusions (Fig. 2): The DNA fragment from the PvuI site within the *repB* to the start codon of *pasA*/*repA* was amplified (using pDR412 or pDR412del1 as templates) with primers FC2lac-PvuI and FC2lacPasA (for *pasA*)/FC2lacRepA (for *repA*) and the fusion verified by sequencing. In-frame fusions of *pasA* and *repA* to a promoterless *lacZ* gene were made by cloning the MunI to PvuI fragment (3889 bp) from pDR412 and the PvuI to BamHI (primer inserted site) PCR amplicon into pMC1403 digested with EcoRI and BamHI. The *lacZ* fusion constructs were transferred to the lower copy number plasmid pGL10 by digestion with SmaI/EcoRI (pGL10) and ScaI/MunI (pMC1403-pDR412 fusion constructs), followed by ligation, to form pGM-MC (vector control), pGMORep (*lacZ* fused to the *repA* start codon), pGMOPas (*lacZ* fused to the *pasA* start codon) and pGM1Rep (*lacZ* fused to *repA* start codon with the upstream *pas* operon deleted).

Plasmid pBADRepAC was constructed by inserting the PCR amplicon produced by primers RepA-pBAD Fwd and RepC-delitn Rev into the SacI and SmaI sites of pBAD28. Iteron sequences overlapping the C-terminal region of the *repC* gene were inactivated by altering every third nucleotide such that the encoded amino acid does not change but the iteron repeat sequence was destroyed. This ensured that results obtained for the effect of pBAD-RepAC *in trans* on plasmid copy number occurred independent of increased handcuffing due to the additional iterons.

2.2. Plasmid stability assay

Plasmid constructs pDR412 and pDR412del3 were co-transformed with pGL10pasOp into *E. coli* DH5α and stability assays carried out as described by Deane and Rawlings (2004). Approximately every 20 generations, 1000–5000 cells were sub-inoculated into fresh 5 ml LB and grown overnight at 37 °C until a total of approximately 260 generations had been achieved. Plasmid maintenance was determined every second day by replica plating 50 colonies of a spread plated serial dilution onto LA with 50 μg/ml kanamycin and LA with 50 μg/ml kanamycin plus 25 μg/ml chloramphenicol.

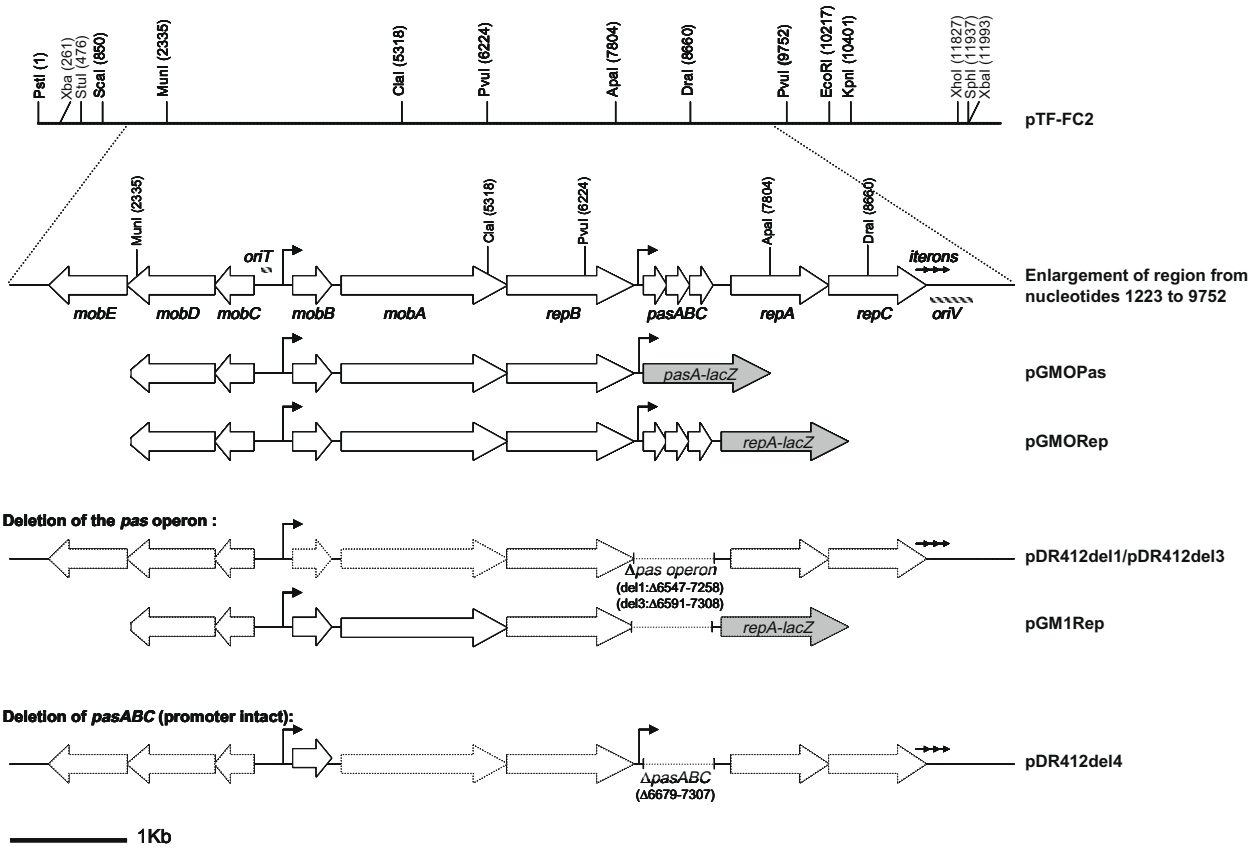


Fig. 2. Arrangement of the pTF-FC2 mobilization and replicon regions illustrating relevant restriction endonuclease recognition sites and the direction of gene expression from promoter regions (small, solid arrows). The translation fusions of *repAC* and *pasA* to the promoterless *lacZ* gene (gray arrows) as well as deletion constructs (deletions illustrated by dashed gray lines) are shown.

2.3. β-Galactosidase assays of lacZ reporter gene fusions

Constructs pGM-MC, pGMORep, pGMOPas and pGM1-Rep were transformed into *E. coli* DH5α with/without pGEMPasProm or pGEMPasOp *in trans*. β-galactosidase assays were carried out as described by Miller (1972) with the following adjustments: Triplicate test tubes, with fresh 5 ml LB containing the relevant antibiotic (50 μg/ml kanamycin, 100 μg/ml ampicillin), were inoculated from overnight cultures of the plasmid containing *E. coli* cells and grown to an OD_{600} of 0.5–0.6 at 37 °C. 100 μl of each sample was removed and 900 μl Z Buffer added. Cells were permeabilized by addition of 10 μl toluene, vortexing vigorously for 10 s and held at 37 °C for 30 min. The assay was done at 28 °C and initiated by addition of the substrate ONPG to a final concentration of 0.8 μg/ml. On development of a yellow color, the reaction was terminated by addition of 500 μl 1 M Na_2CO_3 and the time elapsed noted. Absorbance's at 550 nm and 420 nm were determined and the units calculated according to Miller (1972).

2.4. Plasmid copy number determined by relative quantitative real-time PCR

Duplicate cultures of *E. coli* DH5α containing pDR412, pDR412del3, and pDR412del4 were grown to mid-exponential phase at 37 °C. Total DNA was extracted using the QIAamp DNA Mini kit (Qiagen) and diluted to 2 ng/μl subsequent to quantification using a Nanodrop Spectrophotometer. Real-time PCR was carried out using a LightCycler instrument (version 2.0) and LightCycler software version 3.5. The threshold cycles (Cp) were determined with the second derivative maximum method using the LightCycler software.

Each 20 μl real-time PCR was set up with LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) with an optimal final concentration of 3 mM $MgCl_2$ and 0.5 μM primers. Thermal cycling parameters were : pre-incubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 56 °C for 4 s and primer extension at 72 °C for 15 s. Melting curve analysis (to verify the specificity of amplification products) was done with denaturation at 95 °C, template annealing at 65 °C for 15 s followed by template melting up to a final temperature of 95 °C in 0.1 °C/s increments. Melting curve analysis was followed by sample cooling to 40 °C for 30 s. Fluorescence was measured at the end of each extension step as well as continually over the melting curve analysis.

The housekeeping gene coding for D-glyceraldehyde-3-phosphate dehydrogenase (*gap*) in *E. coli* and the *repB* gene on the pDR412 were selected as the reference and target genes, respectively, and primers were designed accordingly (Table 2). The amplification efficiencies of both *gap* and *repB* PCR products were determined using a serial dilution series of total DNA isolated from cells containing pDR412. Relative quantification of the pDR412, pDR412del3

Table 2
PCR primers used for real-time PCR.

Gene	Primers	Primer sequence	Amplicon size (bp)
gap	K12GAPAF	5′-TGTTAGACGCTGATTACATGG-3′	294
	K12GAPAR	5′-CTTTAACGAACATCGGAGTGT-3′	
cat	Cat-FC2-F	5′-ACCTGGCCTATTTCCCTAA-3′	202
	Cat-FC2-R	5′-TCACAAACGGCATGATGAAC-3′	
repA	RepA-FC2-F	5′-TCATGGGCAAGCGGCTAAA-3′	214
	RepA-FC2-R	5′-TTGTTGACGTGGTGCAGGTA-3′	
repB	RepB-FC2-F	5′-CTACACGCCGCTATCGGA-3′	205
	RepB-FC2-R	5′-GTTCAGGGCATCGCTCA-3′	
repC	pTF-FC2AF	5′-ACGCCATAGAGAAAGATACGA-3′	294
	pTF-FC2AR	5′-GAACTTCTGCCATGTTGATG-3′	

and pDR412del4 was done in duplicate for each set of two cultures (i.e., four replicates for each plasmid set). The resultant Cp values were analysed using REST analysis tool (Pfaffl et al., 2002) and reported as units relative to the wild type condition.

2.5. Expression of repAC in trans

The plasmids pBADRepAC and pDR412ΔRepAC (Table 1) were co-transformed into *E. coli* Top10 (*F-mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 nupG recA1 araD139 Δ(ara-leu)7697 galE15 galK16 rpsL(Str^R) endA1 λ$^-$*) and cultured in 5 ml M63 minimal medium, supplemented with 50 μg/ml leucine, 0.0002% L-arabinose and 20 μg/ml kanamycin, overnight at 37 °C. Two duplicate tubes of 5 ml M63 minimal medium, supplemented with 50 μg/ml leucine and 20 μg/ml kanamycin as well as 0%, 0.000002%, 0.00002%, 0.002% arabinose or 0.2% glucose were inoculated from the overnight culture and grown to OD_{600} of 0.3–0.4. Total DNA was extracted from 2 ml of culture using the QIAamp DNA Mini kit (Qiagen). Plasmid copy number was determined for each culture condition (i.e., varying levels of induction of *repAC* expression) using relative quantitative real-time PCR as described above.

2.6. Relative quantification of mRNA levels

Gene expression profiles of pDR412, pDR412del3 and pDR412del4 were determined by relative quantitative real-time PCR. The plasmids were transformed into *E. coli* DH5α and cultured, in duplicate, to an OD_{600} of 0.6–0.7 in 5 ml Luria broth. Total RNA was extracted from 2 ml of culture using the RiboPure-Bacterial RNA Isolation Kit (Ambion) and treated with DNase to remove contaminating DNA. cDNA was produced from 1 μg of total RNA using random hexameric primers with the Transcriptor First Strand cDNA Synthesis kit (Roche). Real-time PCR was carried out using the LightCycler instrument (version 2.0) with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics) as described above. Amplicons from the chloramphenicol resistance marker on the plasmid backbone (*cat*) was used as the reference for the target genes *repA*, *repB*, and *repC*, respectively. The amplification efficiencies of each amplicon was determined using a 10-fold serial dilution series of cDNA produced from the total RNA extracted from cells containing pDR412. Relative quantification of the pDR412, pDR412del3 and pDR412del4 target genes was done in duplicate for each set of two cultures (i.e., four replicates for each plasmid set). The resultant Cp values were analysed using REST analysis tool (Pfaffl et al., 2002).

3. Results

3.1. Transcriptional strengths of promoters within the replicon region

To determine the relative transcriptional strengths of promoters responsible for the expression of the *repAC* and *pasABC* genes, in-frame fusions of the start codons of *repA* (pGMORep) or *pasA* (pGMOPas) to a promoterless *lacZ* gene were constructed (Fig. 2). Considerably elevated levels of *lacZ* expression and subsequent β-galactosidase activities (7413 Miller units) were observed from the *pasA* fusion (pGMOPas) (Fig. 3A) compared to the *repA* fusion (pGMORep) (484 Miller units). This demonstrated the strength of the *pas* promoter in the absence of regulation as pGMOPas does not encode the structural genes of *pasABC* and no negative feedback regulation of the *pas* promoter could occur. In the case of the *repA-lacZ* fusion (pGMORep), *pasAB* would have been expressed and *repA* expression would have been affected by autorepression of the *pas* promoter. Consequently, as shown for pTC-F14 (Gardner and Rawlings, 2004), the Miller units observed for pGMORep could be attributed primarily to read-through transcription from the upstream *mobB/repB* promoter. This was supported by the similar Miller units obtained for pGM1Rep (Fig. 3A) in which the entire *pas* operon had been deleted (Table 1). Thus transcriptional read-through from *mobB/repB* is equivalent irrespective of the presence or absence of the *pas* operon which was not required for basal level expression of *repAC*.

To confirm that the *pas* operon does play a role in determining the transcriptional levels of *repAC*, the effect of PasAB titration on *repAC-lacZ* transcription with (pGMORep) and without (pGM1Rep) the upstream *pas* operon present was monitored (Fig. 3B). Placement of the *pas* promoter region *in trans* would result in reduced availability of PasAB for binding to, and inhibition of, the *pas* promoter region of pGMORep as any PasAB produced from pGMORep could bind to either pGEMPasProm or pGMORep. This would mimic what would occur under conditions where the pDR412 plasmid copy number has dropped to below normal levels.

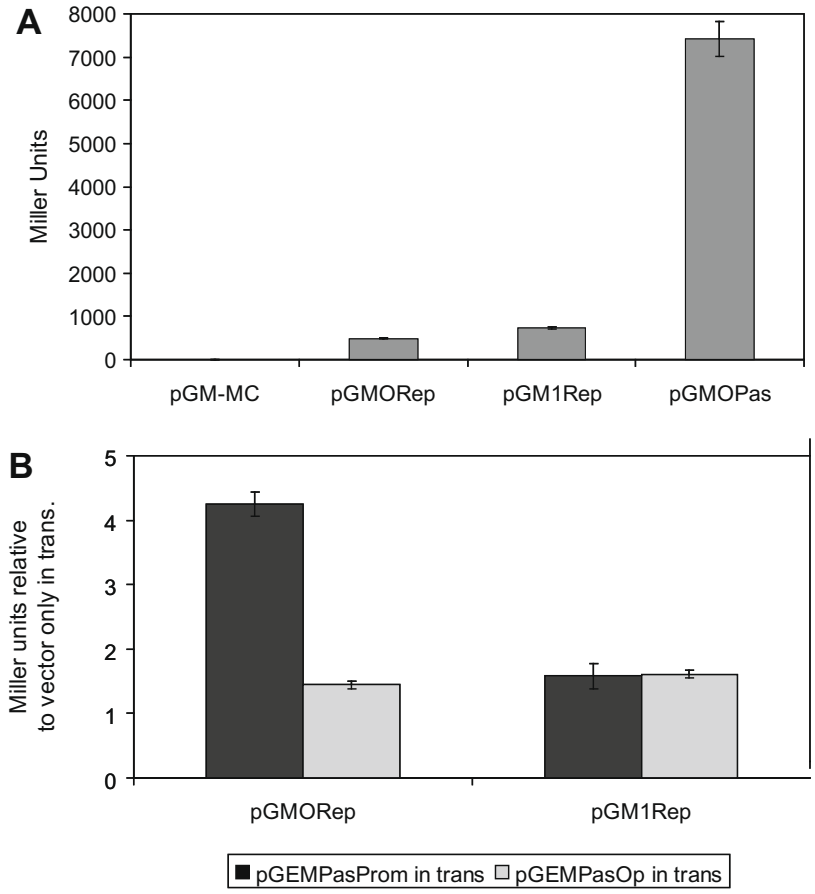


Fig. 3. Reporter enzyme assay of the transcriptional strengths of (A) *repAC* (pGMORep), *pasABC* (pGMOPas), and *repAC* (with upstream *pas* operon absent) (pGM1Rep) and (B) pGMORep and pGM1Rep with an excess of the *pas* promoter region (pGEMPasProm) or the entire *pas* operon (pGEMPasOp) *in trans*. Assays were done via fusion of a promoterless *lacZ* gene to the start codon of *pasA* or *repA* with expression levels of the *lacZ*-fusions monitored via β-galactosidase assays ($n = 4$). In the case of (B) the results are reported as units relative to the Miller units obtained for the promoterless *lacZ* vector (pGM-MC). ($n = 4$).

Under these conditions, the cellular concentrations of PasAB would decrease concomitantly with plasmid copy number as fewer *pasAB* genes would be available to replace PasAB degraded by the cellular protease Lon (Smith and Rawlings, 1998a). Placement of the entire *pas* operon *in trans* would function as a control as pGEMPasOp encodes for its own *pasAB* and consequently would not result in titration of cellular PasAB required for autoregulation of the *pas* promoter in the reporter construct. To this end, pGMORep and pGM1Rep were each co-transformed with either the entire *pas* operon *in trans* (pGEMPasOp) (i.e., no PasAB titration) or with the *pas* promoter region only *in trans* (pGEMPasProm) (i.e., PasAB titration). No difference in the β-galactosidase activity occurred when the *pas* operon of pDR412 was deleted (pGM1Rep) as there was no *pas* promoter region present on the fusion constructs to which the PasAB could bind (Fig. 3B). By contrast, when the *pas* operon was present within the replicon (pGMORep), a 2.7-fold increase in expression of the *lacZ* fused to the *repAC* occurred when the PasAB was titrated by the presence of an *in trans pas* promoter (pGEMPasProm) compared to no titration when the entire *pas* operon was present *in trans* (pGEMPasOp). Thus under low copy number conditions (as mimicked by PasAB titration) the presence of the *pas* operon increased expression of *repAC*.

3.2. Effect of the pas operon on plasmid copy number

Previous studies, using the hybridization method, had estimated the plasmid copy number of pTF-FC2 as 12–15 plasmids per chromosome (Dorrington and Rawlings, 1989). Using relative quantitative real-time PCR, the effect of deleting either the *pas* operon (pDR412del3) or the structural *pasABC* genes whilst retaining the *pas* promoter (pDR412del4) on plasmid copy number relative to the parental form was determined. As illustrated in Table 3, pDR412del3 exhibited a similar copy number to that of pDR412 (note the SEM values in parenthesis in Table 3) thus indicating that the *pas* operon did not play a role in determining the final copy number of pDR412. By comparison, pDR412del4 exhibited a 2-fold decrease in plasmid copies per cell. Thus placement of a strong, but unregulated, promoter upstream of *repAC* reduced plasmid copy number.

3.3. Effect of the pas operon on replicon gene expression profiles

The mRNA expression profiles of the *repBAC* genes of pDR412del3 and pDR412del4 relative to the parental plasmid pDR412 were analyzed by relative quantitative two-step real-time PCR (Fig. 4). Amplicons from the

Table 3
Plasmid copy number of pDR412 deletion constructs relative to the wild type plasmid.

Plasmid	Description	Copy number relative to pDR412 (SEM)[a]	Fold difference in copy number relative to pDR412
pDR412	WT replicon	1 (±0.198)	1
pDR412del3	*pas* operon deleted	1.297 (±0.218)	1.297
pDR412del4	*pasABC* deleted but *pas* promoter retained	0.495 (±0.099)	−2.021

[a] $n = 4$.

chloramphenicol resistance marker were used as the reference to normalize the amount of *repBAC* mRNA produced relative to the plasmid copy number. In this way a decrease/increase in plasmid copy number is compensated for so that variations in gene expression rates were not due to variations in the copy number of the target gene. When the entire *pas* operon was deleted (pDR412del3), expression of all three *rep* genes was slightly reduced (0.7 times the parental) with *repA* mRNA appearing to be a little lower than the *repB* or *repC* mRNA levels. By contrast, when the *pas* genes were deleted but the *pas* promoter retained (pDR412del4), the expression of *repB* was largely unchanged while the expression of *repA* and *repC* was markedly increased in comparison to the parental plasmid pDR412. The especially high *repAC* mRNA levels were not surprising as pDR412del4 retained the strong *pas* promoter region but no longer encoded for the *pasABC* structural genes and thus lacked the autoregulatory effect afforded by the PasAB. The small increase in *repB* mRNA may be due to relief from autorepression by RepB (shown to occur in the related plasmid, pTC-F14, Gardner and Rawlings, 2004) due to the lower copy number of pDR412del4 (Table 3).

As the *pas* operon had such a significant effect on the expression levels of the downstream *repAC* genes (Figs. 3 and 4), the effect of *repAC* expression on plasmid copy number itself was determined. The *repAC* genes were cloned behind the arabinose-inducible promoter of pBAD28 (pBADRepAC) and placed *in trans* to a construct in which both the *repA* and *repC* genes had been truncated (pDR412delRepAC). Replication of pDR412delRepAC was thus reliant on the level of arabinose-induced expression of *repAC* from the pBADRepAC. Even under repressive conditions (i.e., glucose repression of the arabinose promoter), sufficient *repAC* was expressed to allow for plasmid replication albeit at reduced copy numbers (Fig. 5). As the amount of *repAC* expressed was increased, an increase in plasmid copy number occurred. However, an upper limit was rapidly reached after which no further increase in plasmid replication occurred. This can be most likely ascribed to iteron-mediated handcuffing of plasmid copies by RepC dimers. This occurs when a threshold concentration of RepC molecules is reached within the cell resulting in the formation of RepC dimers. These dimers bind to the iteron sequences of adjacent plasmids forming bridges or handcuffs thereby inhibiting further replication of the plasmid (Chattoraj, 2000; Park et al., 2001). Thus the action of iteron/RepC mediated handcuffing regulates plasmid copy number such that it does not get too high. Overexpression of *repAC* from the pBADRepAC, as observed with 0.0002% arabinose (Fig. 5), was detrimental to the plasmid copy number as arabinose-induced overexpression of RepC mimicked what would occur under high plasmid copy number conditions. Thus, the decrease in copy number previously observed for pDR412del4 (Table 3) is likely to be due to an overexpression of *repC* (Fig. 4) driven by an unregulated *pas* promoter.

3.4. Plasmid stability is reduced by deletion of the pas operon, independent of its function as a TA system

Deletion of the entire *pas* operon, as is the case with pDR412del3, resulted in little difference in either the expression profiles of the replicon genes or in the final plasmid copy number under optimal, steady-state conditions. So, aside from its function as a TA system, does the

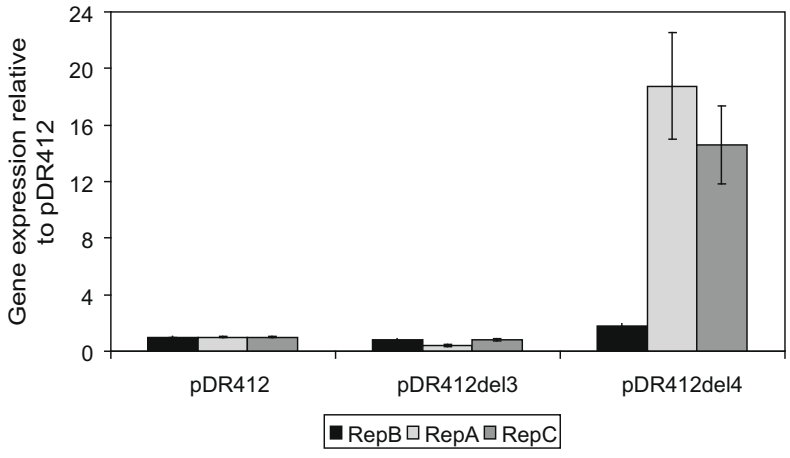


Fig. 4. Gene expression profiles for *repA*, *repB*, and *repC* of pDR412 (wild type plasmid), pDR412del3 (entire *pas* operon deleted) and pDR412del4 (*pasABC* deleted but promoter region remains). Values determined relative to the parental plasmid, pDR412, for each target gene as determined by relative quantitative real-time PCR. The cDNA was generated from mRNA extracted from two independent colonies for each plasmid derivative and each cDNA sample in turn was subjected to real-time PCR in duplicate (thus $n = 4$).

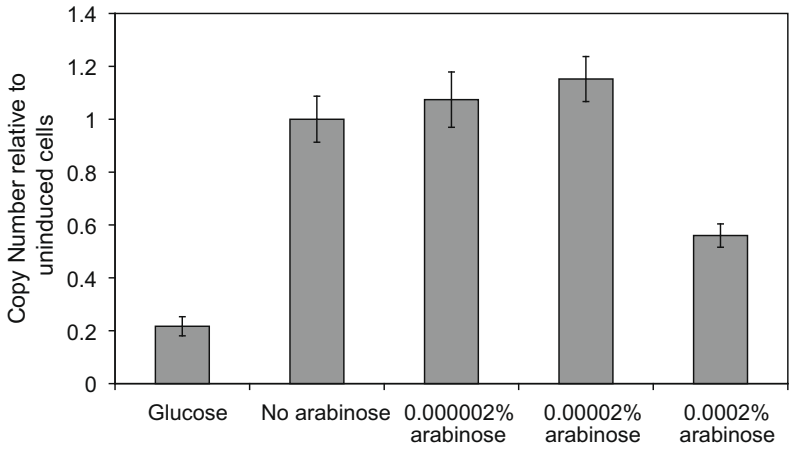


Fig. 5. Plasmid copy number of pDR412 in response to controlled expression of *repAC in trans*. Total DNA was extracted from *E. coli* Top10 cells containing pDR412delRepAC (*repAC* genes inactivated) and pBADRepAC (with *repAC* genes under the regulation of the arabinose promoter of pBAD28), grown in M63 minimal media supplemented with a range of arabinose concentrations. Two independent cultures were assayed for each of the induction conditions and each set of subsequently extracted DNA subjected to real-time PCR in duplicate (thus $n = 4$).

presence of the *pas* operon within the replicon play a role in the maintenance of the plasmid? This question was addressed by examining whether the *pas* enhanced plasmid stability over an extended period of time was independent of its toxin–antitoxin functionality.

The percentage loss of plasmid (pDR412 vs. pDR412del3) was monitored over 260 generations of cell growth by replica plating and assaying for loss of plasmid-encoded antibiotic resistance (Fig. 6). The *pas* operon was placed *in trans* on a low copy number plasmid (pGL10PasOp) to negate plasmid retention due to post-segregational killing by PasA. Thus differences in plasmid maintenance between *pas*-containing and non-*pas*-containing plasmids should be a reflection of the effect of transcription of *repAC* from the upstream promoter regions. As can be seen in Fig. 6, a plasmid from which the *pas* operon was deleted was more readily lost from the bacterial population. This was despite the fact that pDR412 and pDR412del3 have similar copy numbers and gene expression profiles (Table 3, Fig. 4). This is consistent with the hypothesis that when the plasmid copy number drops too low, autoregulation of the strong *pas* promoter results in a brief period of enhanced expression of *repAC* such that the plasmid copy number is more rapidly restored than in the absence of *pas*.

4. Discussion

Plasmids are considered to be modular in nature, with the genes involved in related functions such as replication, plasmid stability, mating pair formation or DNA processing (prior to conjugal transfer), usually grouped together such that a whole module may be inherited or acquired by a different plasmid. Cross-regulation of gene modules is a phenomenon that is widespread in larger plasmids (Thomas, 2000). For example, in the plasmid pTiC58 (from *Agrobacterium tumefaciens*), the quorum sensing system has been shown to not only regulate

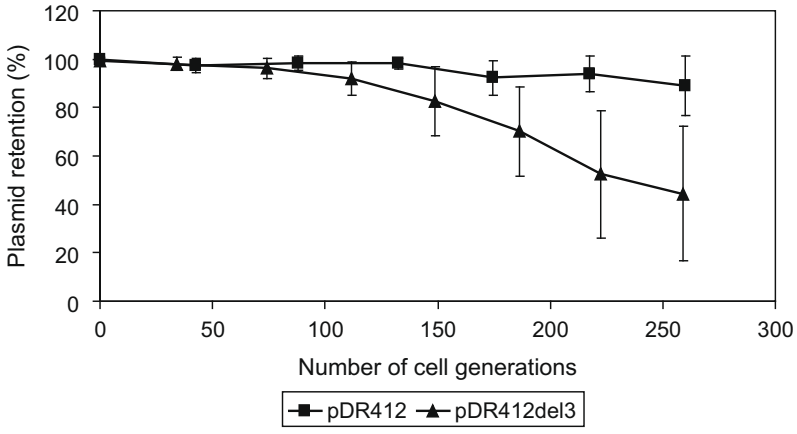


Fig. 6. Stability of parental plasmid (pDR412) and pDR412del3 in the absence of a selection pressure. Cultures containing the relevant plasmids were sub-cultured into fresh media on consecutive days and the generations calculated. Plasmid loss was determined every second day via replica plating onto non-selective and antibiotic-containing agar plates. The *pas* operon was placed at a low copy number (on a pGL10 backbone) *in trans* in order to negate any toxic effect of PasB upon loss of pDR412. ($n = 7$).

expression of conjugative transfer genes but also plasmid copy number (Bingle and Thomas, 2001). The three IncQ-2 type plasmids identified to date, namely pTF-FC2, pTC-F14 and pRAS3, have acquired a toxin–antitoxin module within their replicons such that essential replication genes lie on either side of the TA-module. Plasmids pTF-FC2 and pTC-F14 have related TA-modules but the TA-module of pRAS3 is unrelated although it lies in the same position relative to the replication genes. This suggested that the *pas* TA-module may play a role in the control of plasmid replication. Since deletion of the *pas* genes did not appear to affect the average plasmid copy number, this led us to propose the hypothesis that while upper plasmid copy number is likely to be set by handcuffing via RepC (Chattoraj, 2000), the specific placement of the highly expressed, autoregulated *pas* operon within the replicon may play a role in the rapid correction of the copy number should it fall below a critical threshold. We propose that when the plasmid copy number drops below a threshold level, a concomitant decrease in cellular PasAB protein concentration occurs due to an imbalance between plasmid production of PasAB and cellular Lon-protease driven degradation of PasA (Smith and Rawlings, 1998a). As a result, the strong *pas* promoter is relieved from autorepression by PasAB. The subsequent increase in *pasABC* expression leads to increased read-through transcription of the downstream *repAC* genes thereby "kick-starting" plasmid replication by supplying an initial burst of RepAC.

In the absence of a *pas* operon, transcription of the *repBAC* genes would originate at the relatively weak *mobB* promoter and continue through *mobA/repB* to *repAC* (Gardner and Rawlings, 2004). We have shown that the presence of a *pas* operon between *repB* and *repAC*, greatly increased expression of the downstream *repAC* genes when relieved from negative feedback regulation by PasAB (by providing extra PasAB binding sites *in trans*). Excess *repAC* expression was shown to result in a decrease in plasmid copy number and therefore this increased *repAC* expression from the *pas* promoter is likely to be short-lived. Overexpression would continue only until the normal plasmid copy is re-established and *pas* autoregulatory repression reoccurs. We reconfirmed that deletion of *pas* has minimal or no effect on the final plasmid copy number. Despite the maintenance of average plasmid copy number following the deletion of the entire *pas* operon, plasmid loss over extended generations of cell growth significantly increased in the absence of the *pas*. This occurred independently of the post-segregational killing effect of PasAB (which was supplied *in trans*) and may be explained as follows: As pTF-FC2 lacks a plasmid segregation mechanism, the plasmid may not always be evenly distributed between daughter cells subsequent to each cell division. Thus, while an infrequent occurrence, a proportion of the daughter cells may receive very few copies of the plasmid. This poses no difficulty to the wild type plasmid as the strong autoregulated *pas* promoter, in maintaining cytoplasmic PasAB concentrations, would be more active per copy and rapidly initiates replication by an initial burst of *repAC* read-through expression. This cannot occur in the *pas* deletion construct thus reducing the ability to correct for low plasmid copy number and increasing the potential for plasmid loss as was observed.

The interpretation of this experiment is complicated by the placement of the *pas in trans* on a low copy number vector which was required to prevent inhibition of host cell growth following plasmid loss. Autoregulation of the second *pas* is likely to inhibit expression of the *pas* on the test plasmid but only to a level commensurate with its low copy number (2–4 per chromosome, Robertson et al., 2000). However, the presence of a second *pas* would have an impact on the parental plasmid only and, if anything, would promote plasmid loss. Thus increased stability of the parental plasmid occurred in spite of this complication and the instability of the *pas* deletion construct was independent of the second *pas in trans*. The normal copy number of pTF-FC2 is 12 to 15 per chromosome (Dorrington and Rawlings, 1989) and should its copy number fall as low as 2–4 per chromosome, a *pas* mediated increase in copy number would be predicted to occur in parental plasmid containing cells but not the cells containing *pas* deletion plasmids. This difference is suggested to be the reason for the increased plasmid instability in *pas* deletion plasmids compared with *pas*-containing plasmids.

While a strong promoter upstream of *repAC* may be advantageous for plasmid maintenance, unregulated expression (as demonstrated by pDR412del4) has deleterious results. Thus the *pas* operon, aside from its post-segregational selection activity, has a dual function in its placement within the plasmid replicon. Not only is it responsible for transient increased *repAC* expression to boost initial plasmid replication at low copy numbers but it also prevents deleterious overexpression of *repAC* via stringent autoregulation. The plasmid copy number can be maintained at or near its upper limit as, despite the elevated metabolic burden on the host, this state is reversible. If, however, the plasmid copy number is allowed to drop too low, the plasmid risks the possibility of elimination which is irreversible.

This data collectively substantiates the hypothesis that the strong *pas* promoter plays a role in rapid, transient expression of *repAC* thereby increasing initial plasmid replication rates until the PasAB once again represses its promoter. A similar effect has been reported in plasmid R1 where a TA-module (*parD*) functions to increase plasmid copy number if it falls too low (Pimentel et al., 2005). However, unlike pTF-FC2, the *parD* module of R1 is located externally to the replicon and controls expression of *repA* indirectly by reducing the concentration of the *repA* repressor CopB.

Acknowledgments

Gwynneth Matcher was supported by a Claude Leon Foundation post-doctoral fellowship and the running expenses funded by grants to DER from the National Research Foundation (Pretoria).

References

Bingle, L.E.H., Thomas, C.M., 2001. Regulatory circuits for plasmid survival. Curr. Opin. Microbiol. 4, 194–200.

Casadaban, M.J., Chou, J., Cohen, S.N., 1980. *In vitro* gene fusions that join an enzymatically active β-galactosidase segment to amino-terminal fragments of exogenous proteins: *Escherichia coli* plasmid vectors for the detection and cloning of translational signals. J. Bacteriol. 142, 971–980.

Chattoraj, D.K., 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. 37, 467–476.

Deane, S.M., Rawlings, D.E., 2004. Plasmid evolution and interaction between the plasmid addiction stability systems of two related broad-host-range IncQ-like plasmids. J. Bacteriol. 186, 2123–2133.

Dorrington, R.A., Rawlings, D.E., 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. 171, 2735–2739.

Gardner, M.N., Deane, S.M., Rawlings, D.E., 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. 183, 3303–3309.

Gardner, M.N., Rawlings, D.E., 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiology 150, 1797–1808.

Guzman, L., Belin, D., Carson, M.J., Beckwith, J., 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. 177, 4121–4130.

Hayes, F., 2003. Toxins–antitoxins: plasmid maintenance programmed cell death, and cell cycle arrest. Science 301, 1496–1499.

Jensen, R.B., Gerdes, K., 1995. Programmed cell death in bacteria: proteic plasmid stabilization systems. Mol. Microbiol. 17, 205–210.

L'Abée-Lund, T.M., Sørum, H., 2002. A global non-conjugative TetC plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid 47, 172–181.

Park, K., Han, E., Paulsson, J., Chattoraj, D.K., 2001. Origin pairing ("handcuffing") as a mode of negative control of P1 plasmid copy number. EMBO J. 20, 7323–7332.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 30, e36.

Pimentel, B., Madine, M.A., de la Cueva-Mendez, G., 2005. Kid cleaves specific mRNAs at UUACU sites to rescue the copy number of plasmid R1. EMBO J. 24, 3459–3469.

Miller, J.H., 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Rawlings, D.E., Pretorius, I., Woods, D.R., 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158, 737–738.

Rawlings, D.E., 1999. Proteic toxin–antitoxin, bacterial plasmid addiction systems and their evolution with special reference to the *pas* system of pTF-FC2. FEMS Microbiol. Lett. 176, 269–277.

Rawlings, D.E., 2005. The evolution of pTF-FC2 and pTC-F14, two related plasmids of the IncQ-family. Plasmid 63, 137–147.

Robertson, G.T., Reisenauer, A., Wright, R., Jensen, R.B., Jensen, A., Shapiro, L., Roop II., R.M., 2000. The *Brucella abortus* CcrM DNA Methyltransferase is essential for viability and its overexpression attenuates intracellular replication in murine macrophages. J. Bacteriol. 182, 3482–3489.

Smith, A., Rawlings, D.E., 1997. The poison-antidote stability system of the broad-host-range *Thiobacillus ferrooxidans* plasmid pTF-FC2. Mol. Microbiol. 26, 961–970.

Smith, A., Rawlings, D.E., 1998a. Efficiency of the pTF-FC2 *pas* poison-antidote stability system in *Escherichia coli* is affected by the host strain and antidote degradation requires the Lon protease. J. Bacteriol. 180, 5458–5462.

Smith, A., Rawlings, D.E., 1998b. Autoregulation of the pTF-FC2 proteic poison-antidote plasmid addiction system (pas) is essential for plasmid stabilization. J. Bacteriol. 180, 5463–5465.

Thomas, C.M., 2000. Paradigms of plasmid organization. Mol. Microbiol. 37, 485–491.

JOURNAL OF BACTERIOLOGY, Oct. 2009, p. 6436–6446 Vol. 191, No. 20
0021-9193/09/$08.00+0 doi:10.1128/JB.00864-09

Comparative Biology of Two Natural Variants of the IncQ-2 Family Plasmids, pRAS3.1 and pRAS3.2▽

Wesley Loftie-Eaton and Douglas E. Rawlings*

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Received 1 July 2009/Accepted 6 August 2009

Plasmids pRAS3.1 and pRAS3.2 are two closely related, natural variants of the IncQ-2 plasmid family that have identical plasmid backbones except for two differences. Plasmid pRAS3.1 has five 6-bp repeat sequences in the promoter region of the *mobB* gene and four 22-bp iterons in its *oriV* region, whereas pRAS3.2 has only four 6-bp repeats and three 22-bp iterons. Plasmid pRAS3.1 was found to have a higher copy number than pRAS3.2, and we show that the extra 6-bp repeat results in an increase in *mobB* and downstream *mobA*/*repB* expression. Placement of *repB* (primase) behind an arabinose-inducible promoter in *trans* resulted in an increase in *repB* expression and an approximately twofold increase in the copy number of plasmids with identical numbers of 22-bp iterons. The pRAS3 plasmids were shown to have a previously unrecognized toxin-antitoxin plasmid stability module within their replicons. The ability of the pRAS3 plasmids to mobilize the *oriT* regions of two other plasmids of the IncQ-2 family, pTF-FC2 and pTC-F14, suggested that the mobilization proteins pRAS3 are relaxed and can mobilize *oriT* regions with substantially different sequences. Plasmids pRAS3.1 and pRAS3.2 were highly incompatible with plasmids pTF-FC2 and pTC-F14, and this incompatibility was removed on inactivation of an open reading frame situated downstream of the *mobCDE* mobilization genes rather than being due to the 22-bp *oriV*-associated iterons. We propose that the pRAS3 plasmids represent a third, γ incompatibility group within the IncQ-2 family plasmids.

Plasmids of the IncQ family are small (<20 kb), have a broad host range, and are highly promiscuous due to their ability to be mobilized very efficiently by self-transmissible plasmids such as the IncP plasmids. They have been divided into two families, IncQ-1 and IncQ-2, based on the amino acid sequence relatedness of their RepA (helicase), RepB (primase), and RepC (DNA-binding) replication proteins and because the mobilization proteins of the two families are unrelated, consisting of three or five genes, respectively (31). IncQ-1 group plasmids include RSF1010 and the near-identical R1162, pDN1, pIE1107, pIE1115, and pIE1130, while IncQ-2 plasmids include pTF-FC2, pTC-F14, and pRAS3.

IncQ-2 plasmids pRAS3.1 and pRAS3.2 were isolated in Norway from the fish pathogens *Aeromonas salmonicida* subsp. *salmonicida* and atypical *A. salmonicida*, respectively, while investigating plasmids that conferred resistance to tetracycline (21). The two plasmids encode identical replication and mobilization proteins, with the most important differences in the plasmid backbone being that pRAS3.1 has four 22-bp iterons in its *oriV* region and five 6-bp repeat sequences upstream of its *mobB* gene, whereas pRAS3.2 has only three iterons and four 6-bp repeat sequences. No biological studies were carried out in the initial report of the pRAS3 plasmids. As a contribution to our studies on the evolution of IncQ plasmids, our longer-term aim is to address the question of why two natural versions of the plasmid exist. Here we report on the major differences in the biology of the two plasmids. In addition, we discovered the presence of *repC* and *mobB* genes that were not detected when the sequence of pRAS3 plasmids was previously reported. We also discovered a putative toxin-antitoxin (TA) postsegregational system different from that found in other members of the IncQ plasmids and tested it for functionality.

The IncQ-1 plasmids are subdivided into incompatibility groups α, β, and γ, (31), whereas the IncQ-2 plasmids are subdivided into two incompatibility groups, α and β (14). In this work we also report on the incompatibility between the pRAS3 plasmids and other members of the IncQ-2 plasmid family as well as the IncQ-1 family plasmids. Furthermore, we compare the functional relatedness of the pRAS3 mobilization system with that of previously studied IncQ-2 plasmids.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *Escherichia coli* strains, cloning vectors, and plasmid constructs are shown in Table 1. Cultures of *E. coli* were grown in either Luria-Bertani broth (LB) or on Luria-Bertani agar (LA) plates. The growth medium was supplemented with antibiotics as required at the following concentrations: ampicillin (100 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}), kanamycin (30 μg ml^{-1}), nalidixic acid (35 μg ml^{-1}), streptomycin (35 μg ml^{-1}), tetracycline (10 μg ml^{-1}).

General DNA techniques. Plasmid preparation, restriction endonuclease digestions, gel electrophoresis, and cloning were carried out using standard methods (2, 33). Where no suitable restriction sites were present, single-strand DNA primers were designed and DNA fragments to be cloned were amplified by PCR. An initial denaturation step of 90 s at 94°C was followed by 30 cycles of denaturation (30 s at 94°C), a variable annealing step, and a standard elongation step (240 s at 72°C). Annealing temperatures were based on the average primer annealing temperature, and extension times were altered as required based on primer sequence (Table 2). PCR was performed in a Sprint temperature cycling system (Hybaid) using the Expand high-fidelity PCR system DNA polymerase (Roche Molecular Biochemicals). The sequences of all constructs that required a PCR step were confirmed by DNA sequencing using the dideoxy chain termination method and an ABI Prism 3100 genetic analyzer.

Reverse transcription-PCR (RT-PCR). RNA was isolated using the RiboPure RNA isolation kit (Ambion) from mid-logarithmic *E. coli* DH5α cultures carrying the respective plasmids. The quality of the RNA was assayed on a 1%

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa. Phone: 27-21-808 5848. Fax: 27-21-808 5846. E-mail: der@sun.ac.za.

▽ Published ahead of print on 14 August 2009.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[b]	Reference or source
Strains		
DH5α	ϕ80Δ*lacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp., Madison, WI
S17-1	*recA pro hsdR* (RP4-2 Tc::Mu Km::Tn*7*)	34
CSH56	F$^-$ *ara* Δ(*lac pro*) *supD nalA thi*	Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
EC100D *pir*$^+$	F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80Δ*lacZ*ΔM15 Δ*lacX74 recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK* λ$^-$ *rpsL nupG pir*$^+$(*DHFR*)	Epicentre Biotechnologies
Plasmid vectors		
EZ-Tn*5*	Km^r, R6K γ-*ori*	Epicentre Biotechnologies
pACYC177	Ap^r Km^r, p15A replicon, cloning vector	6
pBAD28	Ap^r Cm^r, arabinose-inducible expression vector, pACYC184 replicon	17
pBR322	Ap^r Tc^r, ColE1 replicon, cloning vector	4
pGEM-T	Ap^r, T-tailed PCR product cloning vector	Promega Corp., Madison, WI
pOU82	Ap^r, *lacZYA*, R1 replicon	15
pUC19	Ap^r, *lacZ′*, ColE1 replicon, cloning vector	41
Plasmid constructs		
pBAD28-mobCDEorf3	Ap^r Cm^r, 2.7-kb ApaLI-ScaI fragment containing pRAS3.1 *mobCDE* and *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-mobDEorf3	Ap^r Cm^r, 2.7-kb HindIII-ScaI fragment from pRAS3.1::mobC containing *mobDE* and *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-orf3	Ap^r Cm^r, 1.25-kb PstI-ScaI fragment from pRAS3.1::mobE containing *orf3* cloned behind P_{BAD} promoter	This study
pBAD28-repAC	Ap^r Cm^r, 2.7-kb SalI-StuI fragment containing pRAS3.1 *repAC* cloned behind P_{BAD} promoter	This study
pBAD28-repB	Ap^r Cm^r, 1,212-bp PCR fragment containing pRAS3.1 *repB* (nt position 9891 to 8688)[a] cloned behind P_{BAD} promoter	This study
pBAD28-repBAC	Cm^r, 3.5-kb PvuI-SphI fragment containing pRAS3.1 *pemIK*-like and *repAC* genes cloned into pBAD28-repB after inactivation of the pBAD28 PvuI site	This study
pBAD28-repC	Ap^r Cm^r, 1,015-bp PCR fragment containing the pRAS3.1 *repC* (nt position 6903 to 5889)[a] cloned behind P_{BAD} promoter	This study
pGEM-OriV3.1	Ap^r, 742-bp PCR fragment containing pRAS3.1 *oriV* (nt position 3123 to 2387)[a] cloned into pGEM-T	This study
pIE1108Cm	Cm^r, pIE1107 replicon with nonessential *oriVa* deleted and St^r and Km^r genes replaced by Cm^r gene	13
pIE1130	Cm^r Km^r Sm^r Su^r, natural 10,687-bp IncQ-like plasmid isolated from piggery manure	35
pOriTF14	Ap^r, 203-bp HindIII-NcoI fragment containing pTC-F14 *oriT* cloned into pUC19	40
pOriTFC2	Ap^r, 208-bp HhaI-HhaI fragment containing pTF-FC2 *oriT* cloned into pUC19	40
pOriT-RAS3	Ap^r, 196-bp PCR fragment containing pRAS3.1 *oriT* (nt position 11,820 through 0 to 176)[a] cloned into pGEM-T	This study
pOU82-TA	Ap^r, 731-bp PCR fragment containing pRAS3.1 *pemIK*-like genes (nt position 8819 to 8088)[a] cloned into pOU82	This study
pR6K.3.1.repC$^\Delta$	Km^r, pRAS3.1::tet with *repC* and *tetR* truncated by NheI-NheI deletion	This study
pR6K.3.2.repC$^\Delta$	Km^r, pRAS3.2::tet with *repC* and *tetR* truncated by NheI-NheI deletion	This study
pRAS3.1	Tc^r, natural 11,851-bp plasmid isolated from *Aeromonas salmonicida* subsp. *salmonicida* with four iterons and five 6-bp repeats	21
pRAS3.1.34	Tc^r, pRAS3.1 derivative with three iterons obtained by random ligation of short iteron fragments after BstEII digestion and four 6-bp repeats from pRAS3.2 by exchange of a 2.9-kb HindIII-PvuI region	This study
pRAS3.1.35	Tc^r, pRAS3.1 derivative with three iterons obtained by random ligation of short iteron fragments after BstEII digestion	This study
pRAS3.1Km	Km^r, pRAS3.1 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.1.44	Tc^r, pRAS3.1 derivative with four 6-bp repeats from pRAS3.2 by exchange of 2.9-kb HindIII-PvuI region	This study
pRAS3.1::mobC	Km^r Tc^r, pRAS3.1 with *mobC* interrupted by EZ-Tn*5* at position 296	This study
pRAS3.1::mobD	Km^r Tc^r, pRAS3.1 with *mobD* interrupted by EZ-Tn*5* at position 1082	This study
pRAS3.1::mobE1	Km^r Tc^r, pRAS3.1 with *mobE* interrupted by EZ-Tn*5* at position 1586	This study
pRAS3.1::mobE2	Km^r Tc^r, pRAS3.1 with *mobE* interrupted by EZ-Tn*5* at position 1614	This study
pRAS3.1::orf3	Km^r Tc^r, pRAS3.1 with *orf3* interrupted by EZ-Tn*5* at position 2089	This study
pRAS3.1::repB	Km^r Tc^r, pRAS3.1 with *repB* interrupted by EZ-Tn*5* at the PvuI site	This study
pRAS3.1::tetAR	Km^r, pRAS3.1 with *tetAR* interrupted by EZ-Tn*5* at the SphI-SphI sites	This study
pRAS3.2	Tc^r, natural 11,823-bp plasmid isolated from atypical *Aeromonas salmonicida* with three iterons and four 6-bp repeats	21
pRAS3.2Km	Km^r, pRAS3.2 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.2::tetAR	Km^r, pRAS3.2 *tetAR* interrupted by EZ-Tn*5* at the SphI-SphI sites	This study
pTF-FC2Cm	Cm^r, natural pTF-FC2 plasmid with chloramphenicol resistance gene cloned into Tn*5467* (called pDR412 in previous manuscripts)	32
pTF-FC2Tet	Tc^r, Cm^r of pDR412 replaced by Tc^r of pACYC184 at the XbaI and EcoRV sites	G. Matcher
pTC-F14Cm	Cm^r, natural pTC-F14 plasmid with Cm^r inserted at the BamHI site	13
pTC-F14Km	Km^r, pTC-F14Cm with Cm^r replaced by Km^r from Tn*5*	40
R6K-OriV3.1	Km^r, pRAS3.1 *oriV* from pGEM-OriV3.1 transferred to EZ-Tn*5*	This study
RSF1010K	Km^r, 1,1704 bp of RSF1010 replaced by Tn*903*	G. Ziegelin

[a] The nucleotide (nt) positions refer to the positions on pRAS3.1 to which the PCR fragments correspond.
[b] Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin; Tc, tetracycline.

morpholinepropanesulfonic acid–EDTA agarose gel and quantified using a NanoDrop spectrophotometer. RNA (1 μg) was converted to cDNA using the Transcriptor first-strand cDNA synthesis kit (Roche Diagnostics).

Qualitative gene expression to verify whether *orf3* is expressed as part of the *mobCDE* operon was assayed using the FastStart *Taq* DNA polymerase (Roche Diagnostics) according to the manufacturer's protocol. Two microliters, or ~100 ng, of the cDNA was used in each reaction mixture with a final $MgCl_2$ concentration of 2.3 mM. The GC-rich solution was added to a 1× final concentration

TABLE 2. Primers used for cloning, quantitative gene expression, and plasmid copy number assays

Primer	Sequence
E.coli GAPA Fwd	5′-TGTTAGACGCTGATTACATGG-3′
E.coli GAPA Rev	5′-CTTTAACGAACATCGGAGTGT-3′
pRAS3A Fwd	5′-GGAGCCACTATCGACTACG-3′
pRAS3A Rev	5′-GAAGCAGCCCAGTAGTAGG-3′
pRAS3MobC Fwd	5′-ACACAACAGAGCAGCTAGA-3′
pRAS3MobC Rev	5′-TCTGGTCAAGCGTGTATCC-3′
pRAS3MobE Fwd	5′-GCATCAGCGGAAGCAGCC-3′
pRAS3MobE Rev	5′-GCCTATCGCACTTCGCC-3′
pRAS3ORF3 Fwd	5′-CCGTTCGATCTGGTAGACC-3′
pRAS3ORF3 Rev	5′-GTTCTTCCATGTCTCGACG-3′
pRAS3OriT Fwd	5′-CTTGCAGGATGAGCCAGAC-3′
pRAS3OriT Rev	5′-TGGTTGCGGAGTTGACAG-3′
pRAS3OriV Fwd	5′-GTCGAATTCGTACATTATGTTTCG-3′
pRAS3OriV Rev	5′-ATAGGTACCAGTCTTTTCCATCC-3′
pRAS3REPB Fwd[a]	5′-ACGAATTCATGTGCGGGAAG-3′
pRAS3REPB Rev[a]	5′-TCACTGCAGTGCAACATTGTA-3′
pRAS3REPB2 Fwd	5′-GCAACTATCAGGCCATCAT-3′
pRAS3REPB2 Rev	5′-TTGGGCTTGCGGTTCTC-3′
pRAS3REPC2 Rev[a]	5′-TATCTGCAGCTTGAACAGGTG-3′
pRAS3REPC3 Fwd[a,b]	5′-TAGAATTC***AGGAGG***AGGGCT**ATG**ACTCAGCAGC-3′
pRAS3SS2 Fwd[a]	5′-GAATTCAGTGGGAGAAGCTGGAAG-3′
pRAS3SS2 Rev[a]	5′-GGATCCGGAATGGTGTAGATCGTT-3′
R6KKANR Fwd	5′-CCATTCTCACCGGATTCAG-3′
R6KKANR Rev	5′-TCACCGAGGCAGTTCCATA-3′

[a] The primer includes an endonuclease restriction site (underlined).
[b] The primer overlaps the start codon (shown in bold) and includes an artificial ribosome binding site (in bold italics).

whenever the pRAS3MOBE forward and reverse primers (Table 1) were used, and 2.5% dimethyl sulfoxide was added to each PCR mixture when the pRAS3MOBC forward or reverse primers were used. The reaction mixtures were subjected to 35 cycles of denaturation, elongation, and extension as described above. The RNA samples were assayed for the presence of contaminating DNA by using ~400 ng of RNA in control reaction mixtures. PCR products were analyzed on a 1% agarose gel.

Quantitative gene expression of the *repB* and *orf3* genes using the pRAS3REPB2 and pRAS3ORF3 primer sets was assayed using a LightCycler as described above. The cDNA was diluted twofold and a total of 50 ng was used in each reaction mixture. The amplification efficiencies were determined as described above using serial dilutions of the cDNA, and the reaction parameters were set as described above for the plasmid copy number determinations. Relative gene expression was determined using the REST analysis tool (30). The R6KkanR primer set was used as a calibrator.

Copy number determinations. Total genomic DNA was prepared from *E. coli* DH5α cultures containing the respective plasmids during exponential growth. Cells were grown overnight (in the presence of antibiotics), reinoculated (1/100) into 50 ml of prewarmed LB medium (no antibiotics), and grown while shaking at 37°C to an optical density at 600 nm of ~0.8. Total genomic DNA was extracted from 1 ml of culture using the QIAamp DNA minikit (Qiagen). The genomic DNA was eluted in 60 μl elution buffer, and the concentration and purity were checked using a Nanodrop spectrophotometer.

Real-time quantitative PCR (qPCR) amplification was performed using a LightCycler (version 2.0) with the LightCycler FastStart DNA master SYBR green I kit (Roche Diagnostics). A total of 4 ng of total DNA was added to each amplification reaction mixture, and the thermal cycling protocol of Lee et al. (22) was followed, except that primer annealing was at 56°C for 4 s and DNA extension was at 72°C for 15 s.

To generate standard curves for plasmid copy number determinations, the *gapA* amplicon (Table 2) was cloned into pGEM-T(Easy). The pGEM-*gapA* and pRAS3.1 plasmids were extracted from *E. coli* DH5α using a Nucleobond AX plasmid DNA purification kit. The concentrations of both DNA samples were determined (six replicates) by using a NanoDrop spectrophotometer. A 10-fold dilution series (10^0 to 10^{-5}) was set up for each plasmid. Samples were amplified with thermal cycle parameters as specified above, and the threshold cycle (C_t) values were plotted against the number of DNA molecules in each sample. The R^2 value for both calibration standard curves was greater than 0.9995. Absolute plasmid copy number was determined by amplification of pRAS3.1 and pRAS3.2 in the same cycle as the calibration curves. The C_t values were used to extrapolate the total amount of chromosome and plasmid present in each sample from the standard curves, using the LightCycler software (version 3.5) according to the calculations of Lee et al. (22). Relative plasmid copy numbers were determined using the same conditions and cycle parameters as described above. All copy numbers were determined relative to pRAS3.1.35 by using the REST analysis tool (30). The same standard curves that were used for absolute copy number determinations were used to calculate the amplification efficiency.

Plasmid copy numbers in the presence of excess replication proteins which were expressed from the P_{BAD} arabinose-inducible promoter of pBAD28 were measured by means of qPCR relative to the same sample with only pBAD28 in *trans.* Addition of arabinose to Luria-Bertani broth resulted in slow cell growth and proved unnecessary, as *rep* genes cloned behind the P_{BAD} promoter were sufficiently expressed to be able to complement their respective *rep* deletion mutants.

Mobilization assay. *E. coli* S17.1 donor and *E. coli* CSH56 recipient cells were cultured separately overnight with appropriate antibiotic selection. Cells were washed three times in phosphate-buffered saline (PBS, pH 7.4) and mixed in a donor-to-recipient ratio of 1:10 or 1:100. One hundred microliters of this mixture was spotted onto an LA plate and incubated at 37°C for 60 min (unless specified otherwise). The agar plug was excised and suspended in 10 ml PBS (pH 7.4) and vigorously shaken, after which 8 ml was collected, pelleted, and resuspended in 1 ml PBS (pH 7.4). Serial dilutions were plated onto donor and transconjugant selective media, and the number of transconjugants per donor was calculated.

Plasmid stability assay. Stability assays using the pOU82-based test system were performed by growing plasmid-containing *E. coli* cells without selection in 5 ml LB at 30°C for 4 days, with transfer of ~1,000 cells to fresh LB at ~20-generation intervals. Samples taken at ~20-generation intervals were diluted in PBS (pH 7.4) and plated onto LA plates supplemented with 40 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and incubated at 37°C. The percentage plasmid loss was determined by calculating the ratio of plasmid-containing (blue) to plasmid-free (white) colonies (15).

Incompatibility assays. Plasmid-containing *E. coli* DH5α cells were transformed with a second plasmid and plated on LA plates with antibiotic selection for both plasmids. Single colonies were picked into LB containing appropriate antibiotics and incubated overnight at 30°C. Survival of the plasmids was then tested by removing selection for both plasmids and growing the cells in 5 ml LB for ~100 generations, with transfer of approximately ~1,000 cells to fresh medium at ~20-generation intervals. Finally, 50 colonies were replica plated to antibiotic-containing LA plates to score for plasmid retention. Cells containing individual plasmids were similarly grown and plated as a control to account for spontaneous plasmid loss.

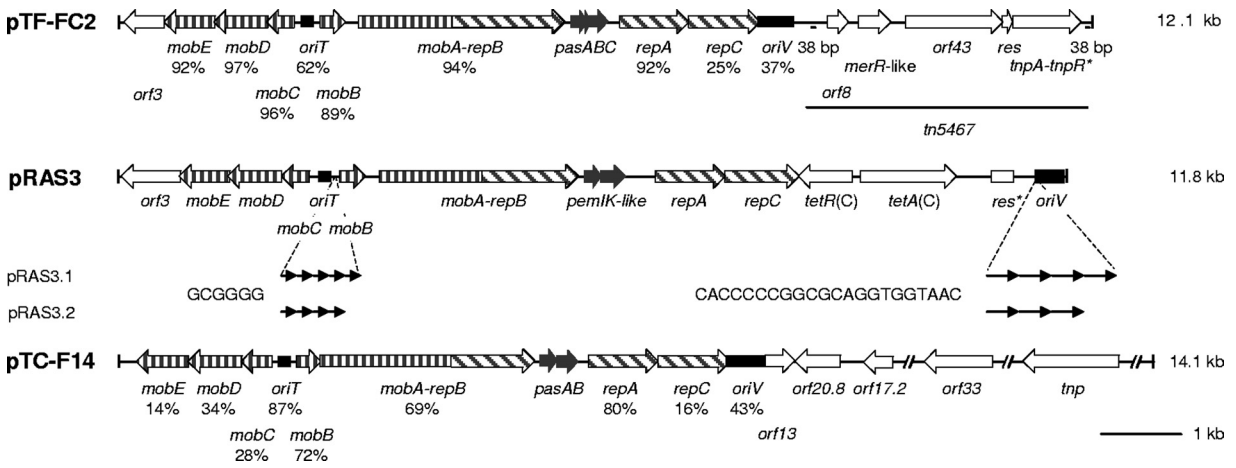


FIG. 1. Comparison of the genetic maps of the pRAS3 plasmids with pTF-FC2 and pTC-F14. Percentages below the plasmid backbone genes of pTF-FC2 and pTC-F14 indicate the percent amino acid sequence identity of the gene product with that of the pRAS3 plasmids. Percentages below the *oriT* and *oriV* regions indicate nucleotide sequence identity. Plasmids pRAS3.1 and pRAS3.2 have different numbers of 6-bp repeats and 22-bp iterons, while the nucleotide sequence of each repeat or iteron is identical, as indicated below pRAS3.

Displacement assays. Competent *E. coli* DH5α cells containing a resident plasmid were transformed with a second incoming plasmid and plated on antibiotic-containing medium that selected only for the incoming plasmid. Sixteen colonies were picked and plated onto three sets of solid media, two containing single antibiotics to separately test for the presence of the resident or incoming plasmid and one containing no antibiotics as a control for cell viability. Controls to check for spontaneous loss of the resident plasmids were carried out using the same procedure except that the initial competent *E. coli* cells containing the resident plasmids were taken through a cycle of growth on solid medium without antibiotic selection before testing for retention of the resident plasmid.

Random knockouts and screening for an incompatibility determinant. Random knockouts of pRAS3.1 were generated using the EZ-Tn*5* transposon system (Epicenter Biotechnologies). The transposon (0.025 pmol) mutagenesis was carried out in vitro using 0.05 pmol pRAS3.1 as per the manufacturer's protocol. The reaction mixture was transformed into electrocompetent *E. coli* EC100D, and all the colonies were scraped off the plates into 100 ml fresh LB medium and incubated for 1 h at 37°C. The plasmid DNA was purified using the Nucleobond AX plasmid purification kit (Macherey-Nagel).

The bank of random knockouts was screened for a compatible phenotype by transforming 1 ng of the plasmid DNA into electrocompetent *E. coli* EC100D cells containing a resident pTF-FC2Cm (pTF-FC2 with Cm^r gene) plasmid. EZ-Tn*5* and pRAS3.1::Tet were used as controls for compatibility and incompatibility, respectively, on double-selective plates. The expression mix was spread on plates containing antibiotic selection for both plasmids, and colonies were replica plated for two rounds of growth on plates selecting for the pRAS3.1 knockouts only. This was followed by selection for cells that still retained pTF-FC2Cm. After restriction analysis of the extracted plasmid DNA, the random knockout plasmids from selected positive colonies were separated from the coresident pTF-FC2Cm plasmid by transforming the extracted DNA into *E. coli* DH5α and selecting for only the pRAS3.1 knockouts that had allowed pTF-FC2Cm to be retained.

Sequence analysis and bioinformatics. The DNA sequence previously deposited as pRAS3.1 (accession number AY043298) and pRAS3.2 (accession number AY043299) by L'Abée-Lund and Sørum (21) was analyzed using a variety of software programs but mainly a combination of the Glimmer 2 (www.tigr.org/softlab) (9) and DNAMAN (Lynnon BioSoft) programs. Comparison searches were performed using the gapped BLAST program at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (1).

RESULTS

Reanalysis of the sequences of pRAS3.1 and pRAS3.2. An analysis of the sequences of the pRAS3 plasmids was previously reported by L'Abée-Lund and Sørum (21). Given the high similarity in gene organization and sequence with plasmid pTF-FC2, we were surprised by the apparent absence of *mobB* and *repC* genes in the pRAS3 plasmids. When the sequence data were reanalyzed, four genes were identified that were not detected previously. These were the *mobB* and *repC* genes, with the amino acid sequences of their products being 87% and 37% identical to the equivalent gene products of pTF-FC2, respectively. In addition two genes were identified for what appears to be a TA postsegregation killing system. The TA genes are distantly related to the previously published *pemIK* (*parDE*) (5, 38) and *mazEF* (*chpAI chpAK*) (24) systems. A comparison of the pRAS3 plasmids with pTF-FC2 and pTC-F14, the only other two plasmids of the IncQ-2 group identified to date, is shown in Fig. 1. In general, the nucleotide sequences of the backbones of the pRAS3 plasmids are more closely related to pTF-FC2, with the exceptions being the sequences of the *oriT* and *oriV* regions, whereas the pRAS3 plasmids were more closely related to pTC-F14. The putative TA system of the pRAS3 plasmids was unrelated to that of either pTF-FC2 or pTC-F14 (which are closely related to each other [8]). However, in all plasmids the TA system is situated in a similar position between the *repB* and *repA* genes.

Copy numbers of pRAS3.1 and pRAS3.2. The absolute and relative plasmid copy numbers (PCN) of pRAS3.1 and pRAS3.2 were determined in *E. coli* DH5α by quantitative real-time PCR using the chromosomal *gapA* gene as a calibration standard. The copy number of pRAS3.1 was found to be 45 ± 13 (n = 11) plasmids per chromosome and that for pRAS3.2 was 30 ± 5 (n = 4). This large difference in copy number was surprising, as pRAS3.2 has three *oriV*-associated 22-bp iterons while pRAS3.1 has four of these identical 22-bp iterons, and one would expect the plasmid with fewer iterons to have the higher copy number. Furthermore, other IncQ-2 family plasmids have been reported to have a considerably lower PCN of 12 to 16 plasmids per chromosome (10, 36), which is two- to threefold lower than determined for pRAS3.2 and pRAS3.1, respectively. The PCN of the well-known cloning vector pBR322 has been determined to be ~18 plasmids per chromosome based on both absolute and relative quantifica-

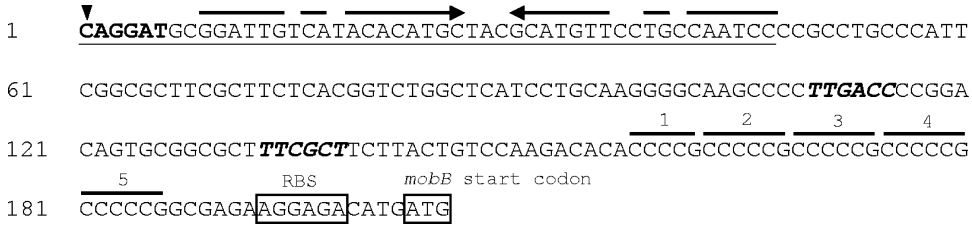


FIG. 2. The intergenic sequence between the *oriT* and *mobB* of pRAS3.1, showing the position of the 6-bp repeats. The *oriT* is underlined and the imperfect inverted repeat within the *oriT* is indicated by broken inverted arrows. The conserved hexameric nick site is indicated in bold with a vertical arrow indicating the putative nick position. The 6-bp CCCCCG repeats are labeled 1 to 5. The first repeat consists of only 5 bp, as it lacks a cytosine base. A putative promoter with a near-consensus −35 region and a weak −10 region is shown in bold italics and is separated by a 17-bp spacer.

tion real-time PCR methods (22). As the tetracycline resistance gene of pBR322 is identical to that of pRAS3.2, the PCN of pBR322 could be quantified using the same primer sets. It was therefore included in an additional set of real-time PCR assays as a copy number control. The relative PCN of pRAS3.1 and pRAS3.2 were 2.3- and 1.7-fold higher than that of pBR322, respectively. Based on a copy number of 18 for pBR322, this is equal to PCN values of approximately 41 for pRAS3.1 and 30 for pRAS3.2.

Reason for the difference in copy number between pRAS3.1 and pRAS3.2. The observation that pRAS3.1 with four 22-bp *oriV*-associated iterons had a higher copy number than pRAS3.2 with three 22-bp iterons was unexpected. We therefore investigated the effect on PCN of the additional 6-bp CCCCCG repeat upstream of the *mobB* gene of pRAS3.1. The repeats are located 6 bp upstream of a putative ribosomal binding site of the *mobB* gene (Fig. 2). Three derivatives of pRAS3.1 (five 6-bp repeats and four 22-bp iterons) were constructed to isolate the effects of the 6-bp repeats and 22-bp iterons on PCN (Table 1). When the number of 22-bp iterons in pRAS3.1 was decreased from four to three (pRAS3.1.35), the calculated PCN increased from approximately 41 to 59 copies (Table 3). The five 6-bp repeats in this construct were then exchanged for those in pRAS3.2 (Table 1) to give a construct still with three 22-bp iterons but now with only four 6-bp repeats (pRAS3.1.34). If the number of 6-bp repeats had an influence on PCN, the PCN of pRAS3.1.34 should fall to that of pRAS3.2, as these two plasmids have equal 6-bp repeat and 22-bp iteron copy numbers. This is what was found, as the relative PCN of pRAS3.1.34 decreased by approximately 47% to 31 copies, the same as that of pRAS3.2. When the number of 22-bp iterons in this lower-copy-number plasmid was increased from three to four (pRAS3.1.44) the PCN decreased further to approximately 23 copies. Therefore, the presence of a fifth 6-bp repeat resulted in the PCN of a plasmid with four 22-bp iterons to increase by 1.77-fold (pRAS3.1.44 compared with pRAS3.1) and a plasmid with three 22-bp iterons increased by 1.89-fold (pRAS3.1.34 compared with pRAS3.1.35). A decrease in the number of 22-bp iterons from four to three also resulted in an increase in PCN. This was 1.45-fold in a plasmid with five 6-bp repeats (pRAS3.1 compared with pRAS3.1.35) and 1.35-fold in a plasmid with four 6-bp repeats (pRAS3.1.44 compared with pRAS3.1.34).

TABLE 3. Effects of the number of 6-bp repeats and 22-bp iterons on plasmid copy number

Plasmid construct	No. of 6-bp repeats	No. of 22-bp iterons	Relative plasmid copy no.[b]	Calculated plasmid copy no.[c]
pRAS3.2	4	3	0.51 ± 0.09	30 ± 5
pRAS3.1	5	4	0.69 ± 0.062	41 ± 4
pRAS3.1.35[a]	5	3	1.0	59
pRAS3.1.34	4	3	0.53 ± 0.002	31 ± 1
pRAS3.1.44	4	4	0.39 ± 0.037	23 ± 2

[a] pRAS3.1.35 served as the reference for the determination of relative copy numbers and standard deviations.
[b] The number of replicates for relative copy number determinations was four to six, and a *P* value of 0.001 was obtained for each qPCR experiment.
[c] Plasmid numbers were calculated to the nearest whole plasmid.

These results suggested that it was the presence of an additional 6-bp repeat upstream of the *mobB* gene that resulted in a higher PCN and raised the question of how the extra 6-bp repeat exerted this effect on PCN. The most obvious possibility was that the 6-bp repeat affected the level of expression of *mobB* as well as the downstream *mobA*/*repB* genes (Fig. 2). To test this, the levels of expression of *mobB*, *mobA*/*repB*, and the divergently transcribed *mobCDE* and *orf3* operon were determined for pRAS3.1 and compared with pRAS3.2 by using qPCR. To carry out the comparison, an R6K *oriV* and kanamycin resistance gene (EZ-Tn*5*) were cloned into the *tetAR* genes of both pRAS3.1 and pRAS3.2, whereafter the native replicons were inactivated through truncation of the *repC* and *tetR* genes. This ensured that both plasmids had the same copy number and allowed the relative levels of gene expression to be determined. The expression of *mobA*/*repB* in the case of a pRAS3.1 equivalent (five 6-bp repeats; pR6K.3.1.repC$^{\Delta}$) was approximately twofold higher (2.0 ± 0.9; $n = 12$; $P = 0.048$) relative to a pRAS3.2 equivalent (four 6-bp repeats; pR6K.3.2.repC$^{\Delta}$). In contrast, expression in the opposite direction (*mobCDE-orf3*) was the same (1.1 ± 0.5; $n = 4$; $P = 0.875$) for both pRAS3.1 and pRAS3.2 equivalents. These results suggested that the reason for the increase in PCN of pRAS3.1 compared to pRAS3.2 was that the additional 6-bp repeat resulted in an increase in *mobB-mobA*/*repB* expression and that increased expression of *repB* was the actual cause.

Effect of increased *repBAC* expression on plasmid copy number. To confirm that the additional *repB* expression resulted in an increased PCN as well as to determine whether the products of the *repA* and *repC* genes affected PCN, the *repC*, *repAC*, and *repB* genes were cloned behind the P_{BAD} promoter of the vector pBAD28. The arabinose-inducible *repB* construct was

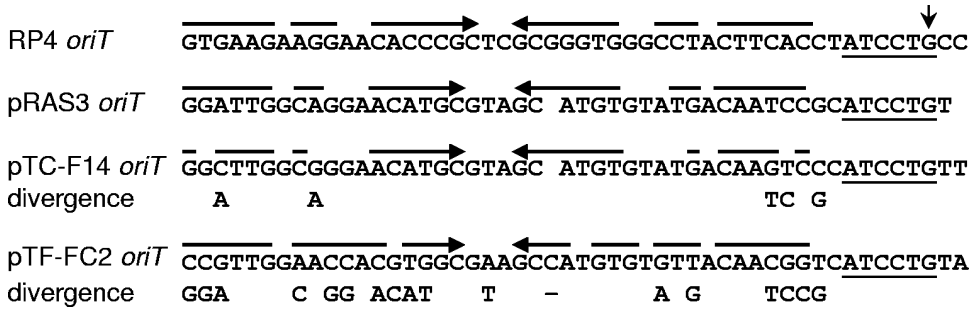


FIG. 3. Alignment of *oriT* regions of IncQ-2 plasmids and the IncPα plasmid RP4, showing the sequence divergence that could be tolerated by the Mob proteins of plasmid pRAS3 while they were still able to mobilize DNA from an *oriT*. A vertical arrow indicates the relaxase *nic* site at which single-stranded cleavage takes place as determined for plasmid RK2/RP4 (29).

placed in *E. coli*(pRAS3.1.34) cells and the copy number was compared with the same cells containing the pBAD28 vector only. With the *repB*-containing construct in *trans*, the PCN of pRAS3.1.34 was increased approximately 2.2-fold ± 0.53-fold ($n = 4$; $P = 0.001$) relative to the vector control. This is approximately equal to the 1.96-fold difference in PCN between plasmids differing only in their number of 6-bp repeats, such as pRAS3.2 (three iterons; four 6-bp repeats) and pRAS3.1.35 (three iterons; five 6-bp repeats), as shown in Table 3. Therefore, the additional *repB* expression raised the copy number of a plasmid which contained four 6-bp repeats to approximately the same as that of a plasmid that had five 6-bp repeats.

To examine the effects of additional *repC* or *repAC* gene products, plasmid pRAS3.1.35 was used, as this plasmid with five 6-bp repeats already had high levels of *repB* expression and it was possible that the *repC* or *repAC* gene products were limiting. Expression of *repC* in *trans* did not result in a change in PCN (1.02 ± 0.22; $n = 4$; $P = 0.874$), whereas expression of *repAC* (pBAD28-repAC) resulted in an approximately 30% reduction in PCN (0.67 ± 0.13; $n = 4$; $P = 0.008$). This reduction in PCN due to overexpression of *repAC* was consistent with that reported by Matcher and Rawlings (25).

Comparison of the mobilization frequencies of pRAS3.1 and pRAS3.2. As the mobilization frequencies of pRAS3.1 and pRAS3.2 were not reported in the study by L'Abée-Lund and Sørum (21), we determined the mobilization frequency between *E. coli* S17.1 donor and *E. coli* CSH56 recipient cells. This was found to be 0.032 ± 0.014 for pRAS3.1 and 0.021 ± 0.013 for pRAS3.2. Experiments using plasmids with different numbers of 6-bp repeats but identical 22-bp iteron numbers, as well as plasmids with identical 6-bp repeats but different 22-bp iteron numbers, showed that neither had any significant effect on the mobilization frequencies. Thus, although increased transcription in the direction *mobB-mobA/repB* compared with *mobCDEorf3* as a result of the extra 6-bp repeat had an effect on plasmid replication, it did not have a marked effect on plasmid mobilization.

Functional relatedness of mobilization regions within the IncQ-2 plasmids. Prior to plasmid conjugation a relaxase cleaves one of the DNA strands at the origin of transfer (*oriT*), forming a covalent protein-DNA complex in the donor cell that is transferred to the recipient. In the case of plasmid F, the relaxase-*oriT* recognition has a high degree of structural and sequence specificity (12, 26). In contrast, the *oriT* region of the IncQ-1 plasmid R1162 is small, structurally simple, and can accommodate base pair changes without a complete loss of function (3, 20, 27). Since the five-protein mobilization operon of the IncQ-2 plasmids has more in common with the IncP plasmids than with the three mobilization protein operons of IncQ-1 plasmids, we tested whether the relaxase-*oriT* recognition of the pRAS3 plasmids was relaxed like that of R1162 or specific like plasmid F and related plasmids. The amino acid sequences of the *mob* gene products of the pRAS3 plasmids were closely related to that of pTF-FC2, with an average amino acid sequence identity of over 90% for the five *mob* gene products (Fig. 1). This contrasted with pTC-F14, where the average amino acid sequence identity was approximately 25% for the MobCDE genes and 70% for the MobAB genes. However, when the nucleotide sequences of the *oriT* regions were compared (Fig. 3), the *oriT* of the pRAS3 plasmids was considerably more related to that of pTC-F14 (87% identity) than to pTF-FC2 (62% identity). We therefore tested whether the *oriT* of the pRAS3 plasmids was able to be mobilized by *E. coli* S17.1 cells containing the conjugative plasmid RP4 by the products of the *mob* genes of pTF-FC2 and pTC-F14. A 196-bp fragment from pRAS3.1 containing the *oriT* was PCR amplified and cloned into the nonmobilizable pUC19 vector to produce pOriT-RAS3, and the sequence was confirmed by DNA sequencing. Plasmid pOriT-RAS3 was mobilized at the saturation frequency within 60 min of mating by *E. coli* S17.1 when either pRAS3.1 or pRAS3.2 was coresident, while no transconjugants were obtained in the absence of coresident pRAS3 plasmids. When either pTF-FC2Cm or pTC-F14Cm was coresident instead of the pRAS3 plasmids, no transconjugants were obtained. However, in the reverse experiments, coresident pRAS3.1 plasmid was able to mobilize pUC19 vector containing the pTF-FC2 and pTC-F14 *oriT*s at frequencies of $1.20\ (\pm 0.44) \times 10^{-1}$ and $3.42\ (\pm 1.64) \times 10^{-4}$ transconjugants per donor in 60 min, respectively. The ability of Mob proteins of pRAS3 to mobilize the *oriT*s pTF-FC2 and pTC-F14 suggested that like R1162 these *oriT*s are also relaxed and that the Mob proteins pRAS3 can mobilize *oriT*s with very different sequences.

The toxin-antitoxin system on pRAS3 is functional. Careful analysis of the sequence of pRAS3.1 and pRAS3.2 revealed the presence of two small tandem ORFs encoding proteins of 74 and 108 amino acids (aa), respectively, situated between *repB*

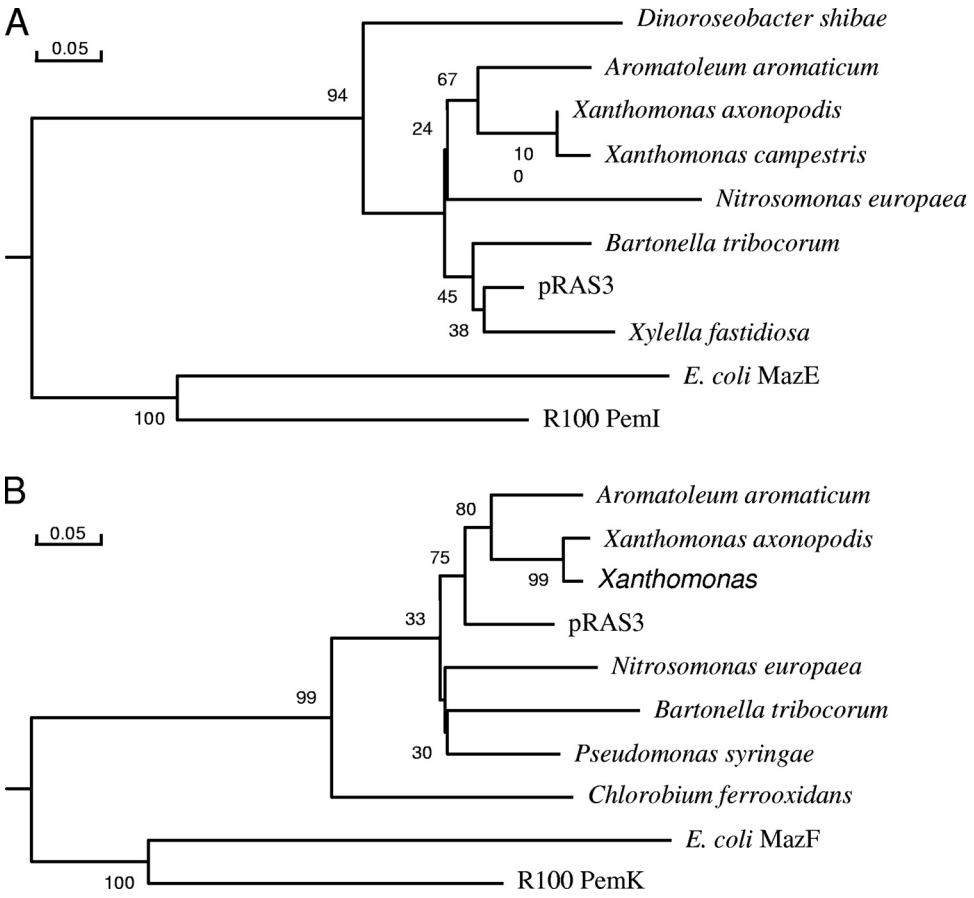


FIG. 4. Phylogenies of toxin-antitoxin proteins of pRAS3 and comparison with closely related proteins as well as the more distantly related PemIK and MazEF proteins. (A) Antitoxins were as follows: *Aromatoleum aromatium*, CAI08016; *Bartonella tribocorum*, CAK00897; *Dinoroseobacter shibae*, YP_001541878; *Nitrosomonas europaea* ATCC 19718, CAD85218; *Xanthomonas axonopolis* pv. *citri strain* 306, NP_644761; *Xanthomonas campestris* pv. *vesicatoria* strain 85-10, CAJ19793; *Xylella fastidiosa* Ann-1, ZP_00682677; *E. coli* MG1165 MazE, AAA69293; plasmid R100 PemI, P13975. (B) Toxins were as follows: *Aromatoleum aromatium*, CAI08015; *Bartonella tribocorum*, CAK00896; *Chlorobium ferrooxidans*, EAT59633; *Nitrosomonas europaea* ATCC 19718, CAD85217; *Pseudomonas syringae* pv. *phaseolicola*, AAZ37969; *Xanthomonas axonopolis* pv. *citri* strain 306, NP_644760; *Xanthomonas campestris* pv. *vesicatoria* strain 85-10, CAJ19792; *E. coli* MG1165 MazF, AAA69292; plasmid R100 PemK, P13976.

and *repA*. BLAST analysis of the proteins from these ORFs indicated that they were most closely related to pairs of proteins of similar size from adjacent ORFs detected in genome sequencing data obtained from a number of other bacteria, such as *Xanthomonas campestris*, *Xanthomonas axonopodis*, *Aeromatoleum aromaticum*, and *Nitrosomonas europaea* (Fig. 4A and B). These proteins are related to toxin-antitoxin post-segregational killing plasmid stability systems and are listed as being either PemIK-like or MazEF-like, although they group in a cluster well separated from either of these protein pairs. No members of the clusters shown in Fig. 4A and B, besides the distantly related PemIK and MazEF, have been tested for toxin-antitoxin activity. Using PCR we amplified the two ORFs from pRAS3.1 and cloned them into the segregationally unstable test plasmid pOU82, to give plasmid pOU82-TA. When grown without plasmid selection for 72 generations, approximately 98% of *E. coli* DH5α cells retained pOU82-TA, whereas only 35% of cells retained pOU82 (Fig. 5). This enhanced plasmid stability suggests that the other untested pro-

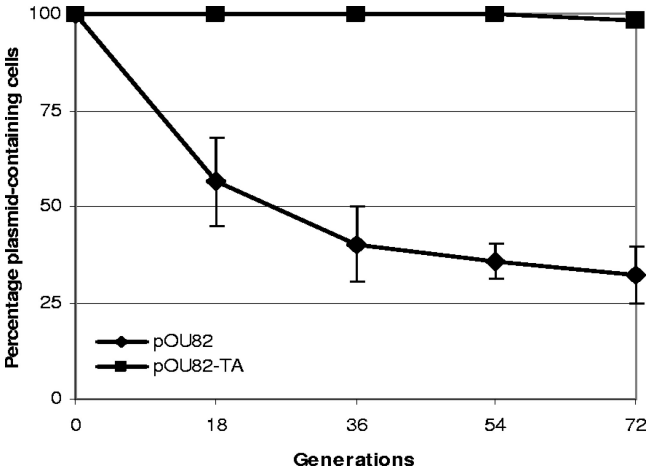


FIG. 5. Loss of the low-copy-number test plasmid, pOU82, with and without the PemIK-like TA genes from pRAS3 in the absence of plasmid selection.

teins shown in Fig. 4A and B are also likely to be toxin-antitoxin pairs.

Compatibility of pRAS3.1 and pRAS3.2 with other IncQ plasmids. We tested whether plasmids pRAS3.1 and pRAS3.2 were compatible with different IncQ-1 and IncQ-2 plasmids. Plasmids RSF1010 (16), pIE1108 (35), and pIE1130 (37) were used as representatives of the α, β, and γ incompatibility groups of InQ-1 plasmids, respectively. The incompatibility assay involved the placement of the two test plasmids into a single *E. coli* DH5α host cell and then testing for how many cells retained both plasmids after approximately 34 generations in the absence of selection. When present on their own, plasmids RSF1010K, pIE1108Cm, and pIE1130 were slightly less stable after 34 generations (94 to 100% retention) than pRAS3.1 or pRAS3.2 (100% retention). When either pRAS3.1 or pRAS3.2 was coresident with plasmid RSF1010K, pIE1108Cm, or pIE1130, the stability of each plasmid was indistinguishable from that when it was present in the *E. coli* host on its own.

When testing for the compatibility of pRAS3.1 and pRAS3.2 with pTF-FC2 and pTC-F14, members of the IncQ-2 α and β incompatibility groups, respectively, a different compatibility assay had to be used. The pRAS3.1 and pRAS3.2 plasmids were so highly incompatible with either pTF-FC2Cm or pTC-F14Cm that we were unable to isolate *E. coli* host cells containing two test plasmids. When attempts were made to transform competent *E. coli* pRAS3.1- or pRAS3.2-containing cells with either pTF-FC2Cm or pTC-F14Cm, no transformants were isolated, although the competent cells could be readily transformed with other plasmids, such as pUC19 and pACYC177. In the reciprocal experiments with either pTF-FC2Cm or pTC-F14Cm being resident in the competent cells prior to transformation with either pRAS3.1 or pRAS3.2, successful transformation by the incoming plasmid was achieved but the resident plasmid was immediately displaced.

Search for the source of strong plasmid incompatibility. Experiments were carried out in an attempt to identify the reason for this strong incompatibility. A functional 751-bp pRAS3.1 *oriV* region containing the four 22-bp iteron DNA was cloned into the *E. coli* pGEM-T vector to give pGEM-OriV3.1. However, this construct was fully compatible with either the pTF-FC2 or pTC-F14 replicons and therefore the iterons and associated DNA were not the cause of the strong incompatibility. The pRAS3.1 *repC*, *repAC*, and *repB* genes were cloned behind the P_{BAD} promoter of the pBAD28 expression vector and shown to be expressed by their ability to complement pRAS3.1 *repC* or *repB* mutants. Again, none of these clones displaced pTF-FC2 or pTC-F14. Next, the region containing the *repBAC* genes, including the genes for the *pemIK*-like TA system, was cloned behind the P_{BAD} promoterto give pBAD28-repBAC. This plasmid could support the replication of the 751-bp pRAS3.1 *oriV* region when cloned into a R6K vector (EZ-Tn*5*), which was unable to replicate in *E. coli* DH5α unless pBAD28-repBAC was present in *trans*. This showed that the *repBAC* fragment was functional, but this fragment also did not displace the pTF-FC2 or pTC-F14 replicons.

As plasmid incompatibility did not appear to be associated with the replicon region, we tested whether we could knock out whatever was responsible for incompatibility using the EZ-Tn*5* transposon mutagenesis system. A bank of random mutants of pRAS3.1 was generated and screened for mutants that did not displace *E. coli* containing a resident pTF-FC2Cm plasmid. Approximately 5% of mutants displayed a compatible phenotype by selection on plates for both plasmids. All 200 compatible pRAS3.1 mutant plasmids tested retained pTF-FC2Cm after two rounds of growth from a single cell to a colony when selecting for only the pRAS3.1 mutants. Restriction endonuclease analysis using BamHI and SalI for 48 of the 200 mutants indicated that the mutations fell into five groups, with all transposons located within a 1.75-kb region. Nucleotide sequencing of a representative of each group indicated that the five insertions were evenly spaced within the region containing the *mobCDE* genes and a previously unreported downstream ORF (called *orf3*), with one insertion in each gene and two in *mobE*. To determine whether *orf3* is expressed from the same transcript as *mobCDE*, mRNA was isolated from *E. coli* DH5α(pRAS3.1) cells and analyzed by RT-PCR. Primer sets to *mobC* and *orf3* or to *mobE* and *orf3* gave positive amplification products of the predicted sizes, while a lack of amplification products in experiments in which reverse transcriptase was omitted indicated that amplification products were not due to DNA contamination (data not shown). We concluded that *orf3* is expressed as part of the *mobCDE* operon. Since *orf3* is the last gene in the series of four genes, we predicted that insertion into the upstream *mob* genes presumably affected expression of *orf3* and that it was *orf3* that was probably responsible for plasmid incompatibility.

Role of *orf3*. *orf3* is 753 bp long and encodes a predicted protein of 250 aa that is preceded by a putative ribosome-binding site (GGAGG) 5 bp upstream of the ATG start. A BLAST analysis against the nonredundant NCBI protein database indicated two strong hits of 98 and 97% amino acid identity along the entire length of a hypothetical protein (240 aa) from an uncultured bacterium and to ORFX (163 aa) of plasmid Rms149 from *Pseudomonas aeruginosa* (18). Rms149 is a 57-kb IncP-6 plasmid that has a different replicon from IncQ plasmids but a 5.6-kb mobilization region that is very similar to that of the pRAS3 plasmids and pTF-FC2. Like the IncQ-2 plasmids this mobilization region includes a *mobB* followed by a *mobA*/*repB* gene fusion and the divergent *mobCDE* genes and has between 86 and 100% amino acid identity to the corresponding gene products of pRAS3 and pTF-FC2. The *orfX* of Rms149 is in the same location as *orf3*, immediately downstream of *mobE*, but appears to have been truncated by the insertion of Tn*1012*. Plasmid pTF-FC2 has an *orf4* encoding a 270-aa product in a similar location to *orf3* and *orfX*, but this ORF is unrelated in nucleotide or predicted amino acid sequence.

Despite the Tn*1012* insertion in *orfX*, the 5.6-kb mobilization region of Rms149 remained functional (18), but without an untruncated *orfX* the effect of *orfX* on mobilization frequency could not be tested. We therefore tested whether the insertion of EZ-Tn*5* into *orf3* of pRAS3.1, which was used to indentify *orf3* as the cause of incompatibility, affected the mobilization frequency. Construct pRAS3.1::tet was mobilized at a frequency of 12.44 ± 3.95 transconjugants per donor, while pRAS3::orf3 was mobilized twofold lower, at 6.04 ± 4.33 transconjugants per donor. We concluded that like *mobD* and *mobE*, *orf3* plays a minor role in mobilization frequency. To

determine whether the product of *orf3* was responsible for the strong plasmid incompatibility, *mobDE-orf3* and *orf3* were cloned behind the P_{BAD} promoter. The genes were shown to be expressed by using RT-PCR and primers specific to the *mobE* and *orf3* genes. However, when these constructs were placed in *trans* with pTF-FC2Tet (or pTC-F14Km) they did not cause plasmid incompatibility. Plasmid pTF-FC2Tet was, however, displaced when the entire operon (pBAD28-mobCDE-orf3) was placed in *trans*, and the reason for strong incompatibility requires further investigation.

DISCUSSION

The low nucleotide sequence identity between the *repC* genes of the pRAS3 plasmids with that of pTF-FC2 and pTC-F14, the other members of the IncQ-2 plasmid group, was unexpected. The iteron-binding protein, RepC, is essential for IncQ plasmid replication and is generally the most conserved Rep protein within the IncQ plasmid family (31). The other genes of the plasmid backbone (*mobEDCBA* and *repBA*) of pRAS3 and pTF-FC2 are highly conserved (Fig. 1), and it is unlikely that differences in the *repC* genes are due to a higher mutation rate. It is more likely that the *repC* gene of the pRAS3 plasmids has been acquired by gene swapping with some as-yet-unidentified IncQ-like plasmid that is different from any so far discovered.

In several other plasmids the total number of *oriV*-associated iterons in a cell has been shown to affect plasmid copy number (7, 23, 39), and our expectation was that pRAS3.1 with four 22-bp *oriV*-associated iterons would have a lower copy number than pRAS3.2, with three 22-bp iterons. Unexpectedly, we found that pRAS3.1 had a copy number considerably greater than pRAS3.2 despite of it having more iterons. This increase in copy number was due to the number of 6-bp repeats in the intergenic region between the *mobCBE-orf3* and the *mobB-mobA/repB* operons, with the higher-copy-number pRAS3.1 plasmid having five 6-bp repeats while plasmid pRAS3.2 had four 6-bp repeats. The additional 6-bp repeat resulted in an increase in expression of the *mobB-mobA/repB* operon and it was the increase in expression of the RepB primase that resulted in a higher plasmid copy number. The altered promoter could either increase the efficiency of initiation of *mobB-mobA/repB* transcription or decrease autorepression by RepB (14). Swapping the region with the repeats between pRAS3.1 and pRAS3.2 resulted in a corresponding change in copy number. We further demonstrated that the level of transcription of the *mobB-mobA/repB* operon was increased approximately twofold in the presence of five 6-bp repeats. An increase in PCN as a result of increased *repB* expression was confirmed by placement of a *repB* gene in *trans* under the control of an arabinose-inducible promoter. This resulted in a 2.2-fold increase in copy number of a plasmid containing four 6-bp repeats to approximately the level of a plasmid containing five 6-bp repeats. This provided strong evidence that the copy number of pRAS3.1 was affected by both the number of 22-bp *oriV*-associated iterons and the level of transcription of the *repB* gene.

The 30% reduction in pRAS3 copy number that occurred on overexpression of *repAC* from a P_{BAD} vector promoter is different from the result obtained by Haring et al. (19), who reported that the copy number of the IncQ-1 plasmid, RSF1010, was increased sixfold upon overexpression of *repAC*. However, the small reduction in copy number of pRAS3 is similar to the reduction in copy number that occurred on overexpression of *repAC* in the case of the related plasmid, pTF-FC2 (25). We observed that when *repAC* from either pRAS3 or pTF-FC2 was expressed from the P_{BAD} promoter, the *E. coli* host cells were slow growing and clearly stressed. Whether this might have affected the copy number is unknown.

Demonstration that the pRAS3 plasmids contain a TA system means that all of the IncQ-2 plasmids so far discovered contain a toxin-antitoxin plasmid stability module situated within the replicon in exactly the same position between the *repB* and *repA* genes. However, although the *pas* toxin-antitoxin systems of pTF-FC2 and pTC-F14 are related to each other and are deep-branching members of the *E. coli relBE* family, they are totally unrelated to the toxin-antitoxin system of the pRAS3 plasmids. As the *repB* and *repA* genes of pRAS3 plasmids are highly related to pTF-FC2 and pTC-F14 and flank the unrelated TA modules (Fig. 1), this implies that the TA modules of the pRAS3 plasmids were acquired independently of pTF-FC2 and pTC-F14. The observation that IncQ-2 plasmid replicons have acquired different TA systems located in the same position suggests that there may be a biological reason for their occurrence in that exact position. Matcher and Rawlings (25) have presented evidence that a strong, autoregulated promoter such as that provided by a TA system within the replicon of pTF-FC2 confers on the plasmid the ability to rapidly respond to a fall in copy number. This is because a transient burst of expression of the TA genes results in a related increase in expression of the downstream *repAC* genes. IncQ plasmids appear not to have an active partitioning system, and a fall in copy number might occur on cell division when one daughter cell could receive many more copies of a plasmid than the other. Similarly, a strongly expressed, autoregulated TA system would allow the rapid expression of *repAC* on arrival in a recipient cell following conjugation.

To date plasmids pRAS3.1, pRAS3.2, pTF-FC2, and pTC-F14 are the only reported representatives of the IncQ-2 plasmids. Previous work demonstrated that pTF-FC2 and pTC-F14 are fully compatible and therefore were placed in the IncQ-2 incompatibility groups α and β, respectively. When we tested the incompatibility of pRAS3.1 and pRAS3.2 against representatives of the IncQ-1 α, β, and γ incompatibility groups, they were fully compatible but were violently incompatible with other members of the IncQ-2 plasmids. Plasmid incompatibility is understood as being the inability of two plasmids to coexist in the same host in the absence of selective pressure and implies that they belong to the same incompatibility group (28). This is clearly not the case with pRAS3 plasmids and pTC-FC2 and pTC-F14, as the strong incompatibility observed would imply that they belong to the same incompatibility group. Strong incompatibility appeared to be due to a phenomenon associated with *orf3* that is still not fully understood. If, however, the region containing *orf3* was interrupted, then the replicon of pRAS3.1 was compatible with pTF-FC2 and pTC-F14. This suggests that the pRAS3 plasmids form a third, γ plasmid incompatibility grouping within the IncQ-2 plasmids. Support for this proposal is that the nucleotide sequence of the 22-bp *oriV*-associated iterons and the protein sequences of the

RepC iteron-binding proteins of the pRAS3 plasmids are very different from pTF-FC2 or pTC-F14.

In previous work, plasmid pTF-FC2 was mobilized at saturation frequency by the RP4 conjugative plasmid that had been integrated into the chromosome of *E. coli* S17.1, while pTC-F14 was mobilized at a frequency 3,500-fold lower (40). This difference in mobilization frequencies was due to differences in the MobD and MobE proteins, because when the genes for these proteins from pTF-FC2 were provided in *trans*, the mobilization frequency of pTC-F14 was raised to near saturation levels. The hypothesis for the differences in mobilization frequency was that pTF-FC2 and pTC-F14 had been adapted for efficient mobilization by two different conjugative plasmids and that the very different amino acid sequences of *mobCDE* gene products between the two plasmids were responsible for this. The pRAS3 plasmids have *mobCDE* genes that are closely related to those of pTF-FC2 (Fig. 1) and appeared to be more efficiently mobilized by RP4 than even pTF-FC2 was. Mating of the pRAS3 plasmids was so efficient that the mating time had to be shortened from 60 to 30 min and the donor-to-recipient ratio decreased from 1:10 to 1:100 to prevent mating from reaching saturation so that the mating frequency could be calculated. The finding that the pRAS3 plasmids could mobilize the *oriT*s of pTF-FC2 and pTC-F14 even though they have different sequences from the pRAS3 plasmids (Fig. 3) is interesting in the context of the work of Meyer (27). He showed that the IncQ-1 plasmid R1162 can initiate transfer from a 19-bp locus that is partly degenerate in sequence and that such sites are likely to occur by chance in a bacterial chromosome. R1162-dependent transfer of chromosomal DNA from such a potential *oriT* was demonstrated, and it was pointed out that this might indicate a previously unrecognized potential for the exchange of bacterial DNA. The relaxed nature of the relaxase and *oriT* interaction of the pRAS3 plasmids suggests that these plasmids also have the potential to mobilize chromosomal DNA from cryptic *oriT*-like sequences. The observations that the nucleotide sequence of the *oriT* of the pRAS3 plasmids is 25% more identical to pTC-F14 but that the pRAS3 plasmids do not complement the mobilization of pTC-F14 as well as pTF-FC2 suggest that conservation of specific base pairs within the *oriT* probably affects the mobilization frequency more than the general level of *oriT* sequence identity.

IncQ family plasmids are highly promiscuous, and related plasmids have been found in very different environments. An illustration of this was obtained while searching for genes with homology to those present on the pRAS3 plasmids. A genomic island containing tetracycline resistance genes has been isolated from seven isolates of the obligate intracellular human and animal pathogen *Chlamydia suis* (11). The tetracycline resistance genes were associated with a plasmid that is almost identical to pRAS3.2. Approximately 10.1 kb of plasmid-like DNA is present, the nucleotide sequence of which is 99% identical to pRAS3.2 and contains large stretches of perfect identity. The biggest difference is that a 1.7-kb fragment containing the *mobA*/*repB* gene fusion extending 64 bp into the antitoxin gene is missing. Although the toxin gene remains, this is not likely to be expressed, as its promoter lies upstream of the antitoxin gene and within the missing region. There is an 8-bp deletion in the region of the operator of the divergent *tetA* and *tetR* genes as well as a deletion in the *oriV* region, such that only one 22-bp iteron remains. The presence of pRAS3-like plasmids in an obligately intracellular parasite such as *Chlamydia suis* illustrates the remarkable promiscuity of IncQ family plasmids and their ability to participate in the horizontal gene pool.

We began this work as part of a study aimed at answering the question of why two versions of what are two highly similar plasmids existed. Although this question has not yet been fully addressed, we have established that the effect of the additional 6 bp upstream of the *mobB-mobA*/*repB* operon in pRAS3.1 was to raise plasmid copy number while the effect of the additional 22-bp *oriV*-associated iteron was to partly reduce the copy number (though this reduction was not down to the level of pRAS3.2). During evolution, plasmid variants would be expected to compete for space in the cytoplasm of the host but in such a way that they do not place an evolutionary significant additional metabolic burden on their host cells. For plasmids that do not have an active partitioning mechanism (such as the pRAS3 plasmids), one would predict that the ideal plasmid copy number would be a compromise between a copy number high enough to minimize plasmid loss that might occur on host cell division but not one so high that the plasmid-associated metabolic burden makes the host noncompetitive. In addition, there may also be selection for a plasmid to evolve in such a way that it is not easily displaced by its sister variant. Future studies will include the construction of a number of plasmid variants that will enable us to address this question.

ACKNOWLEDGMENTS

We thank Trine L'Abée-Lund for the gift of plasmids pRAS3.1 and pRAS3.2.

This work was supported by grants from the National Research Foundation (Pretoria, South Africa) and BHP Billiton Johannesburg Technology Centre (Randburg, South Africa).

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1993. Current protocols in molecular biology. Wiley Interscience, New York, NY.
3. **Becker, E. C., and R. J. Meyer.** 2003. Relaxed specificity of the R1162 nickase: a model for evolution of a system for conjugative mobilization of plasmids. J. Bacteriol. **185:**3538–3546.
4. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
5. **Bravo, A., G. de Torrontegui, and R. Diaz.** 1987. Identification of components of a new stability sysyem of plasmid R1, ParD, that is close to the origin of replication of this plasmid Mol. Gen. Genet. **210:**101–110.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
7. **Chattoraj, D.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. **37:**467–476.
8. **Deane, S. M., and D. E. Rawlings.** 2004. Interaction between the plasmid addiction stability systems of two related broad-host-range plasmids. J. Bacteriol. **186:**2123–2133.
9. **Delcher, A. L., D. Harmon, S. Kasif, O. White, and S. L. Salzberg.** 1999. Improved microbial gene identification with GLIMMER. Nucleic Acids Res. **27:**4636–4641.
10. **Dorrington, R. A., and D. E. Rawlings.** 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171:**2735–2739.
11. **Dugan, J., D. D. Rockey, L. Jones, and A. A. Andersen.** 2004. Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial *inv*-like gene. Antimicrob. Agents Chemother. **48:**3989–3995.
12. **Fekete, R., and L. Frost.** 2000. Mobilization of chimeric *oriT* plasmids by F

and R100-01: role of relaxosome formation in defining plasmid specificity. J. Bacteriol. **182:**4022–4027.
13. **Gardner, M. N., S. M. Deane, and D. E. Rawlings.** 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. **183:**3303–3309.
14. **Gardner, M. N., and D. E. Rawlings.** 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiology **150:**1797–1808.
15. **Gerdes, K., J. E. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
16. **Guerry, P., J. Van Embden, and S. Falkow.** 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. **117:** 619–630.
17. **Guzman, L., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
18. **Haines, A. S., K. Jones, M. Cheung, and C. M. Thomas.** 2005. The IncP-6 plasmid Rms149 consists of a small mobilizable backbone with multiple large insertions. J. Bacteriol. **187:**4728–4738.
19. **Haring, V., P. Scholtz, E. Scherzinger, J. Frey, K. Derbyshire, G. Hatfull, N. S. Willetts, and M. Bagdasarian.** 1985. Protein RepC is involved in copy number control of the broad host range plasmid RSF1010. Proc. Natl. Acad. Sci. USA **82:**60690–60694.
20. **Jandle, S., and R. Meyer.** 2006. Stringent and relaxed recognition of *oriT* by related systems for plasmid mobilization: implications for horizontal gene transfer. J. Bacteriol. **188:**499–506.
21. **L'Abée-Lund, T., and H. Sørum.** 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid **47:**172–181.
22. **Lee, C., J. Kim, S. G. Shin, and S. Hwang.** 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. **123:**273–280.
23. **Lin, L.-S., Y.-J. Kim, and R. J. Meyer.** 1987. The 20 bp, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility *in vivo* and inhibits R1162 DNA replication *in vitro*. Mol. Gen. Genet. **208:**390–397.
24. **Masuda, Y., K. Miyakawa, Y. Nishimura, and E. Ohtsubo.** 1993. *chpA* and *chpB*, *Escherichia coli* homologs of the *pem* locus responsible for stable maintenance of plasmid R100. J. Bacteriol. **175:**6850–6856.
25. **Matcher, G. M., and D. E. Rawlings.** 2009. The effect of the location of the proteic post-segregational stability system within the replicon of plasmid pTF-FC2 on the fine regulation of plasmid replication. Plasmid **62:**98–107.
26. **Matson, S. W., and B. Morton.** 1991. *Escherichia coli* DNA helicase I catalyzes a site- and strand-specific nicking reaction at the F plasmid *oriT*. J. Biol. Chem. **226:**16232–16237.
27. **Meyer, R.** 2009. The R1162 mob proteins can promote conjugative transfer from cryptic origins in the bacterial chromosome. J. Bacteriol. **191:**1574–1580.
28. **Novick, R. P.** 1987. Plasmid incompatibility. Microbiol. Rev. **51:**381–395.
29. **Pansegrau, W., G. Ziegelin, and E. Lanka.** 1988. The origin of conjugative IncP plasmid transfer: interaction of plasmid encoded products and the nucleotide sequence at the relaxation site. Biochim. Biophys. Acta **951:**365–374.
30. **Pfaffl, M. W., G. W. Horgan, and L. Dempfle.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30:**e36.
31. **Rawlings, D. E., and E. Tietze.** 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. **65:**481–496.
32. **Rawlings, D. E., I. M. Pretorius, and D. R. Woods.** 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. **158:**737–738.
33. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
34. **Simon, R., U. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784–790.
35. **Smalla, K., H. Heuer, A. Götz, D. Niemeyer, E. Krögerrechlenfort, and E. Tietze.** 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl. Environ. Microbiol. **66:**4854–4862.
36. **Smith, A. G. S., and D. E. Rawlings.** 1997. The poison-antidote system of the broad host-range *Thiobacillus ferroxidans* plasmid pTF-FC2. Mol. Microbiol. **25:**961–970.
37. **Tietze, E., H. Tschape, and W. Voigt.** 1989. Characterization of new resistance plasmids belonging to incompatibility group Q. J. Basic Microbiol. **10:**695–706.
38. **Tsuchimoto, S., H. Ohtsubo, and E. Ohtsubo.** 1988. Two genes, pemK and *pemI*, responsible for stable maintenance of the resistance plasmid R100. J. Bacteriol. **170:**1461–1466.
39. **Tsutsui, H., A. Fujiyama, T. Murotsu, and K. Matsabara.** 1983. Role of nine repeating sequences of the mini-F genome for expression of F-specific incompatibility phenotype and copy number control. J. Bacteriol. **155:**337–344.
40. **van Zyl, L. J., S. M. Deane, and D. E. Rawlings.** 2003. Analysis of the mobilization region of the broad host-range IncQ-like plasmid pTC-F14 and its ability to interact with a related plasmid, pTF-FC2. J. Bacteriol. **185:**6104–6111.
41. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

JOURNAL OF BACTERIOLOGY, Dec. 2010, p. 6182–6190 Vol. 192, No. 23
0021-9193/10/$12.00 doi:10.1128/JB.00176-10

Evolutionary Competitiveness of Two Natural Variants of the IncQ-Like Plasmids, pRAS3.1 and pRAS3.2[▽]

Wesley Loftie-Eaton and Douglas E. Rawlings*

Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

Received 22 February 2010/Accepted 20 September 2010

Plasmids pRAS3.1 and pRAS3.2 are natural variants of the IncQ-2 plasmid family, that except for two differences, have identical plasmid backbones. Plasmid pRAS3.1 has four 22-bp iterons in its *oriV* region, while pRAS3.2 has only three 6-bp repeats and pRAS3.1 has five 6-bp repeats in the promoter region of the *mobB-mobA/repB* genes and pRAS3.2 has only four. In previous work, we showed that the overall effect of these differences was that when the plasmid was in an *Escherichia coli* host, the copy numbers of pRAS3.1 and pRAS3.2 were approximately 41 and 30, respectively. As pRAS3.1 and pRAS3.2 are likely to have arisen from the same ancestor, we addressed the question of whether one of the variants had an evolutionary advantage over the other. By constructing a set of identical plasmids with the number of 22-bp iterons varying from three to seven, it was found that plasmids with four or five iterons displaced plasmids with three iterons even though they had lower copy numbers. Furthermore, the metabolic load that the plasmids placed on *E. coli* host cells compared with plasmid-free cells increased with copy number from 10.9% at a copy number of 59 to 2.6% at a copy number of 15. Plasmid pRAS3.1 with four 22-bp iterons was able to displace pRAS3.2 with three iterons when both were coresident in the same host. However, the lower-copy-number pRAS3.2 placed 2.8% less of a metabolic burden on an *E. coli* host population, and therefore, pRAS3.2 has a competitive advantage over pRAS3.1 at the population level, as pRAS3.2-containing cells would be expected to outgrow pRAS3.1-containing cells.

Plasmids of the IncQ family are characterized by their relatively small size (~6 to 15 kb), their ability to replicate in a very wide range of bacterial host cells, and by being readily mobilizable by certain self-transmissible plasmids, in particular the broad-host-range IncP plasmids such as RP4 or RK2 (22). IncQ family plasmids have been subdivided into the IncQ-1 and IncQ-2 groups depending on whether their mobilization genes are of the three-gene IncQ type or the five-gene IncP type. Both subgroups of IncQ family plasmids have similar replicons that consist of three genes that encode a helicase (*repA*), a primase (*repB*), a DNA-binding initiator protein (*repC*), and an *oriV* region. The *oriV* region typically contains three complete, identical 22-bp iterons (or 20-bp iterons with 2-bp spacers) that serve as the binding site for RepC proteins (18, 22). Although some IncQ family plasmids may possess more than three copies of the iterons, the additional iterons are either partial copies, have sequence variations, or lack the spacer regions (22). It has been shown that even a single-base-pair replacement in a single iteron could result in a nonfunctional iteron-containing region and the inability of the IncQ-1 plasmid RSF1010 to replicate (19). Furthermore, in IncQ plasmids, the iterons have been shown to serve as the primary incompatibility determinants with cloned iterons on their own being able to displace the plasmid from which they were derived in the absence of selection. This displacement is a result of competition for RepC binding, and the rate at which the IncQ-like plasmid was displaced was dependent on the number of iterons cloned in *trans* (16).

Plasmids pRAS3.1 and pRA3.2 are almost identical IncQ plasmids that were isolated from the fish pathogen *Aeromonas salmonicida* (pRAS3.2) or atypical *A. salmonicida* (pRAS3.1 and pRAS3.2) in Norway (12). The mobilization and replication genes of pRAS3 plasmids are similar in order and sequence to the mobilization and replication genes of both pTF-FC2 (7) and pTC-F14 (9), with greater sequence identity to pTF-FC2 than to pTC-F14 (17). Together these plasmids constitute the only known members of the IncQ-2 subgroup of IncQ plasmids. The plasmid backbones of pRAS3.1 and pRAS3.2 differ in only two features. One of these differences is in the promoter region of the *mobB-mobA/repB* genes where pRAS3.2 has four 6-bp GCGGGG repeats, while pRAS3.1 has a fifth identical 6-bp repeat (Fig. 1). The additional 6-bp repeat was shown to increase the level of transcription of the *mobB-mobA/repB* genes by ~2-fold, and the plasmid copy numbers (PCNs) of two plasmids that were identical except that one plasmid had an additional 6-bp repeat increased by a corresponding ~2-fold. The second difference was in the *oriV* region, where pRAS3.2 has three copies of a perfectly conserved 22-bp iteron typical of the IncQ family plasmids, while pRAS3.1 is unusual in that it has an additional, identical, fourth 22-bp iteron. Comparison of pRAS3 derivative plasmids indicated that the effect of an additional 22-bp iteron was to reduce the copy number of the plasmid by 26 to 30%, provided the number of 6-bp repeats was the same for both plasmids.

The overall effect of pRAS3.1 in having a PCN-increasing additional 6-bp repeat in the promoter region of the *mobB-mobA/repB* genes but a PCN-decreasing additional 22-bp iteron compared with pRAS3.2 was that the PCN of pRAS3.1 was 41 while that of pRAS3.2 was 30 copies per chromosome. This raised the question as to whether one of the two natural, nearly identical pRAS3 plasmids has a competitive advantage

* Corresponding author. Mailing address: Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa. Phone: 27-21-808 5848. Fax: 27-21-808 5846. E-mail: der@sun.ac.za.

▽ Published ahead of print on 1 October 2010.

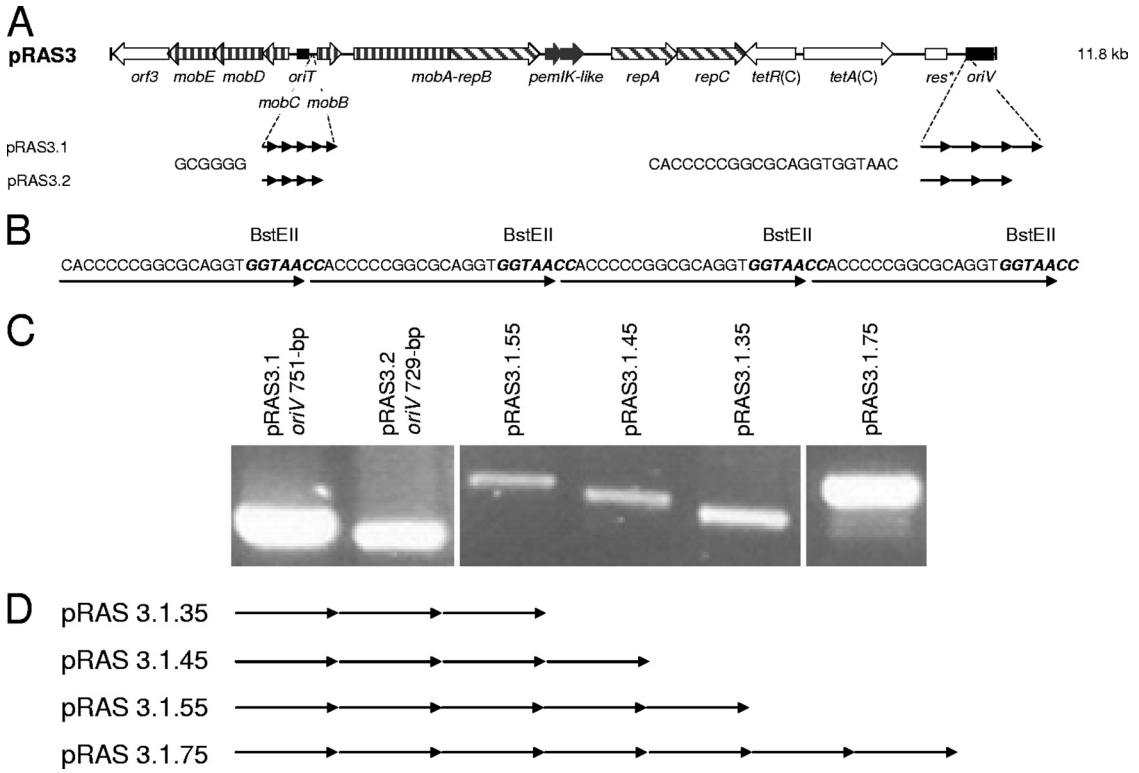


FIG. 1. (A) Genetic map of pRAS3 plasmids with the differences in the plasmid backbones of pRAS3.1 and pRAS3.2 shown below the map. (B) Nucleotide sequence of the four 22-bp repeats of pRAS3.1 indicating the location of the unique BstEII site used to construct plasmids with different numbers of iterons. (C) Agarose gel showing *oriV* regions of pRAS3 containing different numbers of iterons. (D) Diagram showing derivatives of pRAS3.1 with different numbers of iterons.

over the other. For example, did one variant have a stability advantage over the other, would one plasmid be able to displace the other when both were present in the same host cell, and how did the plasmids compare with respect to the metabolic load placed on the host? Were there any observable advantages or disadvantages to having three or four 22-bp iterons besides their effect on copy number? This is a report on the investigation into these questions carried out in *Escherichia coli* host cells.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *Escherichia coli* strains, cloning vectors, and plasmid constructs are shown in Table 1. Cultures of *E. coli* were grown in either Luria-Bertani (LB) broth or on Luria agar (LA) plates. The growth medium was supplemented with the following antibiotics as required at the indicated concentrations: ampicillin, 100 $\mu g \cdot ml^{-1}$; chloramphenicol, 20 $\mu g \cdot ml^{-1}$; kanamycin, 30 $\mu g \cdot ml^{-1}$; nalidixic acid, 35 $\mu g \cdot ml^{-1}$; streptomycin, 35 $\mu g \cdot ml^{-1}$; and tetracycline, 10 $\mu g \cdot ml^{-1}$.

General DNA techniques. Plasmid preparation, restriction endonuclease digestion, gel electrophoresis, and cloning were performed by standard methods (2, 24). Where no suitable restriction sites were present, single-strand DNA primers were designed, and DNA fragments to be cloned were amplified by PCR as described previously (19).

Copy number determinations. The relative and absolute plasmid copy numbers were determined by real-time quantitative PCR (qPCR) using the primers and standard curves described previously (19). Total genomic DNA was prepared from *E. coli* DH5α cultures containing the respective plasmids during exponential growth (optical density at 600 nm [OD_{600}] of 0.8) using a QIAamp DNA minikit (Qiagen); the DNA was prepared, placed in 60 μl of elution buffer, and quantified using a NanoDrop spectrophotometer.

Real-time qPCR amplification was performed using a LightCycler (version 2.0) with the LightCycler FastStart DNA master SYBR green I kit (Roche Diagnostics). Four nanograms of total DNA was added to each amplification reaction mixture, and the thermal cycling protocol of Lee et al. (13) was followed except that primer annealing was performed at 56°C for 4 s and DNA extension was performed at 72°C for 15 s.

Relative plasmid copy numbers were determined with pRAS3.1.35 as a reference standard using the relative expression software tool (REST) analysis software tool (21). The absolute plasmid copy numbers of pRAS3.1 and pRAS3.2 were determined using the threshold cycle (C_T) values to extrapolate the total amount of plasmid and chromosome present in a sample using LightCycler software (version 3.5) by the calculation method of Lee et al. (13).

Plasmid stability assay. The plasmids tested for stability were transformed into *E. coli* DH5α containing a resident pACYC177 vector with or without the *pemIK* system, plated on selective Luria agar plates containing tetracycline, and incubated overnight (O/N) at 37°C. Single colonies were inoculated into 5-ml portions of LB or M9 medium containing antibiotics selecting for both plasmids and incubated at 30°C for 24 h, whereafter the cultures were diluted 1×10^6 times into fresh medium containing an antibiotic selecting for only pACYC177 at 24-h intervals and incubated at 30°C while shaking for a total of 5 days. Each day serial dilutions of the cultures were plated onto plates containing an antibiotic selecting for only pACYC177 and incubated at 37°C. The percentage plasmid retention was then determined by replica plating 50 colonies from each of the plates onto plates containing antibiotics selecting for both plasmids or an antibiotic selecting for only pACYC177.

Relative fitness assays. The fitness of plasmid-containing (P+) cultures was measured relative to the fitness of plasmid-free (P−) cultures (15). Aliquots of competent *E. coli* JM109 cells were split into two samples. One sample was transformed with the plasmid derivatives and plated on selective medium, while the other sample, which was not transformed, was plated on nonselective medium. A single colony from each of the cultures (P+ and P− cultures) was inoculated into 10 ml of Davis minimal (DM) medium supplemented with 125 μl of D-glucose (20%) per liter, 406 μl of magnesium sulfate (1 M) per liter, and 58.8 μl of thiamine (34 mg/ml) per liter and incubated O/N at 37°C (5). The cultures were acclimated to the DM medium for a total of 3 days by serially

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics and/or description	Source or reference
E. coli strains		
DH5α	ϕ80*dlacZ*ΔM15 *endA1 recA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *relA1 supE44 deoR* Δ(*lacZYA-argF*)*U196*	Promega Corp., Madison, WI
JM109	*endA1 gyrA96 hsdR17*(r_K^- m_K^+) *mcrB*$^+$ *recAl relA1 supE44 thi-1* Δ(*lac-proAB*) F′ [*traD36 proAB lacI*^{q}ZΔM15]	26
Plasmid vectors		
pACYC177	Ap^r Km^r; p15A replicon; cloning vector	6
pBAD28	Ap^r Cm^r; arabinose-inducible expression vector; pACYC184 replicon	11
pBR322	Ap^r Tc^r; ColE1 replicon; cloning vector	3
pOU82	Ap^r; *lacZYA*; R1 replicon	10
Plasmid constructs		
pACYC177ΔAp^r	Km^r Amp^s; 683-bp deletion of a BamHI-ScaI fragment from pACYC177	This study
pACYC177-TA(Ap^r)	Ap^r; 731-bp PCR fragment containing pRAS3.1 *pemIK*-like genes (nucleotide positions 8819 to 8088) cloned into the XhoI-BamHI sites of pACYC177	This study
pACYC177-TA(Km^r)	Km^r; 731-bp PCR fragment containing pRAS3.1 *pemIK*-like genes (nucleotide positions 8819 to 8088) cloned into the BamHI-ScaI sites of pACYC177	This study
pBAD28-repC	Ap^r Cm^r; 1,015-bp PCR fragment containing the pRAS3.1 *repC* (nucleotide positions 6903 to 5889) cloned behind the P_{BAD} promoter	17
pOU82-TA	Ap^r; 731-bp PCR fragment containing pRAS3.1 *pemIK*-like genes (nucleotide positions 8819 to 8088) cloned into pOU82	17
pRAS3.1	Tc^r; natural 11,851-bp plasmid isolated from *Aeromonas salmonicida* subsp. *salmonicida* with four iterons and five 6-bp repeats	12
pRAS3.1Km	Km^r; pRAS3.1 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	17
pRAS3.1.34	Tc^r; pRAS3.1.35 derivative with four 6-bp repeats from pRAS3.2 by exchanging the 2.9-kb HindIII-PvuI region	This study
pRAS3.1.35	Tc^r; pRAS3.1 derivative with three iterons obtained by random ligation of short iteron fragments after BstEII digestion	This study
pRAS3.1.35Km	Km^r; pRAS3.1.35 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.1.44	Tc^r; pRAS3.1 derivative with four 6-bp repeats from pRAS3.2 by exchanging the 2.9-kb HindIII-PvuI region	This study
pRAS3.1.55	Tc^r; pRAS3.1 derivative with five iterons obtained by random ligation of short iteron fragments after BstEII digestion	This study
pRAS3.1.55Km	Km^r; pRAS3.1.55 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.1.74	Tc^r; pRAS3.1.75 derivative with four 6-bp repeats from pRAS3.2 by exchanging the 2.9-kb HindIII-PvuI region	This study
pRAS3.1.75	Tc^r; pRAS3.1 derivative with seven iterons obtained by random ligation of short iteron fragments after BstEII digestion	This study
pRAS3.1.75Km	Km^r; pRAS3.1.75 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	This study
pRAS3.2	Tc^r; natural 11,823-bp plasmid isolated from atypical *Aeromonas salmonicida* with three iterons and four 6-bp repeats	12
pRAS3.2Km	Km^r; pRAS3.2 with Tc^r replaced by Km^r from pSKm2 at the BamHI-EcoRV sites	17
pSKM2	Ap^r Km^r; plasmid with the 1.45-kb HindIII-SmaI kanamycin resistance cassette from Tn5 cloned into the HindIII-SmaI sites in pBluescript (SK)	Stellenbosch University laboratory collection (D. E. Rawlings)

diluting the cultures 100-fold into fresh medium every 24 h. Once the cultures were acclimated, 700 μl of each culture was inoculated into 70 ml of fresh medium, mixed, and divided into six 10-ml cultures in 50-ml Erlenmeyer flasks and incubated while shaking O/N at 37°C. After 24 h of growth, the P+ monoculture was mixed with an approximately equal number of P− monoculture cells, determined from colony counts on selective and nonselective media, respectively, and the six mixed cultures were inoculated into fresh DM medium at a 100-fold dilution and incubated at 37°C while shaking. As a control for stability, an approximately equal amount of P+ cells from each of the six cultures was inoculated into DM medium and maintained as a monoculture in the absence of selection for the duration of the assay. The cultures were reinoculated into fresh medium daily for a total of 6 days, and each inoculum, including the time zero (T_0) inoculum representing the original mixed culture and monocultures, was serially diluted and plated onto selective and nonselective media and incubated at 37°C for colony counts.

The selection rate constant was estimated from a linear regression of the daily log ratio of P+ to P− [ln(ratio P+/P−)] cells, and the relative fitness was calculated from the selection rate constant and expressed as a per generation fitness percentage [1 − (selection rate constant/average number of generations per day) × 100] (14). The number of P− cells in a mixed culture was estimated by subtracting the number of P+ cells from the total number of cells on the plates with nonselective medium. Plasmid stability was monitored by plotting the number of P+ cells counted on the selective plates against the total number of cells counted on the nonselective plates. Additionally, to verify the stability data, 50 colonies of each P+ monoculture were replica plated onto selective and nonselective media on days 0, 3, and 6. The relative fitness data were analyzed

TABLE 2. Relative and absolute copy numbers of pRAS3 plasmid derivatives

Plasmid	Relative PCN[a]	SD for relative PCN	Confidence value (*P*) for relative PCN	No. of repeats	Absolute PCN
pRAS3.1.35	1.00	0.090	0.001	4	59
pRAS3.1.45 (pRAS3.1)	0.69	0.062	0.001	6	41
pRAS3.1.55	0.48	0.092	0.001	6	28
pRAS3.1.75	0.32	0.070	0.001	8	19
pBR322	0.30	0.007	0.001	4	18

[a] PCN, plasmid copy number.

using a single-sample *t* test with a constant of 100% (selection rate constant of 0) for each of the samples, and the statistical significance of comparisons between different plasmid samples were determined by the Fisher least-significant difference (LSD) test using Statistica version 9 (StatSoft Inc., OK) software.

Displacement assays. Competent *E. coli* DH5α cells containing a resident plasmid were transformed with a second incoming plasmid and plated on antibiotic-containing media selecting for both plasmids. A single colony was picked and inoculated into LB broth with double antibiotic selection and incubated at 37°C. After growth O/N, the cultures were serially diluted and plated onto nonselective LA plates. The cultures were also diluted 1×10^6 into fresh nonselective LB broth and incubated at 37°C for 24 h before they were serially diluted and spread onto nonselective media. Fifty colonies from each of the day 0 and day 1 nonselective plates were picked and replica plated onto four sets of solid media, two sets containing single antibiotics to test for the presence of the resident or incoming plasmid separately, one set containing two antibiotics to test for the presence of both plasmids, and one set containing no antibiotics. The presence of either or both plasmids was scored and expressed as a percentage of the total number of colonies tested. The same method was used to determine segregation patterns in the presence or absence of additional RepC except that the competent cells also contained either pBAD28-RepC or pBAD28, and selection was provided for the pBAD28 plasmids throughout the duration of the assay. Controls to check for spontaneous loss of the resident plasmids were carried out using the same procedure except that the initial competent *E. coli* cells containing the resident plasmids were taken through equal cycles of growth without antibiotic selection before testing for retention of the resident plasmid.

RESULTS

Construction of plasmids with increased numbers of iterons. To assist in understanding why one of the natural pRAS3 plasmids had the three 22-bp iteron structure typical of the IncQ plasmids and the other had four 22-bp iterons, we amplified the differences by constructing pRAS3 plasmids with even greater numbers of iterons. Examination of the sequences of the pRAS3 plasmids indicated that they contained a recognition site for the BstEII restriction endonuclease within each of the 22-bp iterons and that no other BstEII sites existed. Plasmid pRAS3.1 was therefore digested with BstEII, and a concentrated self-ligation reaction was conducted. *E. coli* DH5α was transformed with the ligation mix, and plasmid DNA was extracted from 80 colonies. Single-strand DNA primers homologous to either side of the *oriV* region were used to PCR amplify the region (751 bp in the pRAS3.1 control) containing the iterons (17); the primers were pRAS3oriV fwd and pRAS3oriV rev (the forward and reverse primers are indicated by fwd and rev, respectively, at the end of the primer designation). Four pRAS3.1 derivatives that appeared to have amplicons of different sizes were identified, and DNA sequencing of the region revealed that plasmids containing 3, 4, 5, and 7 iterons had been constructed (Fig. 1). The plasmids are numbered pRAS3.1.35, pRAS3.1.45, pRAS3.1.55, and pRAS3.1.75 with the last two digits indicating the number of 22-bp iterons and 6-bp repeats, respectively. Using this terminology, construct pRAS3.1.45 is equivalent to the wild-type pRAS3.1 plasmid. Attempts to construct a plasmid containing a single iteron by digesting pRAS3.1 with BstEII and then separating the small 22-bp fragments prior to self-ligation of the remaining 11 785-bp fragments at the single remaining BstII site were unsuccessful. As no plasmids containing either one or two iterons were identified, plasmids with less than three iterons appeared to be nonviable.

Effect of iteron number on plasmid copy number. Unlike plasmids pRAS3.1 and pRAS3.2 that differ in the numbers of both the 22-bp iteron and *mobB-mobA/repB* 6-bp repeat, plasmids pRAS3.1.35, pRAS3.1(0.45), pRAS3.1.55, and pRAS3.1.75 are identical plasmids differing only in the number of 22-bp iterons. The effect of the number of 22-bp iterons on plasmid copy number (PCN) could therefore be determined. The relative copy numbers of plasmids containing three, four, five, or seven 22-bp iterons relative to pRAS3.1.35 were determined using quantitative real-time PCR (Table 2). Plasmid vector pBR322 which has been reported to have a copy number of ~18 as determined by both absolute and relative quantification of real-time PCR methods (13) was included as a standard from which to calculate the absolute PCN of each pRAS3.1 variant. The natural pRAS3.1 (pRAS3.1.45) plasmid containing four 22-bp iterons had a PCN of ~41. A reduction in the number of iterons to three resulted in a copy number ~44% higher (PCN of 59), while increasing the number of iterons to five or seven resulted in a decrease in copy number of ~32% (PCN of 28) and ~54% (PCN of 19), respectively.

The abilities of pRAS3.1, pRAS3.2, and derivatives to displace each other within a host cell. As pRAS3.1 and pRAS3.2 both contain tetracycline resistance genes as their only selectable marker, the kanamycin resistance gene from pSKM2 was inserted into the *tetAR* genes of both plasmids so as to obtain tetracycline- and kanamycin-resistant variants of both plasmids. When plasmids pRAS3.2 and pRAS3.1Km were placed in an *E. coli* DH5α host, the selecting factor was removed, and the host was grown for ~20 generations, plasmid pRAS3.1Km displaced plasmid pRAS3.2 in ~98% of cells (Table 3). Furthermore, the remaining ~2% of cells retained both plasmids in all cases, indicating that although pRAS3.1 readily displaced pRAS3.2, pRAS3.2 was not able to displace pRAS3.1Km. When the antibiotic resistance genes were switched, an identical rate of displacement of pRAS3.2Km by pRAS3.1 was observed (Table 3). This showed that plasmid displacement was not noticeably affected by the antibiotic resistance markers and that a plasmid containing four 22-bp iterons (pRAS3.1) displaced a plasmid with three 22-bp iterons (pRAS3.2). As the natural plasmids pRAS3.1 and pRAS3.2 differ both in the

TABLE 3. Segregation patterns of coresident pRAS3 plasmids and derivatives in an *E. coli* host

pRAS3 plasmid	Coresident plasmid[a]			% colonies with resistance to the following antibiotic(s)[b]:			Direction and strength of segregation bias[c]
	pRAS3.2.Km	pRAS3.1.35.Km	pRAS3.1.Km	Tet only	Km only	Tet and Km	
pRAS3.2			•	0 ± 0	98 ± 3	2 ± 3	3 iterons and 4 6-bp repeats ⋘ 4 iterons and 5 6-bp repeats
pRAS3.1	•			98 ± 0	0 ± 0	2 ± 0	4 iterons and 5 6-bp repeats ⋙ 3 iterons and 4 6-bp repeats
		•		79 ± 4	0 ± 0	21 ± 4	4 iterons ⋙ 3 iterons
			•	23 ± 7	20 ± 8	57 ± 11	Tet ≈ Km
pRAS3.1.35			•	0 ± 0	87 ± 10	12 ± 8	3 iterons ⋘ 4 iterons
		•		24 ± 3	13 ± 1	62 ± 3	Tet > Km
pRAS3.1.34		•		14 ± 0	18 ± 14	66 ± 14	4 6-bp repeats ≈ 5 6-bp repeats
pRAS3.1.44			•	30 ± 6	8 ± 6	60 ± 17	4 6-bp repeats > 5 6-bp repeats

[a] The small black circles indicate the plasmid in the cell in addition to the pRAS3 plasmid used in each experiment. Each plasmid was completely stable on its own for the duration of the assay in the absence of antibiotic.
[b] Tet, tetracycline; Km, kanamycin.
[c] Direction and strength of segregation bias as a result of iteron and/or 6-bp repeat copy number, as well as the influence of the antibiotic resistance genes. Arrowheads indicate the direction of plasmid displacement, and the number of arrowheads is an indication of the strength of displacement. Tet, tetracycline resistance gene; Km, kanamycin resistance gene.

number of 6-bp repeats of their *mobB-mobA/repB* promoter regions and in the number of 22-bp *oriV*-associated iterons, we investigated the effect of the number of 22-bp iterons on plasmid displacement alone by constructing pRAS3.1.35Km. This plasmid has a backbone identical to that of pRAS3.1 except that it has three rather than four 22-bp iterons. After ~20 generations of growth without selection in *E. coli* DH5α, the plasmid with four 22-bp iterons (pRAS3.1) displaced the plasmid with three 22-bp iterons (pRAS3.1.35Km) in 79% ± 4% of host cells, while 21% ± 4% of cells retained both plasmids, and no instances of the 3-iteron plasmid displacing the 4-iteron plasmid were observed. The reciprocal experiment with pRAS3.1.35 and pRAS3.1Km gave largely similar results with the 4-iteron plasmid (pRAS3.1Km) displacing the 3-iteron plasmid (pRAS3.1.35) in 87% ± 10% of host cells and with 12% ± 8% of the host cells retaining both plasmids. The displacement of a 3-iteron plasmid by a 4-iteron plasmid occurred in spite of the observation that when the iteron number of pRAS3.1 was increased from 3 to 4, its PCN decreased from ~59 to ~41 (17). The displacement of a plasmid containing three 22-bp iterons with a higher PCN by a plasmid with an identical structure but with a lower PCN was unexpected. Therefore, a plasmid with three 22-bp iterons (pRAS3.1.35) and a plasmid with four 22-bp iterons (pRAS3.1Km) were placed into the same cell with antibiotic selection to ensure that both plasmids were retained and the relative copy numbers were determined by the gel method of Park et al. (20). The plasmid with three 22-bp iterons (pRAS3.1.35) was barely detected, whereas the plasmid with four 22-bp iterons was clearly visible (data not shown). Therefore, a plasmid with four iterons was able to inhibit the replication of a plasmid with three iterons in spite of the four-iteron plasmid having a lower PCN when on its own in a host cell.

Experiments to determine the effect of an additional 6-bp repeat in the *mobB-mobA/repB* promoter region on plasmid stability when the number of iterons was kept the same were less clear. In order to obtain such plasmids, a 2.9-kb HindIII-PvuI region containing the five 6-bp repeats in each of the pRAS3.1 derivatives was replaced with the same region from pRAS3.2. In *E. coli* host cells containing plasmids pRAS3.1.34 and pRAS3.1.35Km, both of which have three 22-bp iterons but four or five 6-bp repeats, respectively, ~66% ± 14% of the cells contained both plasmids after ~20 generations, while approximately equal numbers of cells retained only one of the two plasmids (14% ± 0% of the cells or 18% ± 14% of the cells, respectively). When both plasmids had four 22-bp iterons but differed by having four or five 6-bp repeats, pRAS3.1.44 or pRAS3.1Km, respectively, then after ~20 generations, ~60% ± 17% of the cells contained both plasmids after ~20 generations, while 30% ± 6% of the cells retained pRAS3.1.44 with four 6-bp repeats and only 8% ± 6% of the cells retained pRAS3.1Km with five 6-bp repeats. Taken together, the results suggest that even though a greater level of *mobB-mobA/repB* expression has been shown to occur in a plasmid with five 6-bp repeats compared with plasmids with four 6-bp repeats (17), this appears not to have an effect on plasmid displacement. This observation is not unexpected, given that the *oriV* regions of the two plasmids are identical and replication proteins are capable of working in *trans*, and therefore, the replication proteins produced by one plasmid variant would also be available to the other plasmid. Under such circumstances, plasmids containing identical numbers of iterons would be expected to initiate replication equally efficiently, and therefore, the PCNs of coresident plasmids with different numbers of 6-bp repeats might be expected to be approximately equal.

Effect of iteron number on the displacement of related plasmids. We had shown that a plasmid with four 22-bp iterons displaced a plasmid with three 22-bp iterons, and this raised the question as to how plasmids with five or seven 22-bp iterons would compete with a typical plasmid with three 22-bp iterons. It is known that the binding of RepC monomers to the iterons is an essential step during the initiation of replication and that iteron binding by DNA-binding proteins may be cooperative (4). That is, DNA-binding proteins may be more effectively sequestered by DNA with larger numbers of tandem iterons. Plasmids with four, five, or seven 22-bp iterons, all plasmids

TABLE 4. Effect of iteron number on coresident plasmid segregation patterns in the absence and presence of excess RepC

pRAS1 plasmid	Coresident plasmids[a]		% colonies with resistance to the following antibiotic(s):			Direction and strength of segregation bias[b]
	pRAS3.1.35.Km + pBAD28	pRAS3.1.35.Km + pBAD28-RepC	Tet only	Km only	Tet and Km	
pRAS3.1.35.Tet	●		53 ± 11	21 ± 6	28 ± 10	Tet > Km
		●	55 ± 8	17 ± 1	29 ± 5	Tet > Km
pRAS3.1	●		91 ± 6	1 ± 1	7 ± 5	4 iterons >>> 3 iterons
		●	88 ± 4	7 ± 3	6 ± 0	4 iterons >>> 3 iterons
pRAS3.1.55.Tet	●		79 ± 3	6 ± 3	14 ± 6	5 iterons ≫ 3 iterons
		●	67 ± 2	16 ± 2	21 ± 1	5 iterons ≫ 3 iterons
pRAS3.1.75.Tet	●		63 ± 14	25 ± 13	15 ± 2	7 iterons > 3 iterons
		●	63 ± 11	26 ± 13	13 ± 4	7 iterons > 3 iterons

[a] The small black circles indicate the two plasmids in the cell in addition to the pRAS3 plasmid used in each experiment. All plasmids were completely stable in the absence of antibiotics for the duration of the assay.

[b] Direction and strength of segregation bias as a result of iteron number in the absence or presence of additional RepC. Arrowheads indicate the direction of plasmid displacement, and the number of arrowheads is an indication of the strength of displacement.

containing identical five 6-bp repeats, were allowed to compete for replication space in an *E. coli* DH5α host containing a coresident plasmid with three 22-bp iterons. Since RepC is an important player in the initiation of plasmid replication, these experiments were repeated with additional RepC DNA-binding protein expressed in *trans* from the arabinose-inducible P_{BAD} promoter on pBAD28 (Table 4).

When two plasmids each with three 22-bp iterons but with different antibiotic markers (pRAS3.1.35 and pRAS3.1.35.Km) were competed, plasmid incompatibility was weak, with the plasmid containing the tetracycline (Tet) marker having a small advantage over the plasmid containing the kanamycin (Km) marker but with a fairly high proportion (28%) of cells retaining both plasmids. The standard deviations were high, supporting the interpretation that the bias in favor of the plasmid with the Tet marker was relatively weak. The pRAS3.1 plasmid, which contained four 22-bp iterons, displaced the pRAS3.1.35Km plasmid, which contained three 22-bp iterons, in 90% of the cells, much like was found in the earlier experiment in which pRAS3.1 displaced pRAS3.2 in 87% of the cells (Table 4). The plasmids with five and seven 22-bp iterons also displaced the plasmid with three 22-bp iterons, although this displacement became progressively weaker as the number of 22-bp iterons increased. Because of the apparent weak bias in favor of plasmids containing the Tet marker, reciprocal experiments to those shown in Table 4 were carried out; in these experiments, the plasmid with three 22-bp iterons was pRAS3.1.35, rather than pRAS3.1.35Km, and the competing plasmids were Km resistant rather than Tet resistant. Similar results were obtained except that the weak displacement of the plasmid with three 22-bp iterons by the plasmid with seven 22-bp iterons had largely disappeared (data not shown). The presence of additional RepC (second row of each data set in Table 4) did not affect plasmid competition but served to confirm the plasmid displacement results obtained without additional RepC.

Stability of pRAS3 plasmids and derivatives. We wished to determine how the metabolic loads that the pRAS3.1 or pRAS3.2 plasmids placed on a host cell differed. Before doing this, it was important to determine how stable these plasmids were in the absence of selection, as the addition of antibiotics to growth medium would itself affect the metabolic load experienced by a plasmid-containing cell. Furthermore, the pRAS3 plasmids contain a toxin-antitoxin (TA) system and should one plasmid be lost at a higher rate than the other, this would result in a greater proportion of the daughter cells of a host with the less stable plasmid being unable to continue to grow and divide. This inability to grow on the loss of a TA-containing plasmid would, of itself, appear to increase the metabolic load. The stability of plasmids pRAS3.1 and pRAS3.2 when present in *E. coli* DH5α host cells was tested in LB for approximately 100 generations in the absence of selection. No loss of either plasmid was detected (data not shown). The stability assay was repeated in M9 minimal medium, and again, 100% of cells appeared to have retained the plasmid after 100 generations. Since the apparent plasmid stability might have been due to the effectiveness of the TA system, the TA system was cloned into vector pACYC177 so that it could be placed in a cell together with the pRAS3 plasmids to neutralize any effect the TA system might have on plasmid loss. Functionality of the pACYC177-TA(Km^r) clone was demonstrated by its ability to eliminate the stabilizing effect of the TA system on an unstable pOU82 test plasmid. Plasmids pACYC177-TA(Km^r) or pACYC177ΔAmp were placed in *trans* with either pOU82-TA or pOU82, and the stability of the test plasmid was determined. When the pRAS3 TA genes were cloned onto the pOU82 test plasmid, the stability of pOU82-TA was 99% after ~90 generations. Neutralization of the TA genes on pOU82-TA by having pACYC177-TA(Km^r) in *trans* decreased plasmid stability to 12% after ~90 generations. This was identical to the high level of instability observed for pOU82 plasmid that lacked the TA system.

The presence of pACYC177-TA(Ap^r) in the same cell as either pRAS3.1 or pRAS3.2 did not affect plasmid stability, and both these pRAS3 plasmids were 100% stable even when their TA systems were neutralized. To further investigate the stability of the pRAS3 plasmids, we tested the stability of the pRAS3 derivatives with the highest copy number (pRAS3.1.35) and the lowest copy number (pRAS3.1.74) with PCNs of ~59 and ~15, respectively. The stability of these

TABLE 5. Metabolic burden of plasmids on *E. coli* host cells relative to plasmid-free cells

Plasmid	Approximate PCN	% relative fitness	SD for relative fitness	No. of cells tested	*P* value[a]
pRAS3.2	30 ± 5	95.30	1.56	6	0.001
pRAS3.1.34	31 ± 1	94.70	1.72	5	0.002
pRAS3.1.35	59	89.12	1.16	6	0.000
pRAS3.1	41 ± 4	92.52	2.64	6	0.001
pRAS3.1.74	15 ± 1	97.36	1.07	6	0.002

[a] *P* value for the metabolic burden of plasmids on *E. coli* host cells compared to plasmid-free cells.

plasmids was tested in *E. coli* DH5α cultures containing plasmid pACYC177ΔKm or pACYC177-TA(Ap^r) in *trans*. Both of these plasmids, pRAS3.1.35 and pRAS3.1.74, were found to be 100% stable for ~90 generations irrespective of whether the TA system was neutralized by having a coresident pACYC177-TA(Ap^r) or not. The stability assays were carried in LB and M9 minimal medium and at 30 and 37°C, and in none of the experiments was there any evidence of instability. It thus seemed that the pRAS3 plasmids were sufficiently stable for metabolic load experiments to be carried out without selection for plasmid maintenance.

Comparative metabolic loads of pRAS3 plasmids and derivatives. The relative effects of pRAS3.1 and pRAS3.2 plasmids on host cell fitness was carried out in *E. coli* JM109 cells and compared with the fitness of an isogenic plasmid-free host as described by Lenski (14) (Table 5). The wild-type plasmid pRAS3.2 had a smaller impact on host cell fitness with a cost of ~4.7% than pRAS3.1 with the cost being slightly higher at ~7.5%. This difference in metabolic load of ~2.8% was small but statistically significant ($P < 0.05$). We next tested the metabolic burden imposed by derivatives of pRAS3.1 with various PCNs as a result of their having different combinations of 6-bp repeats and 22-bp iterons (Table 5). As may be expected, plasmid pRAS3.1.34 with a PCN of ~31, which was approximately equal to the PCN of pRAS3.2 (PCN of ~30), had a similar metabolic load at ~5.3% ($P > 0.05$). Plasmid pRAS3.1.35 with the highest PCN of ~59 also had the highest metabolic load of ~10.9%, and plasmid pRAS3.1.74 with the lowest PCN (PCN of ~15) also had the lowest metabolic load at ~2.6%.

DISCUSSION

The backbone sequences of the two natural plasmid variants, pRAS3.1 and pRAS3.2, are identical with the exception that pRAS3.1 has a fourth perfectly conserved 22-bp iteron in its *oriV* region, whereas pRAS3.2 has only three of the 6-bp repeats within the promoter region of the *mobB-mobA/repB* genes, pRAS3.1 has five of the 6-bp repeats, and pRAS3.2 has four of these repeats. In previous work, we established that the additional 6-bp repeat in the *mobB-mobA/repB* gene promoter region of pRAS3.1 had resulted in an increase in plasmid copy number (PCN) of about ~2-fold (from 30 to 59), whereas the additional 22-bp iteron had resulted in a decrease in PCN of pRAS3.1 (from 59 to 41). The two alterations appear to have been compensatory, with one increasing the PCN and the other decreasing the PCN, at least partially. This raised the question of why two natural variants of the pRAS3 plasmids exist and whether either of the resultant plasmids, pRAS3.1 or pRAS3.2, is more fit evolutionarily.

Plasmid fitness can operate at two levels. One level is competition between related plasmids within a host cell. That is, plasmids with identical (or nearly identical) origins of replication are incompatible when both plasmids are present in an individual host cell. If one of the plasmids has a replication advantage, it will tend to displace the other. The second level is competition at a host cell population level. That is, a population of host cells containing one of the plasmids may be more competitive than a population of the identical host cells containing the other plasmid because of differences in metabolic burden that the two plasmids place on the host.

A high PCN would be expected to decrease the chance of plasmid loss on cell division, but too high a PCN would increase the metabolic load on a cell and thereby reduce host cell competitiveness. In contrast, although a low PCN might decrease the metabolic burden on the host, the host population might have reduced competitiveness should plasmids like the pRAS3 plasmids be lost too easily on cell division. The reason for this is that the pRAS3 plasmids have a functional toxin-antitoxin module and these modules function by inhibiting the growth of cells that fail to inherit a plasmid on cell division. Therefore, should the PCN be so low that a high rate of plasmid loss occurs, cells failing to inherit the plasmid would cease to grow, thereby reducing the competitiveness of the population. At the population level, plasmid copy number is likely to be a compromise between having a high PCN to reduce plasmid loss but with an increased metabolic load and a low PCN with an increased chance of plasmid loss but decreased metabolic load.

The 22-bp-iteron deletion and ligation experiments clearly indicated that the minimum number of 22-bp iterons required to support plasmid replication was three. One would have expected the ligation mix to have contained higher numbers of plasmids with one or two iterons, but since no plasmids of that type were isolated, plasmids with less than three iterons appeared to be nonviable. These findings were consistent with studies by Lin and Meyer (16) using plasmid R1162, an IncQ-1 plasmid that has 20-bp iterons with 2-bp spacers. These workers constructed *oriV* regions containing 0, ½, 2, and 3 iterons on multicopy vectors and found that only plasmids containing 3 iterons were able to replicate in the presence of *repBAC* genes. We succeeded in isolating pRAS3.1 plasmids with three, four, five, and seven 22-bp iterons and found that the number of iterons had a marked effect on the copy number. The PCN decreased from 59 to 19 as the number of iterons in tandem

increased from 3 to 7 (Table 2) or from 30 to 15 with the same increase in iteron numbers but with one less 6-bp repeat (data not shown). A variation in the number of iterons is therefore a highly effective means of selection for plasmids with a suitable PCN that may apply generally to iteron-containing plasmids.

In incompatibility experiments, plasmids with 4 or 5 22-bp iterons strongly displaced plasmids with three iterons in spite of the PCN of plasmids with 5 iterons being half of that of plasmids with 3 iterons (Table 4). Displacement of 3-iteron plasmids by 7-iteron plasmids was not marked. This suggested that plasmids with 4 or 5 iterons were selectively replicated in preference to plasmids with 3 iterons. A possible reason for this is that binding of the RepC replication initiator protein to the 22-bp iterons was enhanced when 4 or 5 iterons were present due to the phenomenon of cooperative binding. Such cooperative binding by the π replication initiator protein to the seven 22-bp iterons of plasmid R6K has been shown (4). This view is supported by the observation that when a 4-iteron plasmid and a 3-iteron plasmid were present in the same cell with antibiotic selection for both plasmids, the presence of the 3-iteron plasmid was barely detected (data not shown). This indicated that whereas the PCN of the 3-iteron plasmid was higher than the 4-iteron plasmid when each was on its own in a host cell, the 4-iteron plasmid inhibited the normal replication of the 3-iteron plasmid when both plasmids were present in the same cell. We speculate that this cooperative binding may favor the initiation of replication from plasmids containing four or five iterons above plasmids containing three iterons in spite of their copy number being lower. In the case of the 7-iteron plasmid, although cooperative binding of RepC to the iterons may be enhanced with respect to 3-iteron plasmids, the much greater difference in copy number eliminates this advantage, and both plasmids were able to compete for replication approximately equally. The provision of additional RepC protein by the placement of a *repC* gene under the control of a P_{ara} promoter on a coresident vector did not noticeably affect the pattern of plasmid displacement. This is either because the additional RepC resulted in inactive RepC dimer formation (23) or because cooperative binding resulted in the distribution of RepC to iterons in the same proportions irrespective of whether the intercellular levels of RepC were elevated.

Next we investigated what the effect of variations in the pRAS3 plasmids was on the fitness of *E. coli* host cells. One might predict that plasmids with four or five iterons had a double advantage over otherwise identical plasmids with three iterons. First, they were able to displace coresident three-iteron plasmids, and second, they had a lower copy number and therefore would be expected to place a lower metabolic burden on the host. As mentioned earlier, a possible disadvantage of having a lower copy number is that if this resulted in increased plasmid loss on cell division, then the host population may be negatively affected, as the TA system present on the pRAS3 plasmids may inhibit the growth of plasmid-free cells. However, when grown in an *E. coli* host for ~90 generations, the loss of pRAS3-derived plasmids was undetectable over a PCN range of 15 to 59 irrespective of whether the TA system was neutralized or the cells were grown in Luria broth or minimal medium. These results are in agreement with a predicted theoretical loss frequency (based on random segregation) of 1 in 1.6×10^4 generations for a plasmid with a copy number of 15 plasmids per chromosome, and even less for plasmids with a higher copy number (25). The high level of TA system-independent stability may be a result of the relatively high PCN of pRAS3 plasmids relative to other IncQ family plasmids which have been reported to have a PCN varying from 10 to 16 (17). The reason for the high PCN of the pRAS3 plasmids is not known. A possible reason is that amino acid sequence alignments show that the RepC proteins of the pRAS3 plasmids are very different from those of the other sequenced IncQ-like plasmids. It is possible that the formation of inactive RepC dimers occurs at a higher concentration in pRAS3 plasmids than in other IncQ family plasmids; however, this requires further investigation.

The contribution of PCN on host fitness was measured by determining the growth rate of a plasmid-containing (P+) *E. coli* JM109 host relative to that of an isogenic plasmid-free (P−) host (14). There are several advantages in measuring fitness using this method as opposed to directly competing cultures containing the respective plasmids. First, a plasmid-bearing culture is competed against a host which does not have the additional metabolic burden, thus amplifying the growth rate differences and allowing a more accurate estimation in a shorter time frame. Second, it negates the need to have different antibiotic selection genes on each of the competing plasmids for identifying the number of each competing plasmid-containing strain at different stages of a competition experiment. Different markers may have resulted in a different, artificial burden that could have influenced the results. Last, as cultures were competed against the same reference strain, the percentage relative fitness allows for comparisons between any of the samples rather than just between the samples that were paired in an assay. A disadvantage is that the conclusions derived from comparing the fitness between the different plasmid derivatives are less direct.

It was found that metabolic load on an *E. coli* host cell population was increased as the PCN increased (Table 5) and that of the two natural plasmids, pRAS3.1 placed a small but significantly higher metabolic load on the host than did pRAS3.2. With this information, we speculate on the evolution of pRAS3.1 and pRAS3.2 and why both plasmids have survived. Although one cannot be certain which plasmid originated first or which of the two differences to the backbones of the plasmids occurred first, a likely scenario is the following. Plasmid pRAS3.2, with three 22-bp iterons plus four 6-bp repeats in the promoter of the *mobB-mobA/repB* genes and a PCN of ~30, was most likely the ancestral plasmid. It more closely resembles the rest of the IncQ plasmids in the number of 22-bp iterons and has a PCN closer to that of typical IncQ family plasmids (although still considerably higher). It is also interesting that the pRAS3-like plasmid that has been integrated into the chromosome of the obligately intracellular parasite *Chlamydia suis* has four 6-bp repeats, similar to pRAS3.2, rather than pRAS3.1 (8). Plasmid pRAS3.1 probably arose from pRAS3.2 in two steps, the first being the acquisition of an additional 6-bp repeat which resulted in an approximately doubling of the PCN to ~59 and eventual random segregation of the two sister plasmids. The additional metabolic burden placed on the host by this high-copy-number intermediate plasmid provided the evolutionary pressure for the selection of a

plasmid with a lower copy number. This was achieved by the acquisition of an additional 22-bp iteron with a corresponding reduction in PCN to ~41. As shown in this study, this plasmid with four 22-bp iterons would be expected to displace the intermediate plasmid with three 22-bp iterons, even though it had a higher PCN. Should an increase in the number of iterons have occurred first with a corresponding reduction in PCN (such a plasmid, pRAS3.1.44, was constructed, and the PCN was found to be ~23) (17), the plasmid with a lower PCN and with four iterons would still have displaced the three-iteron plasmid as shown in Table 3. However, since this lower-copy-number plasmid is perfectly stable, it seems less likely for there to have been evolutionary pressure for this plasmid to have gained an additional 6-bp repeat, thereby increasing its PCN but placing an increased metabolic load on the host. A different possible reason for the acquisition of an additional 6-bp repeat is that not only is the expression of *repB* increased (17) but the expression of the *mobA* and *mobB* genes is also increased, as they are part of the same operon (Fig. 1A). If the additional 6-bp repeat altered the mobilization frequency, this could affect the horizontal spread of one of the variants and thereby provide a selective advantage. However, when mobilized by the RP4 conjugative system, the mobilization frequency of plasmids with either four or five 6-bp repeats and identical iteron numbers was found to be similar (17). Therefore, a change in mobilization frequency is unlikely to have provided selective pressure for acquisition of an altered number of 6-bp repeats.

We would have liked to perform plasmid copy number, displacement, competition, and metabolic load experiments in the two different *Aeromonas* hosts for pRAS3.1 and pRAS3.2, but they are no longer available. Our repeated attempts to transform a different natural isolate of *Aeromonas salmonicida* were unsuccessful. As IncQ plasmids have a broad host range and are highly promiscuous, we carried out our studies in *E. coli* as a representative of one of the many types of bacteria in which pRAS3 plasmids could occur. However, it is possible that other hosts might have placed somewhat different evolutionary pressures on the evolution of the pRAS3 plasmids.

Our overall finding from this study was that although pRAS3.2 has the advantage that it places a slightly lower metabolic burden on an *E. coli* host, it has the disadvantage that it is displaced by pRAS3.1 if both plasmids are present in the same cell. Nevertheless, both plasmids appear to have survived in the natural environment, with pRAS3.1 having been isolated from *Aeromonas salmonicida* in Norway and Scotland and pRAS3.2 having been isolated from atypical *A. salmonicida* in Norway (12) and *A. salmonicida* in Japan (1).

ACKNOWLEDGMENTS

We thank Trine L'Abée-Lund for the gift of plasmids pRAS3.1 and pRAS3.2.

This work was supported by grants from the National Research Foundation (Pretoria, South Africa).

REFERENCES

1. **Aoki, T., and A. Takahashi.** 1986. Tetracycline resistant gene of a non-transferable R-plasmid from fish pathogenic bacteria *Aeromonas salmonicida*. Bull. Jpn. Soc. Scient. Fish. **52:**1913–1917.
2. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1993. Current protocols in molecular biology. Wiley Interscience, New York, NY.
3. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker, and H. W. Boyer.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Bowers, L. M., R. Krüger, and M. Filutowicz.** 2007. Mechanism of origin activation by monomers of R6K-encoded π protein. J. Mol. Biol. **368:**928–938.
5. **Carlton, B. C., and B. J. Brown.** 1981. Gene mutation, p. 222–242. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, DC.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:**1141–1156.
7. **Dorrington, R. A., and D. E. Rawlings.** 1989. Identification and sequence of the basic replication region of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. J. Bacteriol. **171:**2735–2739.
8. **Dugan, J., D. D. Rockey, L. Jones, and A. A. Andersen.** 2004. Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the chlamydial *inv*-like gene. Antimicrob. Agents Chemother. **48:**3989–3995.
9. **Gardner, M. N., S. M. Deane, and D. E. Rawlings.** 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. **183:**3303–3309.
10. **Gerdes, K., J. E. Larsen, and S. Molin.** 1985. Stable inheritance of plasmid R1 requires two different loci. J. Bacteriol. **161:**292–298.
11. **Guzman, L., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
12. **L'Abée-Lund, T., and H. Sørum.** 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid **47:**172–181.
13. **Lee, C., J. Kim, S. G. Shin, and S. Hwang.** 2006. Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. J. Biotechnol. **123:**273–280.
14. **Lenski, R. E.** 1992. Relative fitness: its estimation and its significance for environmental applications of microorganisms, p. 183–198. *In* M. A. Levin, R. J. Seidler, and M. Rogul (ed.), Microbial ecology: principles, methods, and applications. McGraw-Hill, New York, NY.
15. **Lenski, R. E., S. C. Simpson, and T. T. Nguyen.** 1994. Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. J. Bacteriol. **176:**3140–3147.
16. **Lin, L. S., and R. J. Meyer.** 1986. Directly repeated, 20-bp sequence of plasmid R1162 DNA is required for replication, expression of incompatibility, and copy-number control. Plasmid **15:**35–47.
17. **Loftie-Eaton, W., and D. E. Rawlings.** 2009. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. J. Bacteriol. **191:**6436–6446.
18. **Meyer, R.** 2009. Replication and conjugative mobilization of broad host-range IncQ plasmids. Plasmid **62:**57–70.
19. **Miao, D.-M., H. Sakai, S. Okamoto, K. Tanaka, M. Okuda, Y. Honda, T. Komano, and M. Bagdasarian.** 1995. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. Nucleic Acids Res. **23:**3295–3300.
20. **Park, K., E. Han, J. Paulsson, and D. K. Chattoraj.** 2001. Origin pairing ('handcuffing') as a mode of negative control of P1 plasmid copy number. EMBO J. **20:**7323–7332.
21. **Pfaffl, M. W., G. W. Horgan, and L. Dempfle.** 2002. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. **30:**e36.
22. **Rawlings, D. E., and E. Tietze.** 2001. Comparative biology of IncQ and IncQ-like plasmids. Microbiol. Mol. Biol. Rev. **65:**481–496.
23. **Sakai, H., and T. Komano.** 1996. DNA replication of IncQ broad-host-range plasmids in gram-negative bacteria. Biosci. Biotechnol. Biochem. **60:**377–382.
24. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
25. **Williams, D. R., and C. M. Thomas.** 1992. Active partitioning of bacterial plasmids. J. Gen. Microbiol. **138:**1–16.
26. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequence of the M13mp18 and pUC19 vectors. Gene **33:**103–119.

Plasmid 67 (2012) 15–34

Contents lists available at SciVerse ScienceDirect

Plasmid

journal homepage: www.elsevier.com/locate/yplas

Review

Diversity, biology and evolution of IncQ-family plasmids

Wesley Loftie-Eaton, Douglas E. Rawlings *

Department of Microbiology, University of Department of Microbiology, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa

ARTICLE INFO

Article history:
Received 12 July 2011
Accepted 11 October 2011
Available online 19 October 2011
Communicated by Dr. Dhruba K. Chattoraj

Keywords:
IncQ
Broad host-range
Plasmid replication
Plasmid mobilization
Plasmid evolution

ABSTRACT

Plasmids of IncQ-family are distinguished by having a unique strand-displacement mechanism of replication that is capable of functioning in a wide variety of bacterial hosts. In addition, these plasmids are highly mobilizable and therefore very promiscuous. Common features of the replicons have been used to identify IncQ-family plasmids in DNA sequence databases and in this way several unstudied plasmids have been compared to more well-studied IncQ plasmids. We propose that IncQ plasmids can be divided into four subgroups based on a number of mutually supportive criteria. The most important of these are the amino acid sequences of their three essential replication proteins and the observation that the replicon of each subgroup has become fused to four different lineages of mobilization genes. This review of IncQ-family plasmid diversity has highlighted several events in the evolution of these plasmids and raised several questions for further research.

Contents

* Corresponding author. Fax: +27 21 808 5846.
E-mail address: der@sun.ac.za (D.E. Rawlings).

0147-619X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.plasmid.2011.10.001

1. Introduction

Plasmids belonging to the *Escherichia coli* incompatibility group Q (IncQ) family are fairly small in size (5.1–14.2-kb), capable of replication in a very broad range of hosts and are readily mobilizable (Frey and Bagdasarian, 1989; Meyer, 2009; Rawlings and Tietze, 2001). This makes them highly promiscuous. As they are exposed to many potential hosts that are able to grow in a wide variety of conditions, they are subject to recombination with a large number of genetic elements including other plasmids and transposons. The result of this is that several evolutionary lineages exist within the IncQ-like plasmid family and both micro- and macro-evolutionary events can be identified. They are therefore prime candidates for studies on the adaptability and evolution of plasmids.

The advent of high-throughput DNA sequence technology has resulted in a growing number of IncQ-like plasmids in the nucleotide sequence databases. An analysis of these sequences can expand our understanding of IncQ-family plasmid evolution and is one of the reasons for this review. Since several of the IncQ-family plasmids have been studied in some detail, variations in features known to be important can highlight where our understanding of IncQ plasmid biology requires greater insight and can help to identify research questions.

2. Recognition of IncQ-family plasmids

When DNA sequence information is available but very little biological information this raises the question of how to identify a plasmid as belonging to the IncQ-family. Current understanding of the genetic organization of the IncQ plasmids is that the plasmid backbone can be viewed as consisting of at least two modules, a replicon and a mobilization region. Furthermore, all of the IncQ2 plasmids, but none of the IncQ1 plasmids analyzed to date posses a toxin–antitoxin (TA) type plasmid stability system located within the replicon and this may be considered to be a third backbone module. Since the mobilization genes can vary in type (see Section 7) and a toxin–antitoxin system may or may not be present, identification of an IncQ-family plasmid needs to focus on the nature of the replicon.

A feature of the most studied IncQ plasmid family replicon, RSF1010 (R1162, R300B, pSRC15 and pTY474p3 are almost identical and RSF1010 will be used to refer to all five plasmids), is that it replicates by a strand-displacement mechanism (Sakai and Komano, 1996) that is unique. It seems obvious that for a plasmid to be considered to belong to the IncQ-family it would need to share this replication mechanism. The IncQ plasmid replicon is characterized by having three genes, *repBAC*, and a corresponding *cis*-acting locus within the origin of replication (*oriV*) to which each of the RepB (primase), RepA (helicase) and RepC (specific DNA-binding protein) is able to bind during the initiation of replication. An additional easily recognizable feature of all IncQ-family replicons studied to date is that they have a series of at least three identical 22-bp iterons (or 20-bp iterons with 2-bp spaces) within the *oriV* region to which RepC binds (Scholz et al., 1989). The replication process has been recently reviewed by Meyer (2009) and will not be discussed in detail here although a comparison of the features of IncQ replicons is made in Section 6.

3. The identification of putative IncQ-family plasmids

To identify plasmids that potentially belong to the IncQ-family, we have scanned the GenBank and related databases using RepB, RepA and RepC sequences from a number of previously identified IncQ plasmids belonging to both the IncQ1 and IncQ2 subfamilies using the PSI-BLAST program (Altschul et al., 1997). Where similarities that were considerably above the background were identified, we searched for genes encoding one or more of the other two Rep proteins. Finally, the nucleotide sequences of the putative IncQ-family plasmids or DNA fragments into which IncQ-plasmids has been integrated were examined for *oriV*-like iteron sequences. Plasmids identified in this way are listed in Table 1 and maps of representatives of these are shown in Fig. 1. These plasmids are grouped into the recognized subgroups IncQ1 and IncQ2 or the proposed new IncQ3 and IncQ4 subgroups, the motivation for which will be presented when discussing the replicon and plasmid mobilization regions.

Dendrograms indicating the relatedness of putative IncQ-family plasmids based on the amino acid sequences of the RepA-helicase, RepB-primase and RepC-DNA binding proteins are indicated in Fig. 2. In the case of the RepA-helicase, the plasmids appear to be divided into two major groups with the group that includes plasmids previously identified as belonging to subgroup IncQ1 being less diverse than the group containing plasmids previously identified as belonging to subgroup IncQ2. Plasmid pPNAP08 is a deep-branching outlier more closely related to IncQ1 than IncQ2 and which we propose to be a representative of a new IncQ4 subgroup. When comparing RepB-primases (Fig. 2B), the IncQ1 subgroup contains the same plasmids as for RepA, while the RepB primase of pPNAP08 shows very little sequence similarity to that of any of the IncQ-like plasmids. The second group is split into two with the previously identified IncQ2 plasmids grouped together and a new group comprising plasmids pGNB2, pBRST7.6 and pQ7 being clearly distinguished from the IncQ2 plasmids and which we have allocated to a new IncQ3 subgroup. A similar, though less clear division, is apparent when comparing the RepC proteins. Again the IncQ1 plasmids form a cluster with relatively little internal variation

Table 1
List of IncQ-like plasmids, the host from which they were isolated and accessory DNA.

IncQ subgroup	Plasmid[a]	Size (bp)	Source from which isolated	Genes and ORFs in addition to backbone[b]	Reference(s) and NCBI accession number
IncQ-1	P89S	±8180	*E. coli* (clinical)	Su	Saano and Zinchenko (1987))
	pAZ1	±8000	*S. enterica* serovar Typhimurium type 179	Su, Tp (DHFR type III)	Fling et al. (1988)
	PB165	±11,900	*E. coli* (UK)	Sm, Su	Barth and Grinter (1974), Grinter and Barth (1976)
	pCCK1900	10,226	*Pasteurella multocida* (Germany)	*floR*, *strAB*, *sulII*	Kehrenberg et al. (2008), NC_011378
	pCCK381	10,874	Pasteurella multocida 381(UK)	*floR*	Kehrenberg and Schwarz (2005), NC_006994
	pCHE-A	7560	*Enterobacter cloacae* (Canada)	*bla*GES-5, integron mobilization unit (IMU)	Poirel et al. (2009), NC_012006
	pDN1	5112	*Dichelobacter nodosus* (Australia)	None	Whittle et al. (2000), NC_002636
	pFM202	±7100	*Neisseria gonorrhoeae* (Spain)	Ap	Rotger and Nombela (1983)
	pFM739	±9450	*N. sicca* (Spain)	Ap, Sm, Su	Rotger et al. (1986)
	pHD148	±7500	*Haemophilus ducreyi* (Kenya)	Su	Albritton et al. (1982)
	pHD8.1	±8100	*Actinobacillus pleuropneumoniae* (Canada)	Sm, Su	Willson et al. (1989)
	pIE1107	8520	Piggery manure (Germany)	*aph*(3′)-Id, *sat3*, *sulII*[c]	Tietze (1998), NC_002089
	pIE1115	10,687	Piggery manure (Germany)	*linB*-like, *strAB*, *sulII*	Smalla et al. (2000), NC_002524
	pIE1120	±9100	Piggery manure (Germany)	*tetA*(Y), *strAB*	Smalla et al. (2000), AF070999
	pIE1130	10,687	Piggery manure (Germany)	*aph*(3′)-I, *catIII*, *strAB*, *sulII*	Smalla et al. (2000), NC_004973
	pIE639	±11,100	*E. coli* O20:H-	*aph*(3′)-Id, *sat3*, *strAB*, *sulII*	Tietze et al. (1989)
	pIE723	±9500	*E. coli* O147:K88	*ant*(2″)-Ia, *strAB*, *sulII*	Tietze et al. (1989)
	pMS260	8124	*Actinobacillus pleuropneumoniae* (Japan)	*sulII*, *strAB*,	Ito et al. (2004), NC_005312
	pSRC15	8688	*Salmonella enterica* subsp. *enterica* serovar *Typhimurium* (Australia)	*sulII*, *strAB*	Yau et al. (2010)
	pTY474p3	8688	*Salmonella enterica* subsp. *enterica* serovar *Typhimurium* str. ST4/74 (UK)	*sulII*, *strAB*	CP002490[d]
	R1162	±8680	*P. aeruginosa* (Canada)	*sulII*, *strAB*	Bryan et al. (1972)
	R300B	±8680	*S. enteric* serovar Typhimurium (UK)	*sulII*, *strAB*	Barth and Grinter (1974)
	R678	±14,000	*S. enteric* serovar Dublin (Denmark)	*Sm*, *Su*	Barth and Grinter (1974), Grinter and Barth (1976)
	R684	±9500	*Proteus mirabilis*	*Sm*, *Su*	Barth and Grinter (1974), Grinter and Barth (1976)
	RSF1010	8684	*E. coli* strain 3 (USA)	*sulII*, *strAB*	Guerry et al. (1974), NC_001740
IncQ-2	pJA8102-2	11,823	*A. salmonicida* M28102 (Japan)	*tetAR*(C)	Aoki and Takahashi (1986)
	pRAS3.1	11,851	*A. salmonicida* subsp. *salmonicida* (Norway) and *A. salmonicida* subsp. *salmonicida* MT361 (Scotland)	*tetAR*(C)	L'Abee-Lund and Sørum (2002), AY043298
	pRAS3.2	11,823	Atypical *A. salmonicida* (Norway)	*tetAR*(C)	L'Abee-Lund and Sørum (2002), AY043299
	pTC-F14	14,155	*A. caldus* (South-Africa)	*tnp*, ORF13, ORF20.8, ORF17.2, ORF33	Gardner et al. (2001), NC_004734
	pTF-FC2	12,184	*A. ferrooxidans* (South Africa)	*grx*, *merR*-like, ORF43, *tnpR*[c]	Rawlings et al. (1984), M73777, M35249
IncQ-3	pBRST7.6	7621	*Aeromonas hydrophila* strain AO1	*qnrS2*	NC_011207[d]
	pGNB2	8469	Activated sludge (Germany)	*qnrS2*, Tn1721	Bönemann et al. (2006), NC_013773
	pQ7	9042	*E. coli* strain 7 (Switzerland)	*bla*GES-1, *bla*OXA/aac(6′)-Ib, *int3*	NC_014356[d]
IncQ-4	pPNAP08	6459	*Polaromonas naphthalenivorans* CJ2 (USA)	None	Yagi et al. (2009), NC_008764.1

[a] Plasmids are listed in alphabetical order.
[b] If known the exact gene was given, otherwise the type of antibiotic resistance is given. Ap, ampicillin; Cl, clindamycin; Cm, chloramphenicol; Lm, lincomycin; km, kanamycin; Sm, streptomycin; Su, sulfonamide; Tc, tetracycline; To, tobramycin; Tp, trimethoprim. Ap resistance conferred by *bla*; Cl/Lm conferred by *linB*; Cm conferred by *catIII*; gentamicin/km/To conferred by *ant*(2″)-Ia; km/Nm/Tb conferred by *ant*(2″)-Ia; km/Nm conferred by *aph*(3′)-Id; quinolone resistance conferred by *qnrS2*; streptothricin resistance conferred by *sat3*; Sm resistance conferred by *strAB*; Su resistance conferred by *sulII*; Tc conferred by *tetA*(Y) and *tetAR*(C).
[c] Gene truncated.
[d] Unpublished, GenBank accession number for DNA sequence.

while the RepC of pPNAP08 is an even more distant outlier. The pGNB2, pBRST7.6 and pQ7 plasmids again form a group. The IncQ2 plasmids have a high level of variation mainly due to the RepC protein of the pRAS3 plasmids (pRAS3.1 and pRAS3.2 have identical Rep proteins) being an outlier. The reason for this is that *repC* gene and *oriV* region of the pRAS3 plasmids appears to have undergone a gene swopping event which has resulted in the anomalous

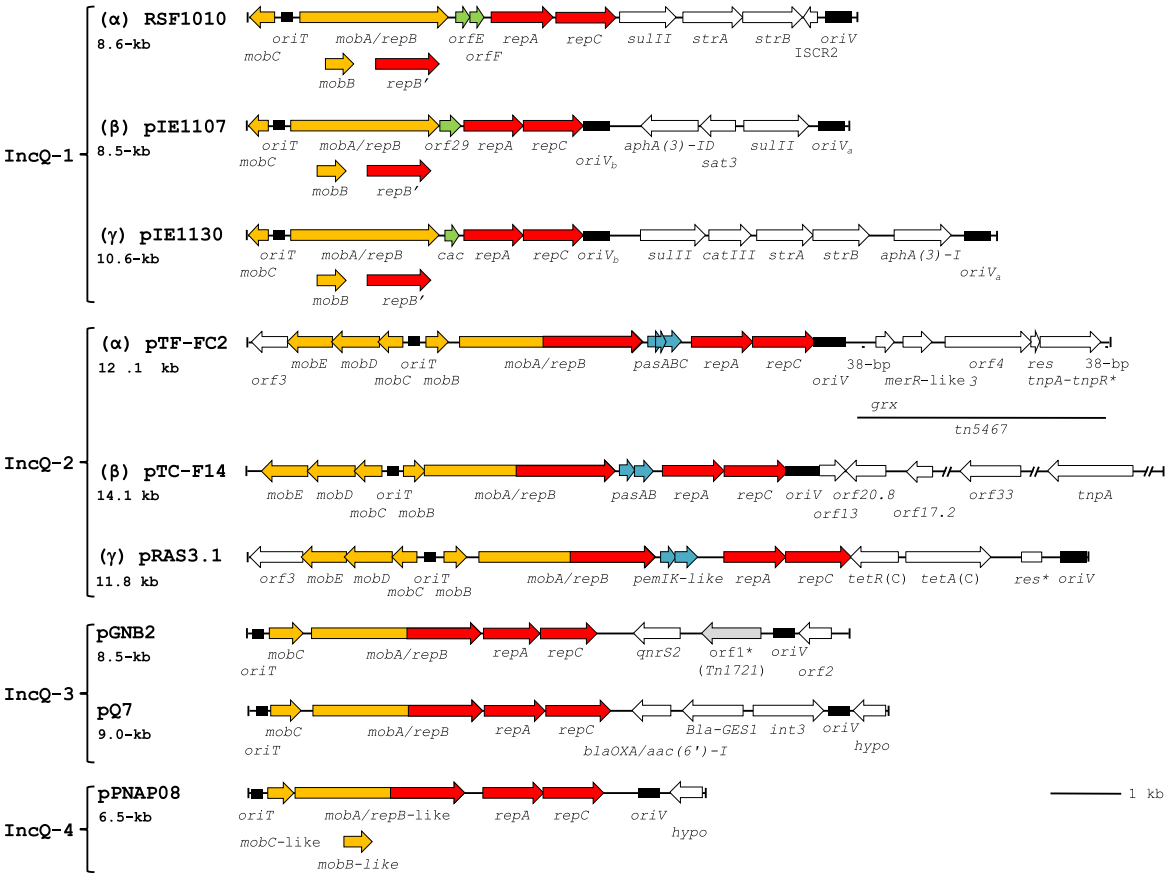


Fig. 1. Maps of representatives of each of the four proposed IncQ plasmid subgroups. Subgroup IncQ1 is represented by RSF1010 (Scholz et al., 1989), pIE1107 and pIE1130 (Smalla et al., 2000); subgroup IncQ2, by pTF-FC2 (Dorrington and Rawlings, 1990), pTC-F14 (Gardner et al., 2001) and pRAS3.1 (L'Abee-Lund and Sørum, 2002); subgroup IncQ3 by pQ7 (Poirel et al., 2010) and pGNB2 (Bönemann et al., 2006); and subgroup IncQ4 by pPNAP08 (accession number NC_008764.1). The α, β and γ incompatibility groups within each IncQ subgroup are indicated in brackets where known. Genes and structural features; *aph*(3′)-ID, kanamycin and neomycin aminoglycoside phosphotransferase; *blaOXA/aac*(6′)-1, gene for OXA-AAC6′-1b fusion protein; *blaGES1*, extended spectrum β-lactamase gene; *E*, *F*, genes for the *cac*, control of *repA* and *repC* regulators, *catIII*, chloramphenicol acetyl transferase; *grx*, glutaredoxin-like gene; *linB*-like, *linB*-like lincosamide nucleotyltransferase; *hypo*, hypothetical protein gene; *int3*, integrase 3; *merR*-like, *merR*-like regulator gene; *mobA*, *B*, *C*, *D* and *E*, mobilization genes; *oriVa* and *oriVb*, origins of vegetative replication; *orf2*, *orf3*, *orf13*, *orf17.2*, *orf20.8*, *orf33* and *orf43* open reading frames of unknown function; *oriT*, origin of transfer; *pasA*, *B* and *C*, plasmid addiction system genes; *pemIK*-like, toxin antitoxin genes; *qnrS2*, quinolone resistance gene; *repA*, *B*, *B′* and *C*, replication genes; *res*, site of cointegrate resolution by transposon resolvase, *strA* and *B*, streptomycin aminoglycoside phosphotransferase; *sulII*, sulfonamide resistant dihydropteroate synthase; *tetR*(C) and *tetA*(C), genes for tetracycline resistance regulator and transporter; *tnpA*, transposase; *tnpR*, resolvase. Genes involved in replication and mobilization are indicated by red and yellow arrows respectively, while the *oriV* and *oriT* regions are indicated by black rectangles. Genes thought to be involved in the control of replication are indicated by green arrows while genes encoding toxin–antitoxin systems are indicated by blue arrows. Accessory genes are indicated by white arrows and genes which have been inactivated by deletions are indicated by an asterisk.

position of these sequences (Loftie-Eaton and Rawlings, 2009) as will be discussed in Section 6.2.5.

To confirm that all of the plasmids shown in Table 1 (Fig. 1) are likely to belong to the IncQ-family, the *oriV* regions containing the RepC-binding target iterons including the region immediately downstream were examined for features previously identified as being characteristic of IncQ-family plasmids. Besides the 22-bp (or 20 plus 2-bp spacer) iterons, these include an A + T-rich region followed by a G + C-rich region and a highly conserved 15-bp region (Rawlings and Tietze, 2001). The alignment of representatives of these *oriV* sequences is shown in Fig. 3 but with the equivalent pPNAP08 and pRAS3 *oriV* regions omitted. It may be seen that the *oriV* regions can be placed into the same three groups identified using the Rep A, B and C protein sequences with a high degree of sequence conservation within each group. There is also a marked amount of overall sequence consensus especially within the A + T rich region and the adjacent highly conserved region. The *oriV* region adjacent to the pRAS3 and pPNAP08 iterons does not align well with the equivalent regions of the *oriV*s discussed above. The pRAS3 plasmids have no clear homology in the G + C or A + T rich regions and a region containing 11 of the normally conserved 15 nucleotides is situated slightly further from the iterons than for the other IncQ-2 plasmids (data not shown). Plasmid pPNAP08 has a 36-bp region with a somewhat A + T-rich (61%) sequence preceding a region similar to the highly conserved sequence (12/15-bp match) but located 46-bp (as opposed to ~86-bp) from the iterons. The 22-bp iterons of all IncQ plasmids have been aligned in Fig. 4. Although there is a considerable amount of sequence variation, there are two highly conserved CCCC and TAAC tetramer (4-mer) sequences shared by most IncQ plasmids. Interestingly, even

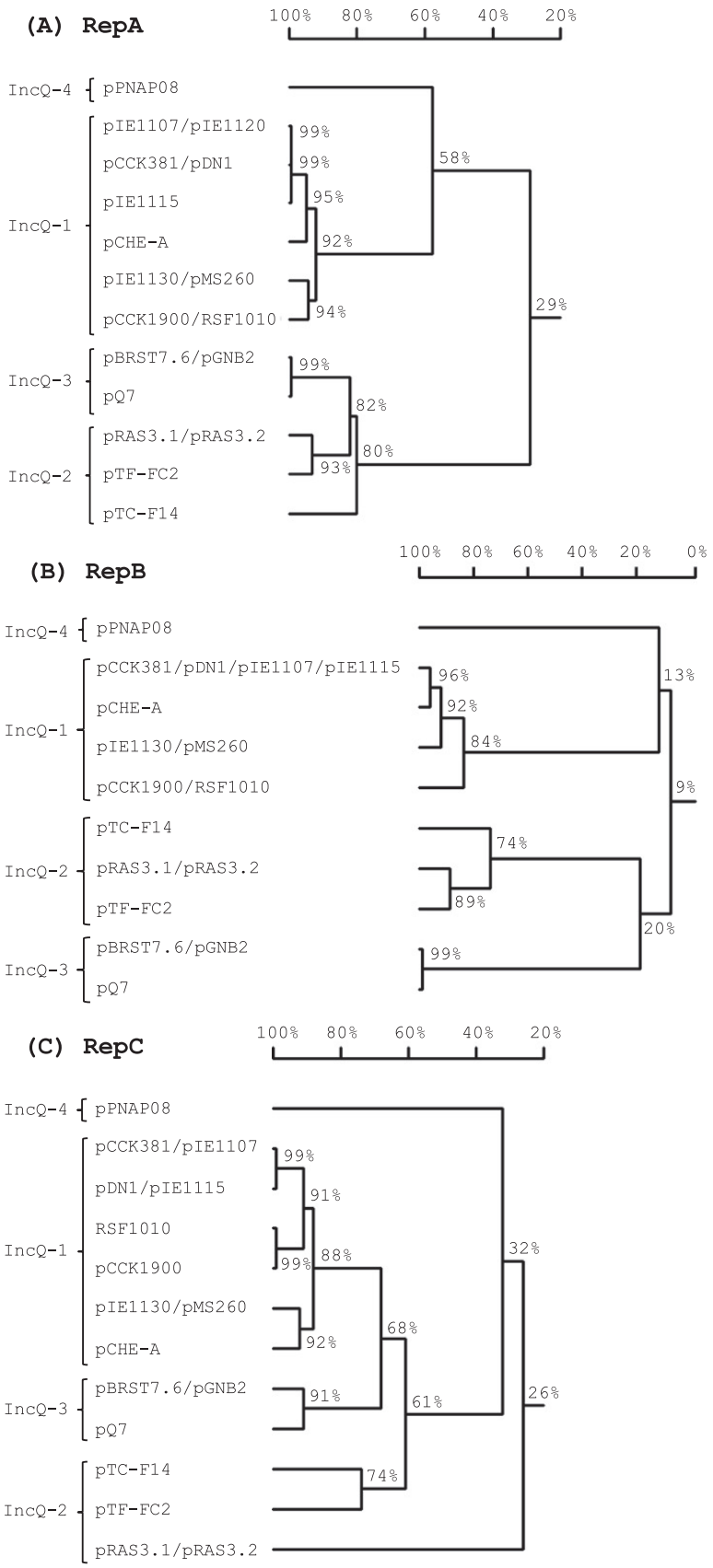


Fig. 2. Phylogenetic relationships between replication proteins of the IncQ plasmid family. Percentages represent amino acid sequence identities. A, RepA helicase; B, RepB primase; C, RepC iteron-binding protein. GenBank accession numbers for the respective plasmid nucleotide sequences from which the protein sequences were obtained are given in Table 1.

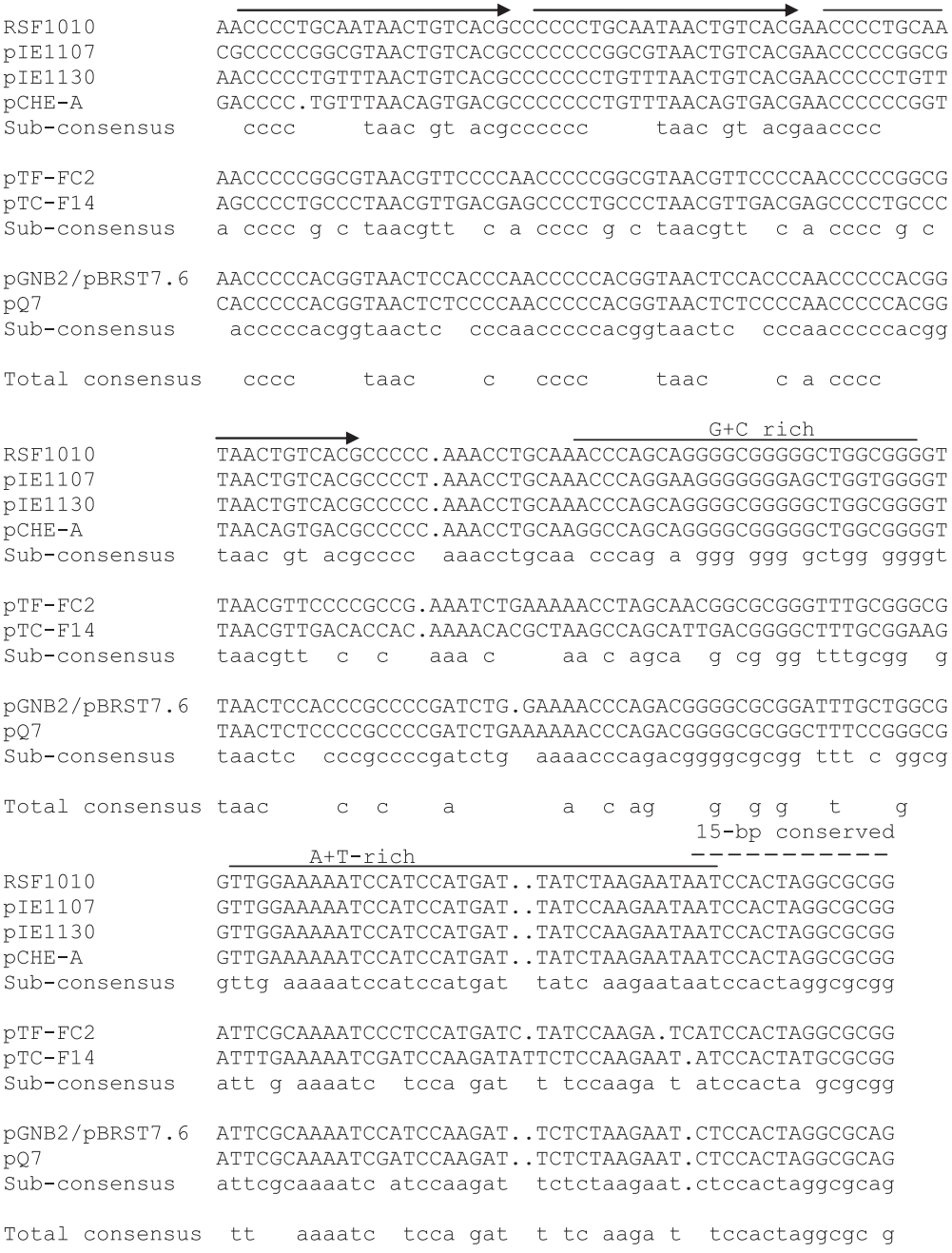


Fig. 3. Nucleotide sequence alignment of the highly conserved region within the functional *oriV* of representatives of IncQ1, IncQ2 and IncQ3 subgroup plasmids. Direct repeats are shown by arrows above the sequence, G + C and A + T regions by solid lines and the highly conserved 15-bp region by a broken line above the sequence. Consensus sequence within each subgroup and the overall consensus between all sequences are shown. Sequences of the pRAS3 plasmids and pPNAP08 have been omitted as they do not have clear G + C, A + T and the highly conserved 15-bp region (see text). GenBank accession numbers for the respective plasmid nucleotide sequences are given in Table 1.

the pRAS3 plasmids whose iteron sequence does not align well with the rest of the IncQ plasmids also have CCCC and TAAC elements within the iterons (although the spacing is different). Similarly in pPNAP08, the CCCC sequence is present in the iterons but the TAAC sequence is replaced by TATC.

4. Diversity and ecology

Information regarding the size, geographical location of where the host was sourced and accessory DNA of IncQ-like plasmids is summarized in Table 1. As will be noted, IncQ-family plasmids are not very large with pDN1 being the smallest at only 5 112-bp and with pTC-F14 the largest at 14 155-bp. The unique strand-displacement mechanism of replication is considered to place a size limitation on IncQ-family plasmids as a result of structural instabilities that may arise during this type of replication as will be explained in Section 6.1.

Also apparent from Table 1 is that where the host has been identified, most IncQ plasmids have been isolated from proteobacteria and in particular from the γ group. These include bacteria of widely different physiological properties such as the obligately anaerobic, heterotrophic,

IncQ-1	(α) pCCK1900/pIE1120/RSF1010	CCCCTGCAATAACTGTCACG
	(β) pCCK381/pDN1/pIE1107/pIE1115	CCCCCGGCGTAACTGTCACG
	(γ) pIE1130/pMS260	CCCCCTGTTTAACTGTCACG
	pCHE-A	CCCCCTGTTTAACAGTGACG
	Sub-consensus	cccc taac gt acg
IncQ-2	(α) pTF-FC2	CCCCCGGCGTAACGTTCCCC
	(β) pTC-F14	CCCCTGCCCTAACGTTGACG
	Sub-concensus	cccc g c taacgtt c
	(γ) pRAS3.1/pRAS3.2	CCCCCGGCGCAGGTGGTAAC
IncQ-3	pBRST7.6	CCCCCACGGTAACTCCACCC
	pQ7	CCCCCACGGTAACTCTCCCC
	Sub-concensus	ccccacggtaactc ccc
IncQ-4	pPNAP08	CCCCCTGTTTATCGTTCCCC
	Total consensus	cccc a

Fig. 4. Comparison of the nucleotide sequences of the 22-bp iterons. GenBank accession numbers for the respective plasmid nucleotide sequences are given in Table 1.

Dichelobacter nodosus, the typically aerobic, acidophilic, autotrophic, iron- and sulfur-oxidizing *Acidithiobacillus ferrooxidans* and the sulfur-oxidizing, moderately thermophilic, *Acidithiobacillus caldus*. The microbial origin of IncQ-family plasmids from piggery manure and activated sludge is unknown and the limited range of sources listed in Table 1 may be a reflection of the mainly antibiotic resistance selectable markers that have been used in plasmid isolation rather than where IncQ plasmids exist in the environment. Plasmids pDN1 and pPNAP08, for example, are cryptic IncQ-like plasmids that were identified only upon analysis of the genome sequences of *D. nodosus* and *Polaromonas naphthalenivorans* strain CJ2. *D. nodosus* was isolated in a consortium of other bacteria responsible for foot rot in sheep in Australia (Whittle et al., 2000) and pDN1 does not contain any antibiotic resistance genes. *P. naphthalenivorans* strain CJ2 is an aromatic hydrocarbon-degrading, facultatively chemolithotrophic β-proteobacterium that was isolated from coal tar waste-contaminated sediment and pPNAP08, at 6.4-kb, was the largest of 8 plasmids that was identified during the *P. naphthalenivorans* genome sequencing project (Yagi et al., 2009) and also does not contain any selectable markers.

Cloning vectors made using IncQ plasmid replicons have been reported to be capable of replication in widely different bacteria such as cyanobacteria (e.g. *Synechocyctis*), *Desulfovibrio desulfuricans*, *Bdellovibrio bacteriovorans*, *Chlorobium tepidum* and even Gram-positive bacteria such as *Mycobacterium smegmatis*, *Brevibacterium methylicum*, *Arthrobacter* sp. and *Streptomyces lividans* (reviewed in Rawlings and Tietze, 2001). Although the ability to replicate under laboratory conditions does not guarantee the ability to be stably maintained in a natural environment, IncQ plasmids may be very much more widely spread than thus far reported. A particularly interesting example suggestive of IncQ plasmid promiscuity is the identification of a tetracycline resistance genomic island bearing 99% sequence identity to the IncQ2 plasmid pRAS3.2 over 10.1-kb of shared DNA on the chromosome of *Chlamydia suis* R19 (Dugan et al., 2004). This *Chlamydia* is an obligatory intracellular pathogen that was isolated from pigs in the United States and how this plasmid was acquired by a pathogen that is not able to grow outside of its mammalian host cell is far from clear.

Not only have different IncQ family plasmids been isolated from different hosts and continents but IncQ-family plasmids with identical or nearly identical plasmid backbones have been isolated from different organisms and continents, indicating the promiscuity of a particular plasmid. This is illustrated by the almost identical plasmids RSF1010, R300B, R1162, pSRC15 and pTY474p3. RSF1010 was isolated from a colicinogenic *E. coli* strain during 1973 in Wisconsin, USA, (Guerry et al., 1974; Niedenzu et al., 2001), R1162 from *Pseudomonas aeruginosa* strain 1162 during 1972 in Alberta, Canada (Bryan et al., 1972) and R300B from pathogenic *Salmonella typhimurium* serovar *Typhimurium* in London, UK, during 1974 (Barth and Grinter, 1974). The two most recently sequenced variants, pTY474p3 (unpublished) and pSRC15 (Yau et al., 2010), were isolated from *Salmonella typhimurium* serovar *Typhimurium* strains in the UK (pTY474p3) and from both human and bovine sources in Australia (pSRC15), and thus demonstrates the ability of these plasmids to persist unaltered in the environment for more than 30 years.

Another clear example of the ability of IncQ-like plasmids to spread and persist in the environment is the isolation of three tetracycline resistance plasmids, that are also either identical or close to identical, from different locations around the world. Plasmid pJA8102-2 was isolated from *Aeromonas salmonicida* M28102 in Japan during 1981 (Aoki and Takahashi, 1986). Approximately 20 years later two nearly identical plasmids, pRAS3.1 and pRAS3.2, of which pRAS3.2 has an identical restriction pattern to pJA8102-2, were isolated from multiple strains of *Aeromonas salmonicida* subsp. *salmonicida* and atypical *Aeromonas salmonicida*, respectively, in Norway (L'Abee-Lund and Sørum, 2002). The same researchers also isolated pRAS3.1 from an *A. salmonicida* subsp. *salmonicida* MT361 strain originally from Scotland. In each of the instances the *A. salmonicida* strains were isolated from aquaculture farms where they are responsible for causing furunculosis in the salmon.

In spite of the apparent bias for bacteria of the γ-group of proteobacteria to serve as hosts, plasmids of the IncQ family are considered to be truly broad host-range. This view is supported by a recent study by Suzuki et al. (2010), in which a trinucleotide genomic signature from almost 2000 completely sequenced plasmids was used to predict the major evolutionary host based on the genomic signatures of 817 completely sequenced prokaryotic genomes available at the time of the study. No genomic signatures corresponding to those of the six IncQ plasmids that were included in the study could be found among the prokaryotic genomes supporting the idea of the promiscuity of IncQ plasmids.

5. Accessory genes

Antibiotic resistance has been the major means by which IncQ plasmids have been selected from the environment. For example the IncQ-like plasmids pIE1107, pIE1130, pIE1120 and pIE1115 were all isolated from uncultured bacterial communities found within piggery manure slurry in Germany by means of biparental matings

using the manure slurry as donor and *E. coli* and *P. putida* as the recipient hosts with antibiotic selection for recipients and plasmids (Smalla et al., 2000; Tietze, 1998). Likewise many IncQ plasmids have been isolated from bacteria from clinical, animal or fish pathogen samples. As a result the accessory genes shown in Table 1 are dominated by a wide range of antibiotic resistance genes. Particularly noticeable is the high prevalence of streptomycin and sulfonamide resistance genes. In the few cases where IncQ-family plasmids have been isolated as a result of genome sequencing projects or from environments that are not pathogen associated, plasmids lacking accessory genes (pDN1 and pPNAP08) or plasmids containing accessory genes for which the functions remain cryptic have been identified (pTF-FC2 and pTC-F14).

Plasmids pTF-FC2 and pTC-F14 were isolated from *A. ferrooxidans* and *A. caldus*, the genus was *Thiobacillus* at the time of plasmid isolation in 1984 and 1998, respectively (Gardner et al., 2001; Rawlings et al., 1984; Rawlings et al., 1986). These extremophiles form part of a consortium of organisms responsible for the leaching of metals at a bioleaching plant in South Africa. As antibiotics are not used in this environment it does not come as a surprise that there were no antibiotic resistance genes on these plasmids. A Tn*21*-like transposon was found to be integrated into pTF-FC2 and although the *tnpR* and *tnpA* genes coding for the resolvase and transposase enzymes respectively, are inactive, the transposon retained the ability to be resolved when a functional *tnpR* from Tn*21* was supplied *in trans* (Clennel et al., 1995). Located between the 38-bp terminal inverted repeats (IR) of the transposon is a functional glutaredoxin-like gene which was able to complement an *E. coli trxA*$^-$ mutant, a *merR*-like gene which potentially encodes a MerR regulator but no *merA*-like gene, as well as ORF8 and ORF43 both of which did not have any sequence similarity to any known proteins in the NCBI database. ORF43 appeared to encode a 12-loop transmembrane protein similar to multidrug transporters but whose function is unknown (Rawlings and Tietze, 2001). No MerR and ORF43 protein products were produced in *E. coli*, but this does not mean that these two ORFs are not expressed in the native *A. ferrooxidans* host. Plasmid pTC-F14 has 5 ORFs all of unknown function in the same location but they are unrelated to those of pTF-FC2 (Gardner et al., 2001).

6. Replicons of IncQ-family plasmids

6.1. Common features of IncQ-family replicons

Some of the features common to IncQ-family replicons have been referred to earlier when describing the criteria used to identify IncQ-like plasmids in the sequence databases. These included the presence of *repB*, *repA* and *repC* genes (encoding the primase, helicase and iteron-binding protein, respectively). In addition, common functional elements within the *oriV* of IncQ-like plasmids are the three 22-bp iterons, followed by a G + C-rich region, an A + T-rich region (as shown in Fig. 3 and described in Section 3) and two inverted repeats (IR) known as single-strand initiation sites A and B (*ssiA* and *ssiB*, respectively) (Rawlings and Tietze, 2001; Scherzinger et al., 1991; Scholz et al., 1989). The function of the 15-bp highly conserved sequence following the A + T-rich region remains unknown (Rawlings and Tietze, 2001).

Although replication by the strand-displacement mechanism has been shown for only RSF1010 it is likely that all IncQ-like plasmids replicate by a similar mechanism as shown in Fig. 5. During replication, RepC binds to the iterons creating a bend that is dependent in the number of iterons bound (Miao et al., 1995). Bending of the DNA results in melting of the dsDNA at the A + T-rich region and stimulates RepA helicase monomers to assemble around the exposed ssDNA and catalyze further unwinding of the DNA in the 5′ → 3′ direction on both strands. It is thought that the G + C-rich region located between the iterons and the A + T-rich region, in addition to having a structural role in the bending of the DNA, momentarily halts unwinding of the R-strand until both the *ssiA* and *ssiB* initiation sites are exposed (Kim and Meyer, 1991). When exposed, inverted repeats within the *ssi* site of each strand form a stem-loop structure which contains the start point for DNA synthesis at its base and which is recognized by the RepB′ primase (Honda et al., 1993; Miao et al., 1993).

Once short primers have been synthesized by the RepB primase, the plasmid DNA is replicated by the DNA polymerase III holoenzyme continuously in either one direction initially (L strand), or both directions simultaneously (L and R strands) (De Graaff et al., 1978). A D-loop is initially formed during replication and as the single-stranded R-strand is displaced, a theta-type (θ) intermediate is formed which is visible by electron microscopy (Scherzinger et al., 1991). This single-strand displacement mechanism of replication, which lacks Okazaki fragment synthesis, is unique to IncQ-like plasmids. At the point when the two replication forks pass each other, the replication intermediate will consist of a maximum amount of displaced single stranded DNA and from that point onwards the amount of single stranded DNA will decrease until both strands have been fully replicated. Termination of replication occurs at the same position at which replication was initiated, and thus there are no specific termination signals (Kok et al., 1989).

Although this replication mechanism appears to be highly successful, the existence of a partially single stranded intermediate can result in structural instabilities that are believed to put a limitation on the size of plasmids that replicate by the strand displacement mechanism (Table 1). During the search for sequences similar to the three replication proteins described in Section 3, all the similarity hits were to proteins from plasmids of less than 15-kb (Table 1). This supports the view that there is a size constraint to IncQ-family plasmids. Although the exact upper size limit is unknown, IncQ plasmids are likely to be smaller than 20-kb.

In all IncQ-family plasmids discovered to date, the *repA* and *repC* genes are physically linked with the stop codon of the *repA* gene frequently, but not always, overlapping with the start of *repC*. It was shown that the cloned *oriV* region of the IncQ2 plasmid, pTC-F14, was able to replicate if a gene encoding its own *repC* plus the IncQ2 plasmid pTF-FC2 (to provide *repA* and *repB*) were placed *in trans*.

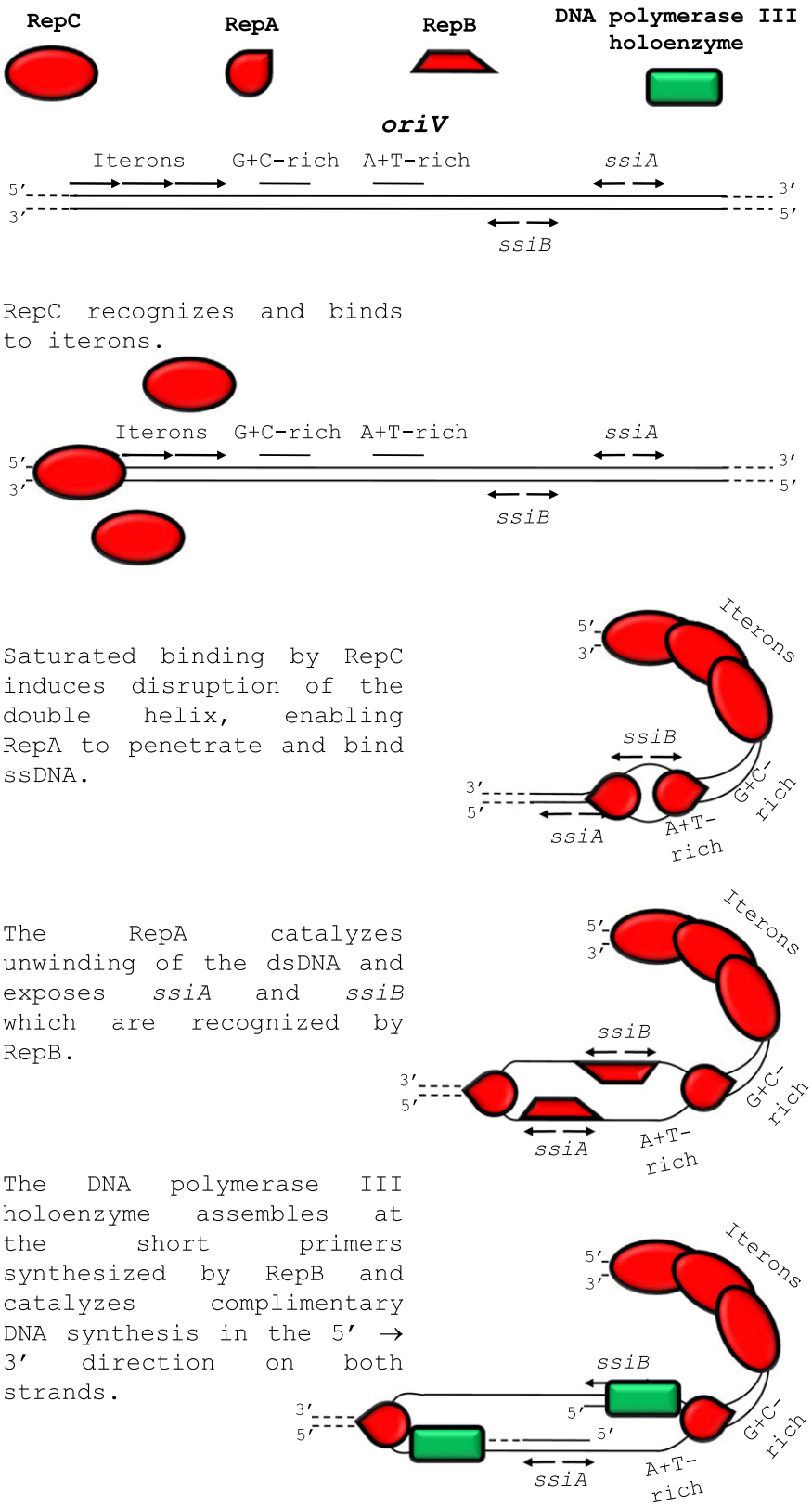


Fig. 5. Model for the initiation of replication at the oriV of IncQ plasmids.

(Gardner and Rawlings, 2004; Rawlings, 2005) Therefore, *repA* and *repC* may be separated. However, although replication with a RepA helicase and RepC iteron-binding protein from different plasmids was possible, this experiment did not test whether close coupling of the two genes improved the competitive fitness of the IncQ-family plasmids compared to if they were uncoupled.

In all IncQ-like plasmids, the gene for the RepB primase is not only upstream of the *repAC* genes but also fused to a relaxase that is an essential enzyme in DNA processing

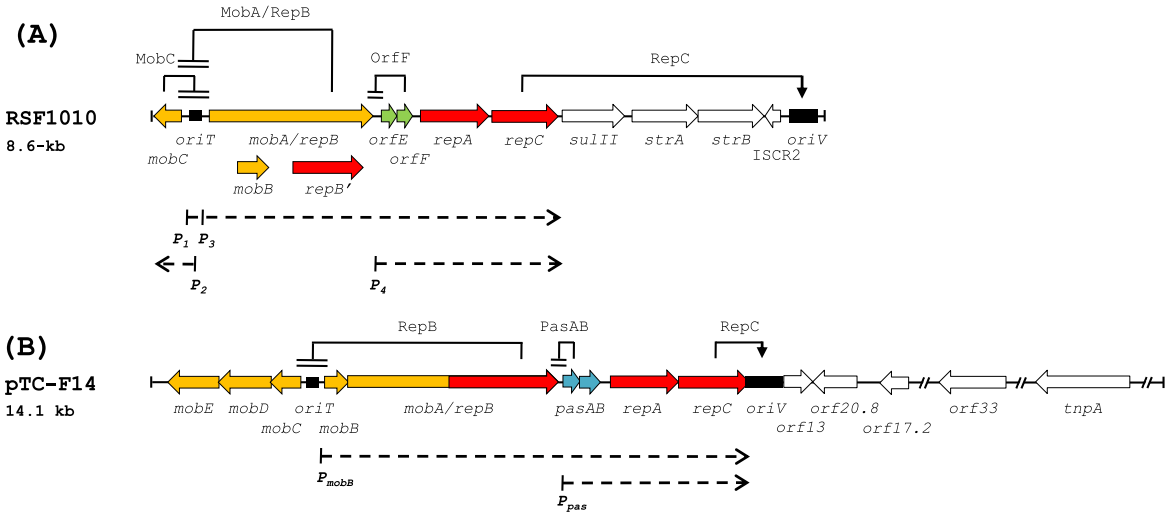


Fig. 6. Regulatory networks within the IncQ-1 and IncQ-2 plasmids (A) RSF1010 and (B) pTC-F14. The mRNA transcripts and the direction of transcription are indicated in broken lines. Negative regulators and the approximate regulatory positions at which they bind are indicated by double horizontal lines. The small silencing RNA produced by RSF1010 that binds and inhibits translation of the complimentary *repA* mRNA is indicated by a reversed half arrow. Positive regulators and the approximate position at which they bind are indicated by pointed arrows. A was adapted from Meyer (2009) and B from Gardner (2003).

prior to its mobilization by conjugative transfer. It has been shown in the case of the IncQ1 plasmid R1162 that the primase activity is required to prime vegetative plasmid DNA replication in an *E. coli* host and functions as a specific primase of DNA replication on the conjugative transfer of a single strand of DNA to a recipient cell (Henderson and Meyer, 1999). However, in certain IncQ-family plasmids (e.g. pTF-FC2) the primase fragment of the MobB-RepB fusion protein can be separated from the relaxase fragment and the respective fragments can function independently in plasmid vegetative replication and mobilization (Dorrington and Rawlings, 1990; Rohrer and Rawlings, 1992). It has not been tested which primase primes single stranded DNA replication in the recipient when the primase fragment has been removed from the relaxase fragment, but the primase of the conjugative IncP plasmid that is required to mobilize pTF-FC2 or the *E. coli* host *dnaG* product are possible candidates. Though both domains are capable of functioning independently of each other, the relaxase-primase fusion was found to be transferred along with the single stranded plasmid DNA during mobilization of R1162. The likely advantage would be that the primase is immediately available for priming complimentary strand DNA synthesis upon entry into the recipient host (Henderson and Meyer, 1996).

Although it is physiologically possible for IncQ replicons to function independently of the mobilization genes, the replicons of all IncQ plasmids identified to date occur downstream of a plasmid mobilization system. Put differently, no non-mobilizable IncQ plasmids have yet been found. Furthermore, since the RepB primase is required for plasmid replication and this is fused to a relaxase that is essential for mobilization, regulation of the *rep* genes is largely from a promoter that controls expression of a large transcript that includes some of the mobilization and all of the essential replication genes (Fig. 6). Regulation of this large transcript has been studied in only RSF1010 and pTC-F14, and to a lesser extent pTF-FC2 and pRAS3. Details of the regulation of RSF1010 have been reviewed in Meyer (2009) and the effect of autoregulation at P_4 of RSF1010 and at P_{mobB} of pTC-F14 is described in Section 6.2.3. Expression of the *mobA/repB* fusion gene from the P_{mobB} in pRAS3.1 was found to be 2.2-fold stronger than in its isogenic equivalent pRAS3.2 due to an additional 6-bp repeat in the *mobB* promoter region of pRAS3.1. The increased *repB* expression resulted in an increase in RepB availability and an increase in the copy number of pRAS3.1 relative to pRAS3.2, thus demonstrating a role of this promoter region in copy number control.

6.2. Diversity among IncQ-family replicons

6.2.1. Structure of the oriV region

Besides the overall similarity in the structure of most of the *oriV* regions there are some differences beyond those already discussed. In most IncQ-plasmids the 22-bp iterons are located immediately downstream of the *repC* gene. In some cases this association is so close that the *repC* gene terminates at a TAA stop codon located within the conserved TAAC site and sometimes at the same site of the second (pTF-FC2) or third iteron (pTC-F14) as each TAAC site is in a different reading frame. In other IncQ-family plasmids (e.g. RSF1010, pCCK381 and the pRAS3 plasmids) the *oriV* region has become dislocated from *repC* by the insertion of genes between the end of *repC* and the iterons. Remnants of transposons frequently remain that suggest that transposon activity was the cause of this separation. Examples of this are the insertion of *sulII*, *strA* and *strB* genes of RSF1010, the *tetR* and *tetA* genes of pRAS3, the *qnrS2* gene of pGNB2 and the *blaOXA* and *blaGES* genes of pQ7 (Fig. 1). In all cases the orientation of the *oriV* region relative to the *repBAC* genes has been retained. This orientation is unlikely to be a necessary requirement of IncQ-plasmid replication (cloned *oriV* regions can function when

Rep proteins are provided *in trans*) although an inverted *oriV* region might affect plasmid competitiveness. The reason for the frequent insertion of accessory genes into this position is probably because there are only two places where accessory genes may be inserted into an IncQ plasmid backbone without affecting the biology of the plasmid and that is on either side of the *oriV* region.

6.2.2. Variation in iteron sequence and plasmid incompatibility.

The iterons are the primary binding sites recognized by the RepC replication initiator and are unique to each plasmid so as to insure plasmid identity among competing plasmids and thus are the major incompatibility determinants in iteron-containing plasmids (Chattoraj, 2000; Lin and Meyer, 1986; Rawlings and Tietze, 2001). When two plasmids with very similar or identical iteron sequences are co-resident within the same host, the replication proteins are unable to distinguish between the two *oriV*s during initiation of replication. This results in copy number fluctuations for one or both of the plasmids and thus the inevitable loss of one of the plasmids (Novick, 1987). The 20-bp directly repeated sequence of each 22-bp iteron within an *oriV* is always fully conserved while the 2-bp spacers are not always conserved. A single point mutation within one of the three 20-bp conserved repeats of an *oriV* can result in an inability or significantly reduced ability, depending on where and in which iteron the mutation is located, to exert incompatibility or initiate replication (Lin et al., 1987; Miao et al., 1995). Plasmid pCHE-A is atypical of IncQ plasmids in that it has only 19-bp in the first of its otherwise identical three iterons (Fig. 4). Sequence conservation within the iterons is usually critical for functional interaction with the RepC, however, individual nucleotides may have a unique, unequal, functional contribution.

As replicon-associated plasmid incompatibility is a function of relatedness it can be used, in addition to DNA sequence homology, as a means to group plasmids into families. The incompatibility status or grouping of many of the IncQ-like plasmids has been determined in relation to other IncQ members by means of plasmid displacement studies in *E. coli* (Rawlings and Tietze, 2001). Currently the IncQ1 plasmids have been divided into three incompatibility groups, with RSF1010 (R1162, R300B, pSRC15 and pTY474p3) being placed in the IncQ1α subgroup, pIE1108 (pIE1107 with *oriVa* deleted), pIE1115 and pDN1 into a β subgroup and pIE1130 into a γ subgroup. Plasmid pCHE-A has not been tested but based on iteron sequence is likely to belong to a fourth subgroup with pMS260 possibly a fifth subgroup. Iteron sequence identity suggests that IncQ1 plasmids pCCK1900 and pCCK381 belong to the α and β subgroups respectively. In the case of IncQ2 plasmids, three incompatibility subgroups have also been identified with pTF-FC2, pTC-F14 and the pRAS3 plasmids being allocated to α, β and γ subgroups respectively. The incompatibility of the plasmids belonging to the proposed new IncQ3 group have not been tested, but an examination of their iteron sequences suggests that pGNB2 and pBSRT7.6 belong to the same subgroup while pQ7 belongs to a second IncQ3 incompatibility subgroup. Neither the replication genes nor the iterons of pPNAP08 share high levels of similarity to that of any of the plasmids within the existing IncQ1, IncQ2 or the proposed new IncQ3 groups and pPNAP08 is, therefore, unlikely to exhibit any replicon-mediated incompatibility to these IncQ-like plasmids.

The necessity of incompatibility testing as a means of plasmid classification has been diminished by the availability of sequence data. The sequence of the iterons is a good indication of incompatibility grouping although where an iteron sequence may differ by only one or two base pairs, incompatibility may not be predictable. Furthermore, although a symmetrical pattern of segregation was observed with most IncQ plasmids, there were exceptions to this. Plasmid RSF1010 was unable to coexist with plasmids pIE1107, pIE1115 and pIE1130, with RSF1010 being displaced by those plasmids but not *vice versa*. Sequence analysis of pIE1107 (also pIE1115 and pIE1130) revealed that it contained a second non-functional *oriV*, referred to as *oriVa*, that was similar to the *oriV* of RSF1010, and when deleted, to give pIE1108, the two plasmids were compatible (Smalla et al., 2000; Tietze, 1998). The non-functional *oriV* contained three iterons that were identical in nucleotide sequence to the iterons of RSF1010. This meant that pIE1107 was able to replicate as its RepC was able to specifically bind to its own iterons without interference by RSF1010. In contrast, RepC of RSF1010 would be expected to bind both its own iterons and non-productively to the identical iterons on pIE1107, thereby reducing the copy number of RSF1010 and resulting in its exclusion from the host.

Incompatibility phenotypes were observed when IncQ2 plasmids pTF-FC2 and pTC-F14 were coresident with the IncQ1 plasmids pIE1108 (pIE1107) or RSF1010. Both resident pTF-FC2 and pTC-F14 plasmids were displaced by pIE1108 as an incoming plasmid, but neither was able to displace a resident pIE1108 during reciprocal transformation experiments. RSF1010, as an incoming plasmid, was able to displace pTC-F14, but not pTF-FC2, and neither was able to displace RSF1010 (Gardner et al., 2001). Later it was demonstrated that most-likely non-productive binding of the RepA or RepC proteins of RSF1010 rather than its *oriV* was responsible for the incompatibility phenotype with pTC-F14 (Gardner and Rawlings, 2004). The IncQ2 plasmids pRAS3.1 and pRAS3.2 aggressively displaced pTF-FC2 and pTC-F14, and this incompatibility was also not associated with the iterons but rather with a small *orf* of unknown function downstream of the *mobCDE* genes (Loftie-Eaton and Rawlings, 2009). Together these examples represent situations where, instead of closely related plasmids being incompatible, plasmids such as pTF-FC2 and pIE1108 or pRAS3 and pTC-F14 are strongly incompatible even though they are more distantly related and for reasons not necessarily related to competition between iterons for RepC binding protein. This illustrates a difficulty in using plasmid incompatibility as the sole means of determining the relatedness between plasmids.

6.2.3. Number of iterons and effect on copy number.

Most of the IncQ-like plasmids sequenced to date have 3 perfectly conserved iterons and an average plasmid copy

number (PCN) between 10 and 16 plasmids per chromosome in *E. coli* (Rawlings and Tietze, 2001). In the event that a functional *oriV* contains more than 3 × 22-bp iterons the additional iterons are partly deleted, contain point mutations or are incorrectly spaced (not shown). The IncQ2-like plasmid pRAS3.1 is an exception as, unlike the other IncQ-like plasmids which have been characterized to date, it contains 4 fully conserved and correctly spaced iterons within its *oriV*. Deletion of one of the 4 × 22-bp iterons from the *oriV* of pRAS3.1 resulted in a 44% increase in PCN. Increasing the number of iterons from 4- to 5- and 7 × 22-bp iterons resulted in a 32 and 54% decrease in PCN, respectively (Loftie-Eaton and Rawlings, 2010). These results demonstrated that the number of fully conserved iterons within the *oriV*s of these plasmids had a direct influence on their respective copy numbers.

In addition to PCN being dependent on the number of iterons within the *oriV*, the copy number of the two pRAS3 plasmids was also found to be dependent on the strength of P_{mobB} (Fig. 6). A stronger P_{mobB} resulted in an increase in PCN through increased *repB* transcription, while the PCN decreased with increasing iteron copy number. Pairwise growth competition experiments between *E. coli* hosts containing pRAS3 derivative plasmids varying in iteron copy number and or P_{mobB} strength verified that an increase in PCN resulted in a decrease in host competitive fitness (Loftie-Eaton and Rawlings, 2010). Thus it was concluded that the fourth iteron in the *oriV* of pRAS3.1 was acquired to compensate for the high PCN brought about by the increased transcriptional activity of P_{mobB}, which would otherwise have resulted in reduced host cell competitive fitness. Interestingly, the fourth iteron in the *oriV* of pRAS3.1 also enabled the plasmid to effectively displace a competing plasmid with only three iterons from a cell, thereby giving pRAS3.1 an additional advantage over pRAS3.2. Derivative plasmids with 5 or 7 iterons, however, were increasingly unable to displace a competing three iteron plasmid due to these plasmids having lower copy numbers and presumably a diminished ability to effectively initiate replication.

6.2.4. Differences in copy number regulation

Strict regulation of PCN is essential for maintenance and spread during vegetative growth of the host. In the absence of a partitioning system, plasmids may not be inherited by daughter cells when the PCN is too low. If the PCN is too high the additional metabolic load carried by the host might reduce the growth rate and render it uncompetitive, thus also resulting in eventual loss of the host from the population, and therefore, a system that prevents runaway replication is also needed (Bingle and Thomas, 2001). It has been well-argued that with most IncQ plasmids, stability is primarily dependant on the maintenance of a relatively high copy number (Meyer 2009). Furthermore, the level of antibiotic resistance conferred by certain antibiotic resistance genes is copy number dependent and lowering the plasmid copy number was shown to lead to the selection of higher copy number mutants so as to confer higher levels of resistance (Becker and Meyer, 1997). Plasmid copy number thus appeared to be a compromise between the resistance level required, the imposed metabolic burden and the need to prevent plasmid loss during periods of non-selection. Although a PCN of 10 to 16 plasmids per chromosome is typical of most IncQ plasmids (Rawlings and Tietze, 2001), there may or may not be differences in different bacterial hosts as well as between different plasmids. The copy number of pTC-F14, for example, was found to be 12 to 16 plasmids per chromosome in both *E. coli* and *A. caldus* while that of the RSF1010 equivalent R300B was 10–12 in *E. coli* and 29–34 in *P. aeruginosa* (Barth and Grinter, 1974; Gardner et al., 2001; Lewington and Day, 1986). The IncQ2-like plasmids pRAS3.1 and pRAS3.2 were maintained at a PCN of 41 ± 4 and 30 ± 5 plasmids per chromosome, respectively, in *E. coli* which is much higher than for the other IncQ plasmids tested.

The copy number regulatory systems of IncQ-like plasmids have not yet been completely elucidated but a number of regulatory systems, such as the promoter system and its negative regulators mentioned above, have been identified for RSF1010 (reviewed in Meyer, 2009) and some members of the IncQ2 plasmids as illustrated in Fig. 6. In the case of RSF1010 a mutation that inactivated P_2, one of three promoters in the origin of transfer (*oriT*) region that is negatively regulated by MobC and MobA/RepB, resulted in an increase in PCN in both *E. coli* and *P. putida*. The change in copy number in *P. putida*, however, was not as pronounced as it was in *E. coli* (Frey and Bagdasarian, 1989). As described for pRAS3.1 and pRAS3.2, the ~1.4-fold higher PCN of pRAS3.1 compared to pRAS3.2, in spite of having 4 as opposed to 3 × 22-bp iterons, was found to be as a result of an approximately 2.2-fold higher level of production of RepB primase in pRAS3.1 due to it having a more active promoter (P_{mob}) upstream of the *mobB-mobA/repB* operon.

The region between the *repB* and *repA* genes has been shown to be involved in the modulation of *repA* and *repC* gene expression and in this region there are clear differences between and within IncQ plasmid subgroups. In RSF1010, a small 7.2-kDa (68 aa) protein encoded by *F*, is responsible for negative feedback regulation of its *repAC* genes at the upstream P_4 promoter (Maeser et al., 1990; Scholz et al., 1989). The *F* gene is the second of two genes that are located between P_4 and the 5′-end of the *repA* gene (Scholz et al., 1989) that are known as *cac* for control of *repA* and *repC*. The first gene, *E*, is also expressed, however the function of the small 5-kDa protein remains undefined and it was shown that repression of the P_4 promoter requires the presence of F and not E. A similar 68 aa protein

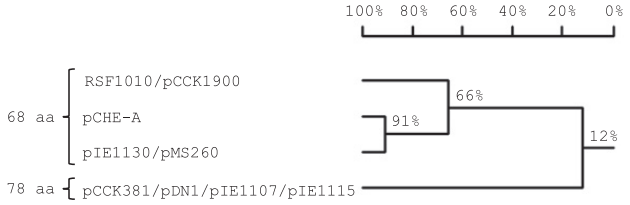


Fig. 7. Phylogenetic relationships between the amino acid sequences of the small putative repressors within plasmids of the IncQ1 subgroup. Percentages represent amino acid sequence identities. GenBank accession numbers for the respective plasmid nucleotide sequences from which the protein sequences were obtained are given in Table 1.

is encoded in the same location on the closely related plasmids pCCK1900, pCHE-A, pMS260 and pIE1130, although these proteins form a different alignment cluster (Fig. 7). The IncQ1 plasmids pCCK381, pIE1107, pIE1115 and pDN1, have a single *orf* that encodes a 78 aa protein in the same location as *cac*, however, it bears only 12% aa identity to F.

Each of the three IncQ2 plasmids also have small *orf*s between *repB* and *repAC* but these are very different from the other families of IncQ plasmids and encode for toxin–antitoxin (TA) systems. Two of these plasmid addiction systems (*pasABC* of pTF-FC2 and *pasAB* of pTC-F14) are related with the PasA antitoxins and PasB toxins having 81% and 72% amino acid sequence identity respectively (Smith and Rawlings, 1997; Gardner et al., 2001). These two systems can functionally interact (Deane and Rawlings, 2004). Plasmid pRAS3, however, encodes an unrelated *pemIK*-like TA system in the same location (Loftie-Eaton and Rawlings, 2009). This TA system is most closely related to unstudied genes found on the chromosomes of bacteria such as *Bartonella tribocorum* and *Nitrosomonas europa*. Like the *pasAB* genes, when the pRAS3 plasmid *pemI*- and *pemK*-like genes were cloned into an unstable test plasmid, they were able to stabilize the plasmid in an *E. coli* host. As two unrelated TA systems had been acquired by IncQ2 plasmids in exactly the same position between the *repB* and *repAC* genes (see Fig. 1), this suggested that the TA genes might have an additional role beyond the toxin and antitoxin they encode.

This was shown to be the case (Matcher and Rawlings, 2009). One of the characteristics of TA systems is that they are autoregulatory with the unstable antitoxin serving both to neutralize the stable toxin and as a repressor of TA gene expression. Reporter gene studies showed that the unregulated strength of the P_{pas} promoter of the *pasAB* genes of pTC-F14 was approximately 100-fold greater than the second strongest P_{mobB} promoter of the plasmid backbone (Fig. 6) (Gardner and Rawlings, 2004). Working with the PasA-PasB complex of pTF-FC2 it was observed that by having the promoter region present *in trans* on a high copy number vector resulted in upregulation of P_{pasABC} and a 2.7-fold increase in expression of *repAC*. Also, when the *repAC* genes of pTF-FC2 were deleted and instead supplied *in trans* from behind a concentration-dependent arabinose inducible promoter, it was found that the PCN increased rapidly in response to small increases in induction and quickly reached an upper limit (Matcher and Rawlings, 2009). The presence of a strong autoregulated promoter upstream of the *repAC* genes is believed to play a role under low copy number conditions such as unequal distribution of plasmids on host cell division or after conjugative transfer to a new host. When the copy number of the plasmid falls below a threshold, autorepression of the TA system is relieved, and expression from the strong promoter results in a rapid increase in the production of RepA and RepC and a corresponding increase in the frequency of initiation of replication. This rapid but transient increase in expression of the *repAC* genes would quickly restore the plasmid to its normal copy number. This is likely to be important in plasmids such as the IncQ plasmids that have no active partitioning systems and are dependent on copy number for their segregation stability as explained earlier. In support of these findings, it was noticed that the PCN of R1162 remained unchanged when the P_4 promoter (located in an equivalent position to P_{pas} of pTF-FC2) was deleted, but that the plasmid was less successful at becoming established after transformation into a new host (Meyer, 2009). It therefore seems likely that having an autoregulated promoter between the 3′-terminus of *repB* and 5′-terminus of *repA* in these IncQ-like plasmids exists not to control the upper copy number limit, but rather to allow for rapid expression of the replication genes when the copy number is too low. This finding helped to resolve the mystery of why deletion of the TA system appeared to have no effect on the average copy number of pTF-FC2 (Smith and Rawlings, 1997). The use of an autoregulated promoter to jump-start replication when the copy number is low has a strong resemblance to the CopB system of plasmid R1 (Nordström, 2006).

Plasmids of the IncQ3 subgroup do not encode any *orf*s between the *mobA/repB* and *repA* genes, while the IncQ4 plasmid, pPNAP08 has an *orf* of 113 aa in this region that lacks a clear promoter and ribosome binding site. It has not been established whether the single *orf*s between the *repB* and *repAC* genes of many of the other IncQ plasmids also autoregulate strong promoters that enable the plasmid to jump start replication at low copy number nor how the IncQ3 and IncQ4 plasmids that appear to lack such *orf*s manage situations of low copy number.

There appear to be differences in which Rep proteins are limiting in different IncQ plasmids. Stringent regulation of P_1 and P_3, the two promoters upstream of *mobB* in RSF1010, insures that all three Rep proteins are limiting under physiological conditions. Up-regulation of this promoter area of RSF1010 resulted in a 4-fold increase in PCN (Frey et al., 1992). An excess supply of RepB mediated only a 1.6-fold increase in PCN and it was suggested that the increased transcription though the *repAC* operon must have had the most significant contribution. Similarly, the increase in transcription that was observed from P_{mobB} in a pRAS3.1 derivative compared to pRAS3.2 mediated a 2.2-fold increase in PCN (when both plasmids had three iterons). Unlike RSF1010, however, the increased PCN was not due to an increase in *repAC* transcription as an excess supply of these proteins did not have the effect of increasing copy number. Instead it was found that the increased PCN of pRAS3.1 was due to increased levels of *repB* transcription (Loftie-Eaton and Rawlings, 2009). That the physiological availability of RepAC in the pRAS3 plasmids was not limiting to plasmid replication as for RSF1010 could explain why the pRAS3 plasmids have a PCN that is higher than that which has been determined for other IncQ-like plasmids.

6.2.5. Evolution of new replicons.

It is possible that evolution from a single ancestor plasmid has given rise to the diversity between replicons found among IncQ-family plasmids, although convergent evolution by the assembly of genes for a primase, helicase and DNA-binding proteins together with *oriV* regions from more than one source is also a possibility. Nevertheless, within IncQ subgroups, it is likely that evolution of different

plasmid incompatibility groups has arisen by evolution from a common ancestor. An example of this was illustrated by a study of the compatible replicons of the IncQ2 plasmids pTF-FC2 and pTC-F14. Replication from the *oriV* regions pTF-FC2 and pTC-F14 could be supported by the RepB primase and RepA helicase of the other plasmid when the relevant genes were placed in the same host as the *oriV* regions (Gardner and Rawlings, 2004). However, the RepC-binding protein of each plasmid was specific and each RepC could support the replication of only its own *oriV*. Therefore, it is most likely that replicon compatibility evolved due to changes in the iteron sequences that occurred together with corresponding evolution of the RepC-binding protein. Since a minimum of three copies of the 22-bp iterons of which 20-bp is highly conserved and all three iterons are required, how all iterons accumulate sequence changes simultaneously is still a matter of speculation.

A model for the diversification of plasmid replicons based on plasmid cointegrate formation has been proposed by Sýkora (1992) and applied to the IncQ-plasmids (Rawlings and Tietze, 2001). A cointegrate plasmid with two compatible replicons has an advantage over two single plasmids in that each of the single plasmids may be eliminated by invasion of an incompatible plasmid, whereas the cointegrate plasmid could be saved by the alternate replicon on the compatible plasmid. However, in most situations one would expect one of the two cointegrate replicons to dominate plasmid replication and control the cointegrate plasmid copy number. This would allow the other replicon the ability evolve new *rep* genes and incompatibility groups. Should the dominant replicon become inactivated, or the cointegrate become resolved, the newly evolving replicon would come under strong selection to function as an efficient independent replicon. The discovery of IncQ1 plasmids with two *oriV* regions, such as pIE1107 (Fig. 1), could be taken as evidence of cointegrate formation. In this case, oriV_b was functional and had iteron sequences that were different from the non-functional oriV_a with iterons identical to RSF1010. However, the rest of the RSF1010 plasmid was missing and so this might represent acquisition of the second *oriV* by a different means.

The pRAS3 plasmids suggest that the evolution of IncQ replicons might also occur by *repC* gene and *oriV* swapping with plasmids that might even be different from IncQ plasmids. When the RepB and RepA proteins of the pRAS3 plasmids were examined, they were found to be highly related (97 and 93% amino acid sequence identity respectively) to those of pTF-FC2 (Fig. 2). However, their RepC sequences and *oriV* regions are outliers when compared to any of the other IncQ plasmid groups so far indentified (Loftie-Eaton and Rawlings, 2009). Since it is highly unlikely that the mutation rate of the *repC* gene is markedly higher than for the *repA* or *repB* genes, the most likely explanation is that the *repC* gene and the *oriV* to which the RepC protein specifically binds was obtained by DNA recombination with a plasmid that has still to be identified.

The acquisition or exchange of DNA within a replicon was described earlier where the pRAS3 plasmids had acquired a TA-system that was very different from the *pas* TA system of either pTF-FC2 or pTC-F14 while the genes that lie on either side of the TA genes are highly related. Similarly, among the IncQ1 plasmids, the presence of genes for small proteins with very little sequence relatedness within the *mobA/repB* and *repAC* intergenic regions suggests that these might have been acquired from different sources.

Taken together these findings suggest that evolution within replicons is likely to be by at least two mechanisms. These are mutation-based evolution within *rep* genes, *oriV* regions and possibly also some aspects of regulation and then there is the more dramatic, non-incremental, evolution by DNA acquisition or recombination.

7. Mobilization

7.1. Association with a diversity of type IV secretion systems

Large conjugative plasmids encode all the genes required for efficient plasmid transfer from the donor bacterium to the recipient bacterium. That is, all of the proteins required for mating pair formation (Mpf) as well as DNA transfer and replication (Dtr) are provided for by the plasmid itself (Lawley et al., 2004). IncQ plasmids encode only the Dtr genes and rely on conjugative plasmids to provide the Mpf components. The occurrence of IncQ-like plasmids is therefore often associated with the occurrence of larger conjugative plasmids. For example, the pRAS3 plasmids were always found to be co-resident with a large conjugative IncU plasmid, pRAS1, irrespective of the *A. salmonicida* strain from which they were isolated and irrespective of the geographical location (Aoki and Takahashi, 1986; L'Abee-Lund and Sørum, 2002). Two plasmids belonging to the IncN family and one belonging to the IncPα family were isolated together with pIE1107, pIE1120, pIE1115 and pIE1130 from the piggery manure bacterial cultures (Smalla et al., 2000). R1162 was mobilized from *P. aeruginosa* 1162 to *E. coli* at low frequencies and although a conjugative plasmid was not isolated at the time, a transfer factor responsible for pilus formation was present (Bryan et al., 1972).

Although there was no conjugative plasmid present in the *E. coli* host from which RSF1010 was isolated, it was shown to be mobilized at equally high frequencies from both *E. coli* and *P. aeruginosa* by IncP plasmids, relatively efficiently from *E. coli* by IncF, IncFVI, IncIα, IncM and IncX plasmids, and less efficiently by IncN or IncW plasmids (Cabezón et al., 1994; Francia et al., 2004; Guerry et al., 1974; Willitts and Crowther, 1981). A 27.6-kb plasmid was associated together with pTF-FC2 in the *At. ferrooxidans* host from which it was isolated, however, this plasmid was never captured and it is not known whether it was a conjugative or mobilizable plasmid (Rawlings et al., 1984). Nonetheless, even though no conjugative partner plasmids were identified at the time of isolation for pTF-FC2, pTC-F14 and pDN1, they were all efficiently mobilized by RP4, an IncP conjugative plasmid (Van Zyl et al., 2003; Whittle et al., 2000; Rawlings et al., 1986). Meyer (2009) has argued that one of the reasons IncQ plasmids such as RSF1010 have been so successful is their ability to associate with and be conjugatively transferred by many varieties of type IV secretion systems.

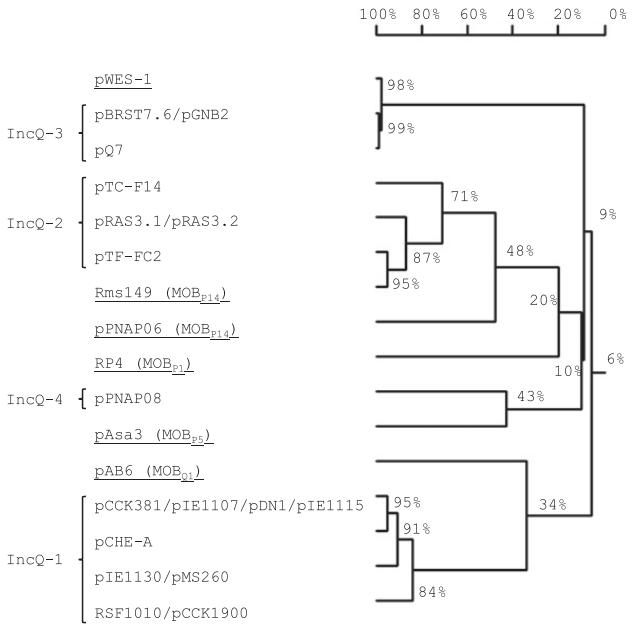


Fig. 8. Phylogenetic relationships between the MobA relaxases of the IncQ plasmids and the most closely related non-IncQ plasmid representative of different MOB relaxase families (Garcillán-Barcia et al., 2009). Percentages represent amino acid sequence identities. GenBank accession numbers for the respective IncQ plasmid sequences are given in Table 1. Additional NCBI accession numbers are; pAB6, AAD31795; pAsa3, NP_861566; pPNAP06, YP_973972; Rms149, YP_245456; RP4, CAA38336; pWES-1, YP_002332250.

7.2. Diversity of mobilization systems

As described in Section 6.1, one of the characteristics of all plasmids containing IncQ-family replicons identified to date is that they are linked to a mobilization system in such a way that their RepB primase is fused to the relaxase that forms an essential enzyme in the processing of DNA prior to conjugative plasmid transfer. In RSF1010 and the IncQ2 plasmids this has been termed the *mobA/repB* gene fusion. It has been shown that only 184 amino acids of the amino terminal region was required for in vitro binding to *oriT* single stranded DNA (Becker and Meyer, 2002). Exactly how much of the 5′ portion of the fusion gene product is required to encode for a polypeptide capable of both binding and cleavage is unknown. However, a good indication comes from work with pTF-FC2 where a natural *Cla*I site cleaved the product of the *mobA/repB* gene fusion into two fragments, the 5′ portion of which encoded a 409 amino acid relaxase fragment (Rohrer and Rawlings, 1992) and the 3′ portion a 422 amino acid primase fragment (Dorrington et al., 1991), both of which appeared to be fully functional. Using 300 amino acids of the N-terminus of the product of the *mobA/repB* fusion as containing the relaxase activity, the relaxases of all of the IncQ replicons were aligned as shown in Fig. 8. The IncQ1 plasmids form a group with relaxases that have 84% amino acid sequence identity within the group. The IncQ2 plasmids (pTC-F14, pRAS3 and pTF-FC2) form a group with 71% sequence identity while the proposed IncQ3 group (pBRST7.6, pGNB2 and pQ7) form a distinct grouping with high internal sequence identity (99%). IncQ-family plasmid pPNAP08 is an outlier that appears to be a representative of a fourth IncQ plasmid group. The relaxases of all four subgroups have very little sequence identity between each other and appear to be relaxases of very different lineages. Garcillán-Barcia et al. (2009) have argued that relaxases can be used for plasmid classification and divided conjugative and mobilizable plasmids into six MOB families. Based on this classification, members of the IncQ1 plasmids all belong to group MOB_{Q1} (distantly related to subgroup MOB_{P1}). The IncQ2 plasmids and the proposed IncQ4 plasmid, pPNAP08, belong to different subgroups of MOB_P, that is, subgroups P14 and P5 respectively. The MOB family to which plasmids of the IncQ3 family belongs is not as clear and either they belong to a currently unrecognized group or are deep-branching members of an existing group.

As mentioned in Section 7.2.1, in the case of RSF1010, MobA and MobC were essential whereas MobB was only stimulatory. It is therefore possible that all mobilization systems may have an equivalent of MobC. However, it is also possible that some families of relaxases might require a MobC equivalent and others not. We have attempted to identify a MobC equivalent for each of the IncQ subgroups and compared these to the most similar mobilization proteins of non-IncQ-like plasmids as a means of identifying whether these proteins indicate similar MOB family relationships as for the MobA relaxases. In addition, mobilizable plasmids also require an *oriT* region for their DNA to be transferred. The *oriT* is the site at which the relaxosome, consisting of all the proteins involved in mobilization, is assembled prior to conjugal transfer of the plasmid DNA and is also the site at which transfer is terminated (Brasch and Meyer, 1987; Bhattacharjee et al., 1992). The R1162 *oriT* was characterized as a 38-bp region containing an inverted repeat, with each arm consisting of 10-bp separated by 3-bp, followed by an 8-bp conserved region which leads up to the *nick*-site (Brasch and Meyer, 1987). We therefore examined the IncQ plasmids to determine whether other features of the mobilization system supported the division into subgroups as suggested by the amino acid sequences of their relaxases. In general, similar relationships were found.

7.2.1. IncQ1 subgroup

The plasmids of this subgroup that have been studied in detail have three mobilization genes that encode three mobilization proteins known as MobA, MobB and MobC. The *mobB* gene is located within *mobA* but translated in a different reading frame, while the *mobC* gene is divergently transcribed from *mobA* and *mobB* (see Fig. 1). The functioning of the MobA (relaxase), the MobB (stimulatory accessory protein) and the MobC (required for DNA cleavage in vitro) proteins has been reviewed by Meyer (2009) and will not be enlarged on further.

When the amino acid sequences of the MobC proteins within the IncQ1 subgroup were compared (Fig. 9), they grouped together in a pattern very similar to the relaxases (Fig. 8). The MobCs were most related to the 5.6-kb MOB_{Q1} plasmid pAB6. Similarly, the *oriT* sites were highly conserved for all members of IncQ1 subgroup including the less studied members of the subgroup (Fig. 10).

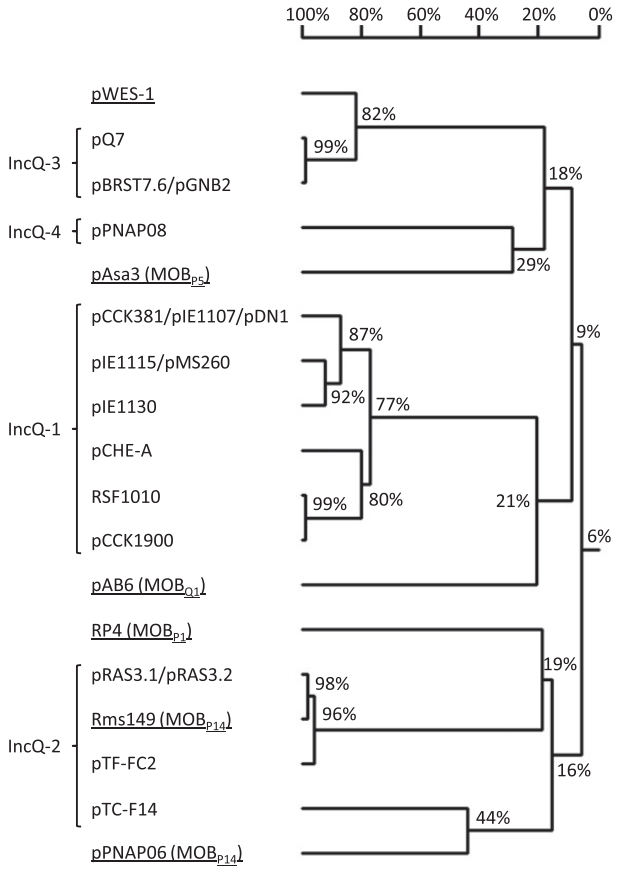


Fig. 9. Phylogenetic relationships between the MobC and putative MobC relaxase accessory proteins of the IncQ plasmids and the most closely related proteins from plasmids that do not belong to the IncQ family. Percentages represent amino acid sequence identities. GenBank accession numbers for the respective IncQ plasmid sequences are given in Table 1. Additional NCBI accession numbers are; pAB6, AAO45531; pAsa3, NP_861565; pPNAP06, YP_973974; pRms149, YP_245459; RP4, AAA26422; pWES-1, YP_002332249.

Fig. 10. Nucleotide sequence alignments of the nick-sites within the *oriT* regions of IncQ plasmid subgroups and the nick-sites of the non-IncQ plasmids to which the relaxases are most closely related. GenBank accession numbers for the respective IncQ plasmid sequences are given in Table 1. Additional NCBI accession numbers are; pAsa3, NC_004924; Rms149, NC_007100; RP4, RP4TRAJ; pWES-1, NC_011604. The *Bacteriodes fragilis* chromosomal transfer factor cLV25 sequence was obtained from Bass and Hecht (2002).

7.2.2. IncQ2 subgroup

In contrast, the mobilization system of the IncQ2 plasmids consists of five genes, a similar number to the *dtr* (DNA transfer) systems of the IncP plasmids (RP4/RK2 and R751). The *mobA* and *mobB* genes are transcribed in the same direction as the rep genes while *mobC*, *mobD* and *mobE* are transcribed in the opposite direction (see Fig. 1). The functions of each mobilization protein has not been studied in as much detail as for RSF1010, but MobC appears to be essential (together with the MobA relaxase) for plasmid mobilization (Rohrer and Rawlings, 1992). MobB is related to TraJ of plasmid RP4 and therefore is likely to have a similar function which is thought to be binding to DNA thereby altering its structure and allowing the relaxase access to its binding site (Pansegrau et al., 1990; Zechner et al., 2000). In pTF-FC2 and pTC-F14, the non-essential MobD and MobE proteins were found to affect only the frequency of mobilization (Rohrer and Rawlings, 1992; van Zyl et al., 2003). Interestingly, there are substantial differences in the relatedness of the amino acid sequences of mobilization proteins within the IncQ2 subgroup. The amino acid sequences of the MobB and MobA proteins of all three IncQ2 plasmids (pTF-FC2, pTC-F14 and pRAS3) are highly related to each other at over 70% amino acid identity (Fig. 8 and Loftie-Eaton and Rawlings, 2009). However, although the MobC, MobD and MobE proteins of pTF-FC2 and the pRAS3 plasmids are closely related at over 96% amino acid sequence identity, the MobC, MobD and MobE proteins of pTC-F14 are only distantly related with amino acid sequence identities of 21–23%, 30% and 9% respectively (Fig. 9). It has been proposed that these large differences are best be explained by a genetic recombination event whereby the *mobC*, *mobD* and *mobE* genes of pTC-F14 were acquired from a different, as yet, unidentified plasmid (van Zyl et al., 2003).

Unlike the IncQ2 MobA relaxases which clustered together, the MobCs of the IncQ2 plasmids pTF-FC2 and pRAS3 were most closely related to the 57.1-kb MOB$_{P14}$ plasmid Rms149 (96% identity) while the MobC of pTC-F14 was the most closely related to that of the 21.6-kb MOB$_{P14}$ plasmid pPNAP06 (different from the IncQ4, pPNAP08). Like the IncQ1 subgroup, the nucleotide sequences of *oriT* regions within the IncQ2 subgroup are well-conserved. However, when compared to the plasmid representatives of the MOB$_P$ plasmids (subgroup P14), this sequence conservation is a lot less marked although a consensus ANCCTG is retained in the highly conserved region immediately preceding the *nick* site (Fig. 10).

7.2.3. IncQ3 subgroup

The mobilization system of the IncQ3 subgroup have not been studied, but the system is clearly different from subgroups IncQ1 or IncQ2. There appear to be only two mobilization genes, one encoding the equivalent of a MobC protein and the second the MobA relaxase. In contrast to the IncQ1 and IncQ2 subgroups were the equivalent of *mobC* is divergently transcribed, in the IncQ3 subgroup it is transcribed in the same direction as *mobA* (Fig. 1). A

search for the equivalent of the IncQ1 *mobB* gene (137 amino acid product) within the *mobA/repB* gene fusion of the IncQ3 subfamily was inconclusive. While there is an open reading frame that could encode a protein of 227 amino acids almost in the middle of the gene fusion, a BLASTP search gave no hits to other proteins of a similar size. Examination of the alignment of the MobC proteins indicated that, like the MobA alignment, the MobC proteins of the IncQ3 subgroup form a tight cluster that is unrelated to any of the other MobC clusters. Interestingly, based on both the MobA relaxase and the MobC protein by far the closest relative to plasmids of the IncQ3 subgroup is a 10.9-kb plasmid pWES-1 that does not have an IncQ-type replicon (Fig. 9 and Doublet et al., 2009).

The *oriT* sites of the IncQ3 subgroup plasmids were fully conserved between all members of the subgroup (Fig. 10). The putative *nick* site had a high level of conservation with the *nick* site of the *Bacteriodes fragilis* chromosomal transfer factor cLV25 (Bass and Hecht, 2002). As for the MobA relaxase, neither the MobC or *oriT* sequences enabled the identification of a relaxase MOB family to which the IncQ3 plasmids belong. They therefore remain as representatives of an unreported or unrecognized group of plasmids based on the relaxase classification system.

7.2.4. IncQ4 subgroup

Like the mobilization system of the IncQ3 subgroup, the mobilization system of pPNAP08, currently the only member of the proposed IncQ4 subgroup, appears also to consist of only two genes, both orientated in the same direction. There is an open reading frame within the *mobA/repB* gene fusion which could encode a 134 amino acid protein of very similar size and position to the 137 amino acid MobB of RSF1010. In this case, a BLASTP search gave hits of 32% (E value 0.14) and 31% (E value 1.8) to 161 amino acid products from genes annotated as *mobB* of plasmid pUCD5000 from *Pantoea citrea* and *mobB* of *Serratia marcescens* respectively, although no plasmid was identified in the case of *Serratia marcescens*. Since the mobilization system of pPNAP08 has not been studied, research would be needed to confirm the function of this putative MobB. When the RepC protein of pPNAP08 was aligned with the equivalent proteins of other IncQ plasmids, the result was very similar to the MobA relaxase. The RepC of plasmid pPNAP08 was a deep-branching outlier compared to any plasmids of the other IncQ subgroups and most closely related to the 5.6–kb MOB_{P5} plasmid pAsa3.

The *oriT* region of pPNAP08 has also not been studied. However a putative *nick* site with very high nucleotide sequence identity to pAsa3, was identified (Fig. 10). Similarity between the MobA, MobC and *oriT nick* site of pPNAP08 with pAsa3, clearly supports the placement of the proposed IncQ4 plasmids into the MOB_{P5} subgroup.

8. Evolution of the linkage of mobilization systems to IncQ replicons.

With respect to the evolution of mobilization systems it is clear that IncQ plasmid replicons have become linked to diverse mobilization systems with each IncQ subgroup being linked to a mobilization system that is only distantly related to the others. This raises the question of whether this linkage has taken place within the *mobA/repB* fusion such that the *mobA* (relaxase) and the other mobilization genes are related to a different set of plasmids from the *repB* (primase) and the other replication genes or whether the relaxase and primase portions have been inherited together. An examination of the dendrograms of RepA and RepC proteins (Fig. 2) indicated that the percentage identity of these two proteins between all IncQ subgroups is considerably higher than for RepB (with the exception of the outlier pRAS3 plasmids probably for the reason explained in Section 6.2.5). In contrast the dendrograms of the RepB and MobA alignments are a lot more deeply branched, but very similar to each other. This would seem to support the idea that the *mobA/repB* gene fusion has been acquired as a unit and that the r*epA* and *repC* genes have also been inherited as a unit (except for the pRAS3 plasmids).

This raises a second question of why IncQ replicons have become linked to such a variety of mobilization systems. A possible answer is suggested by studies on pTF-FC2 and pTC-F14 where, as pointed out in Section 7.2.2, there seems to have been a genetic recombination event so that the *mobC*, *mobD* and *mobE* genes of pTC-F14 appear to be from a different source to the same genes of pTF-FC2. It was observed that pTC-F14 was much less efficiently mobilized by RP4 than pTF-FC2 and that the frequency of mobilization of pTC-F14 could be raised to almost the same as for pTF-FC2 when the *mobC*, *mobD* and *mobE* genes of pTF-FC2 were provided *in trans* (van Zyl et al., 2003). This suggested that since these three mob genes were required to allow high frequency mobilization by RP4, the presence of a different set of these genes on pTC-F14 was because this plasmid had become adapted to mobilization by different self-transmissible plasmid (Rawlings, 2005). Although, only some of the *mob* genes were exchanged in this case, we speculate that having different sets of mobilization genes may allow different IncQ plasmid subgroups to be mobilized most efficiently by different self-transmissible plasmids. This testable hypothesis awaits experimental support.

9. Summary/conclusions

This review has focussed largely on diversity and differences among IncQ plasmids rather than the mechanisms of IncQ plasmid biology as this was expertly reviewed by Meyer (2009) though focussed mainly on IncQ1 plasmids. We have suggested that identifying IncQ plasmids should be based on the nature of their replicons which allow plasmid replication by a strand displacement mechanism that does not require the synthesis of Okazaki fragments. This mechanism is unique and places an upper limit on the size of IncQ plasmids of approximately 15-kb. Besides their shared replication mechanism, all IncQ plasmids appear to be mobilizable and all have a relaxase-primase gene fusion that may encode a single polypeptide with two domains or two separate polypeptides (there may be

additional internal polypeptides in different reading frames).

We have proposed that, based on current information, IncQ plasmids can be divided into four subgroups that have common features within each subgroup but that differ from other IncQ plasmids. Division into four subgroups is supported, not only by the higher degree of relatedness of the replication proteins within each subgroup, but more importantly by the mobilization system with which each plasmid subgroup is associated. As described in Section 7.2, these differ substantially from each other. Several other characteristics of each subgroup further support these divisions. These include the observation that the IncQ1 subgroup has one or more small *orfs* between their *repB* and *repA* genes at least some of which are involved in the regulation of replication. The IncQ2 subgroup has at least two unrelated TA systems in a similar position that appear to jumpstart replication when the copy number falls below a certain threshold. Subgroups IncQ3 and IncQ4 appear to lack *orfs* in this region. Another feature that supports the division into subgroups is that, IncQ1 and IncQ2 plasmids have divergently transcribed mobilization genes while IncQ3 and IncQ4 plasmids do not. With relatively few regulatory studies having been carried out among IncQ plasmids other than the IncQ1 plasmid RSF1010 and to a lesser extent the IncQ2 plasmids pTF-FC2 and pTC-F14, further similarities and differences await to be discovered. The sample size for comparison for the proposed new subgroups is still small with only three members of the IncQ3 subgroup having been identified, two of which have identical backbones but different accessory genes, while only a single representative of the IncQ4 subgroup has been identified. Discovery of new IncQ-like plasmids will help to clarify the features of these subgroups and possibly identify still other IncQ subgroups.

There is a need for comparative studies between the different IncQ subgroups to better understand how these different regulatory mechanisms contribute to copy number control. As explained within this review the different types of genes, or lack thereof, between the *mobA/repB* and *repA* genes are likely to represent different regulation strategies for the respective *repAC* genes. Furthermore, seeing as plasmid replication is likely to be dependent upon transcription from the promoter within the *oriT* region for all of the subgroups, the acquisition of different mobilization systems together with their regulatory sequences would imply a difference in regulation strategies or if nothing else, a difference in the strength of the promoters. Such differences may result in plasmid copy number differences within and between plasmid groups and between different hosts. As most regulatory studies on IncQ-like plasmids have been carried out in *E. coli*, there is a need for comparative studies in different hosts.

During this review, we have highlighted several steps in the evolution of IncQ plasmids. These include incremental mutational changes such as found within the iterons and *repC* genes of IncQ1 and IncQ2 subgroups and that have given rise to different incompatibility groupings. Then there are more substantial changes within subgroups such as the acquisition of different *orfs* in the *repB/repA* regulatory region of the IncQ1 plasmids and the acquisition of unrelated TA systems in the same region of the IncQ2 plasmids. Within the IncQ2 subfamily there is evidence of gene swapping as illustrated by the different *repC* genes and *oriV* regions of the pRAS3 plasmids compared with other members of the IncQ2 subgroup and the different *mobCDE* genes of pTC-F14 compared with the other IncQ2 plasmids. Finally, there is the fusion of IncQ-type replicons to different lineages of mobilization genes as described.

The accessory genes found on IncQ plasmids are highly varied and probably biased by the ease of selection of antibiotic resistance genes. However, as additional plasmids are discovered during whole genome sequence projects, a more balanced view of the genes carried should be obtained. The same can be said for the hosts in which IncQ plasmids have been found in nature. These are dominated by members of the Proteobacteria and particularly members of the γ-Proteobacteria. This is in spite of the replicon having been shown to be capable of both replication in, and mobilization between, a wide variety of Gram-negative and Gram-positive bacteria. Whether this is a real bias or as a result of certain bacteria being more highly researched or easier to cultivate than others, awaits further genome and especially metagenome sequencing data.

In addressing the question of why IncQ plasmids have been so successful and are so widespread, Meyer (2009) drew the analogy between the shared attributes of IncQ plasmids and successful actors. He suggested that these were; not making too many demands on the audience (minimizing the metabolic load); not allowing themselves to be written out of the play (high copy number to insure stability); willingness to work at a moment's notice on any stage (initiate replication in many different hosts) and to know where the exits are and how to use them (successful co-optation to different type IV secretion systems). As a result of the observations made in this review we would suggest two others. The flexibility to work with many different co-actors (the apparent recombination of different plasmid regions or parts of regions with other plasmids while remaining functionally competitive) and to appropriate strategies used by others to serve a different purpose (the use by some IncQ plasmids of the strong autoregulation properties of TA and other systems to quick-start replication when the copy number falls below a threshold).

Acknowledgments

The authors acknowledge the support of the National Research Foundation (Pretoria) and the University of Stellenbosch.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25, 3389–3402.

Aoki, T., Takahashi, A., 1986. Tetracycline-resistant gene of a non-transferable R plasmid from fish-pathogenic bacteria *Aeromonas salmonicida*. Bull. Jpn. Soc. Scient. Fish. 52, 1913–1917.

Albritton, W.L., Brunton, J.L., Slaney, L., MacLean, I., 1982. Plasmid-mediated sulfonamide resistance in *Haemophilus ducreyi*. Antimicrob. Agents Chemother. 21, 159–165.

Barth, P.T., Grinter, N.J., 1974. Comparison of the deoxyribonucleic acid molecular weights and homologies of plasmids conferring linked resistance to streptomycin and sulfonamides. J. Bacteriol. 120, 618–630.

Bass, K.A., Hecht, D.W., 2002. Isolation and characterization of cLV25, a *Bacteroides fragilis* chromosomal transfer factor resembling multiple *Bacteroides* sp. Mobilizable transposons. J. Bacteriol. 184, 1895–1904.

Becker, E.C., Meyer, R.J., 1997. Acquisition of resistance genes by the IncQ plasmid R1162 is limited by its high copy number and lack of a partitioning mechanism. J. Bacteriol. 179, 5947–5950.

Becker, E.C., Meyer, R.J., 2002. MobA, the DNA strand transferase of plasmid R1162. J. Biol. Chem. 277, 14575–14580.

Bhattacharjee, M., Rao, X.M., Meyer, R.J., 1992. Role of the origin of transfer in termination of strand transfer during bacterial conjugation. J. Bacteriol. 174, 6659–6665.

Bingle, L.E.H., Thomas, C.M., 2001. Regulatory circuits for plasmid survival. Curr, Opin.

Bönemann, G., Stiens, M., Pühler, A., Schlüter, A., 2006. Mobilizable IncQ-related plasmid carrying a new quinolone resistance gene, *qnrS2*, isolated from the bacterial community of a wastewater treatment plant. Antimicrob. Agents Chemother. 50, 3075–3080.

Brasch, M.A., Meyer, R.J., 1987. A 38 base-pair segment of DNA is required in cis for conjugative mobilization of broad host-range plasmid R1162. J. Mol. Biol. 198, 361–369.

Bryan, L.E., Elzen, H.M.V., Tseng, J.T., 1972. Transferable drug resistance in *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 1, 22–29.

Cabezón, E., Lanka, E., de la Cruz, F., 1994. Requirements for mobilization of plasmids RSF1010 and ColE1 by the IncW plasmid R388: *trwB* and RP4 *traG* are interchangeable. J. Bacteriol. 176, 4455–4458.

Chattoraj, D.K., 2000. Control of plasmid DNA replication by iterons: No longer paradoxical. Mol. Microbiol. 37, 467–476.

Clennel, A.M., Johnston, B., Rawlings, D.E., 1995. Structure and function of Tn5467, a Tn21-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid pTF-FC2. Appl. Environ. Microbiol. 61, 4223–4229.

Deane, S.M., Rawlings, D.E., 2004. Interaction between the plasmid addiction stability systems of two related broad host range plasmids. J. Bacteriol. 186, 2123–2133.

De Graaff, J., Crosa, J.H., Heffron, F., Falkow, S., 1978. Replication of the nonconjugative plasmid RSF1010 *in Escherichia coli* K-12. J. Bacteriol. 134, 1117–1122.

Dorrington, R.A., Bardien, S., Rawlings, D.E., 1991. The origin of replication of the broad-host-range plasmid pTF-FC2 requires a primase-like protein for autonomous replication in *Escherichia coli*. Gene 108, 7–14.

Dorrington, R.A., Rawlings, D.E., 1990. Characterization of the minmum replicon of the broad-host-range plasmid pTF-FC2 and similarity between pTF-FC2 and the IncQ plasmids. J. Bacteriol. 172, 5697–5705.

Doublet, B., Granier, S.A., Robin, F., Bonnet, R., Fabre, L., Brisabois, A., Cloeckaert, A., Weill, F.X., 2009. Novel plasmid-encoded ceftazidime-hydrolyzing CTX-M-53 extended-spectrum beta-lactamase from Salmonella enterica serotypes Westhampton and Senftenberg. Antimicrob. Agents Chemother. 53, 1944–1951.

Dugan, J., Rockey, D.D., Jones, L., Andersen, A.A., 2004. Tetracycline resistance in *Chlamydia suis* mediated by genomic islands inserted into the Chlamydial inv-like gene. Antimicrob. Agents Chemother. 48, 3989–3995.

Fling, M.E., Kope, J., Richards, C., 1988. Characterization of plasmid pAZ1 and the type III dihydrofolate reductase gene. Plasmid 19, 30–38.

Francia, M.V., Varsaki, A., Garcillán-Barcia, M.P., Latorre, A., Drainas, C., de la Cruz, F., 2004. A classification scheme for mobilization regions of bacterial plasmids. FEMS Microbiol. Rev. 28, 79–100.

Frey, J., Bagdasarian, M., 1989. The molecular biology of IncQ plasmids. In: Thomas, C.M. (Ed.), Promiscuous Plasmids of Gram Negative Bacteria. Academic Press, London, pp. 79–93.

Frey, J., Bagdasarian, M.M., Bagdasarian, M., 1992. Replication and copy number control of the broad-host-range plasmid RSF1010. Gene 113, 101–106.

Garcillán-Barcia, M.P., Francia, M.V., de la Cruz, F., 2009. The diversity of conjugative relaxases and its application in plasmid classification. FEMS Microbiol. Rev. 33, 657–687.

Gardner, M.N., Deane, S.M., Rawlings, D.E., 2001. Isolation of a new broad-host-range IncQ-like plasmid, pTC-F14, from the acidophilic bacterium *Acidithiobacillus caldus* and analysis of the plasmid replicon. J. Bacteriol. 183, 3303–3309.

Gardner, M.N. 2003. An investigation into the replicon of a broad host range mobilizable plasmid from the moderately thermophilic bacterium *Acidithiobacillus caldus*. Thesis (PhD). University of Stellenbosch.

Gardner, M.N., Rawlings, D.E., 2004. Evolution of compatible replicons of the related IncQ-like plasmids, pTC-F14 and pTF-FC2. Microbiol. 150, 1797–1808.

Grinter, N.J., Barth, P.T., 1976. Characterization of SmSu plasmids by restriction endonuclease cleavage and compatibility testing. J. Bacteriol. 128, 394–400.

Guerry, P., Van Embden, J., Falkow, S., 1974. Molecular nature of two nonconjugative plasmids carrying drug resistance genes. J. Bacteriol. 117, 619–630.

Henderson, D., Meyer, R.J., 1996. The primase of broad-host-range plasmid R1162 is active in conjugal transfer. J. Bacteriol. 178, 6888–6894.

Henderson, D., Meyer, R., 1999. The MobA-linked primase is the only replication protein of R1162 required for conjugal mobilization. J. Bacteriol. 181, 2973–2978.

Honda, Y., Akioka, T., Takebe, S., Tanaka, K., Miao, D., Higashi, A., Nakamura, T., Taguchi, Y., Sakai, H., Komano, T., Bagdasarian, M., 1993. Mutational analysis of the specific priming signal essential for DNA replication of the broad host-range plasmid RSF1010. FEBS Lett. 324, 67–70.

Ito, H., Ishii, H., Akiba, M., 2004. Analysis of the complete nucleotide sequence of an *Actinobacillus pleuropneumoniae* streptomycin-sulfonamide resistance plasmid, pMS260. Plasmid 51, 41–47.

Kehrenberg, C., Schwarz, S., 2005. Plasmid-borne florfenicol resistance in *Pasteurella multocida*. J. Antimicrob. Chemother. 55, 773–775.

Kehrenberg, C., Wallmann, J., Schwarz, S., 2008. Molecular analysis of florfenicol-resistant *Pasteurella multocida* isolates in Germany. J. Antimicrob. Chemother. 62, 951–955.

Kim, Y.J., Meyer, R.J., 1991. An essential iteron-binding protein required for plasmid R1162 replication induces localized melting within the origin at a specific site in AT-rich DNA. J. Bacteriol. 173, 5539–5545.

Kok, M., Arnberg, A.C., Witholt, B., 1989. Single-stranded circular DNA generated from broad host range plasmid R1162 and its derivatives. Plasmid 21, 238–241.

L'Abee-Lund, T.M., Sørum, H., 2002. A global non-conjugative Tet C plasmid, pRAS3, from *Aeromonas salmonicida*. Plasmid 47, 172–181.

Lawley, T., Wilkins, B.M., Frost, L.S., 2004. Bacterial conjugation in Gram-negative bacteria. In: Phillips, G., Funnell, B.E. (Eds.), Plasmid Biology. ASM Press, Washington, DC, pp. 203–226.

Lin, L.S., Meyer, R.J., 1986. Directly repeated, 20-bp sequence of plasmid R1162 DNA is required for replication, expression of incompatibility, and copy-number control. Plasmid 15, 35–47.

Lin, L.S., Kim, Y.J., Meyer, R.J., 1987. The 20 bp, directly repeated DNA sequence of broad host range plasmid R1162 exerts incompatibility *in vivo* and inhibits R1162 DNA replication *in vitro*. Mol. Gen. Gen. 208, 390–397.

Lewington, J., Day, M.J., 1986. A rapid electrophoretic method for the measurement of plasmid copy number. Lett. Appl. Microbiol. 3, 109–112.

Loftie-Eaton, W., Rawlings, D.E., 2009. Comparative biology of two natural variants of the IncQ-2 family plasmids, pRAS3.1 and pRAS3.2. J. Bacteriol 191, 6436–6644.

Loftie-Eaton, W., Rawlings, D.E., 2010. Evolutionary competitiveness of two natural variants of the IncQ-like plasmids, pRAS3.1 and pRAS3.2. J. Bacteriol 192, 6182–6190.

Maeser, S., Scholz, P., Otto, S., Scherzinger, E., 1990. Gene F of plasmid RSF1010 codes for a low-molecular-weight repressor protein that autoregulates expression of the *repAC* operon. Nucl. Acids Res. 18, 6215–6222.

Matcher, G.F., Rawlings, D.E., 2009. The effect of the location of the proteic post-segregational stability system within the replicon of plasmid pTF-FC2 on the fine regulation of plasmid replication. Plasmid 62, 98–107.

Meyer, R., 2009. Replication and conjugative mobilization of broad host-range IncQ plasmids. Plasmid 62, 57–70.

Miao, D.M., Honda, Y., Tanaka, K., Higashi, A., Nakamura, T., Taguchi, Y., Sakai, H., Komano, T., Bagdasarian, M., 1993. A base-paired hairpin structure essential for the functional priming signal for DNA replication of the broad host range plasmid RSF1010. Nucl. Acids Res. 21, 4900–4903.

Miao, D.M., Sakai, H., Okamoto, S., Tanaka, K., Okuda, M., Honda, Y., Komano, T., Bagdasarian, M., 1995. The interaction of RepC initiator with iterons in the replication of the broad host-range plasmid RSF1010. Nucl. Acids Res. 23, 3295–3300.

Niedenzu, T., Röleke, D., Bains, G., Scherzinger, E., Saenger, W., 2001. Crystal structure of the hexameric replicative helicase RepA of plasmid RSF1010. J. Mol. Biol. 306, 479–487.

Nordström, K., 2006. Plasmid R1 - Replication and its control. Plasmid 55, 1–26.

Novick, R.P., 1987. Plasmid incompatibility. Microbiol. Mol. Biol. Rev. 51, 381–395.

Pansegrau, W., Balzer, D., Kruft, V., Lurz, R., Lanka, E., 1990. *In vitro* assembly of relaxosomes at the transfer origin of plasmid RP4. Proc. Natl. Acad. Sci. USA 87, 6665–6669.

Poirel, L., Carattoli, A., Bernabeu, S., Bruderer, T., Frei, R., Nordmann, P., 2010. A novel IncQ plasmid type harbouring a class 3 integron from *Escherichia coli*. J. Antimicrob. Chemother. 65, 1594–1598.

Poirel, L., Carrër, A., Pitout, J.D., Nordmann, P., 2009. Integron mobilization unit as a source of mobility of antibiotic resistance genes. Antimicrob. Agents Chemother. 53, 2492–2498.

Rawlings, D.E., Pretorius, I., Woods, D.R., 1984. Expression of a *Thiobacillus ferrooxidans* origin of replication in *Escherichia coli*. J. Bacteriol. 158, 737–738.

Rawlings, D.E., Pretorius, I.M., Woods, D.R., 1986. Expression of *Thiobacillus ferrooxidans* plasmid functions and the development of genetic systems for the Thiobacilli. Biotechnol. Bioeng. Symp. 16, 281–287.

Rawlings, D.E., 2005. The evolution of pTF-FC2 and pTC-F14, two related plasmids of the IncQ family. Plasmid 53, 137–147.

Rawlings, D.E., Tietze, E., 2001. Comparative biology of IncQ and IncQ-Like plasmids. Microbiol. Mol. Biol. Rev. 65, 481–496.

Rohrer, J., Rawlings, D.E., 1992. Sequence analysis and characterization of the mobilization region of a broad-host-range plasmid, pTF-FC2, isolated from *Thiobacillus ferrooxidans*. J Bacteriol. 174, 6230–6237.

Rotger, R., Nombela, C., 1983. Characterization of penicillin-resistant β-lactamase-producing strains of Neisseria gonorrhaoeae isolated in Spain (1978–1981). Microbiol. Esp. 36, 115–122.

Rotger, R., Rubio, F., Nombela, C., 1986. A Multi-resistance plasmid isolated from commensal *Neisseria* species is closely related to the Enterobacterial plasmid RSF1010. J Gen Microbiol 132, 2491–2496.

Saano, A.K., Zinchenko, V.V., 1987. A new IncQ plasmid R89S: Properties and genetic organization. Plasmid 17, 191–201.

Sakai, H., Komano, T., 1996. DNA replication of IncQ broad-host-range plasmids in gram-negative bacteria. Biosc. Biotech. Biochem. 60, 377–382.

Scherzinger, E., Haring, V., Lurz, R., Otto, S., 1991. Plasmid RSF1010 DNA replication *in vitro* promoted by purified RSF1010 RepA, RepB and RepC proteins. Nucl. Acids Res. 19, 1203–1211.

Scholz, P., Haring, V., Wittmann-Liebold, B., Ashman, K., Bagdasarian, M., Scherzinger, E., 1989. Complete nucleotide sequence and gene organization of the broad-host-range plasmid RSF1010. Gene 75, 271–288.

Smalla, K., Heuer, H., Götz, A., Niemeyer, D., Krögerrecklenfort, E., Tietze, E., 2000. Exogenous isolation of antibiotic resistance plasmids from piggery manure slurries reveals a high prevalence and diversity of IncQ-like plasmids. Appl. Environ. Microbiol. 66, 4854–4862.

Smith, A.G.S., Rawlings, D.E., 1997. The poison-antidote system of the broad-host-range *Thiobacillus ferroxidans* plasmid pTF-FC2. Mol. Microbiol. 25, 961–970.

Suzuki, H., Yano, H., Brown, C.J., Top, E.M., 2010. Predicting plasmid promiscuity on genomic signature. J. Bacteriol. 192, 6045–6055.

Sýkora, P., 1992. Macroevolution of plasmids: A model for plasmid speciation. J. Theor. Biol. 159, 53–65.

Tietze, E., 1998. Nucleotide sequence and genetic characterization of the novel IncQ-like plasmid pIE1107. Plasmid 39, 165–181.

Tietze, E., Tschäpe, H., Voigt, W., 1989. Characterization of new resistance plasmids belonging to incompatibility group IncQ. J. Basic Microbiol. 29, 695–706.

Van Zyl, L.J., Deane, S.M., Rawlings, D.E., 2003. Analysis of the mobilization region of the broad-host-range IncQ-like plasmid pTC-F14 and its ability to interact with a related plasmid, pTF-FC2. J. Bacteriol. 185, 6104–6111.

Whittle, G., Katz, M.E., Clayton, E.H., Cheetham, B.F., 2000. Identification and characterization of a native *Dichelobacter nodosus* plasmid, pDN1. Plasmid 43, 230–234.

Willitts, N., Crowther, C., 1981. Mobilization of the non-conjugative IncQ plasmid RSF1010. Genet. Res. 37, 311–316.

Willson, P.J., Deneer, H.G., Potter, A., Albritton, W., 1989. Characterization of a streptomycin-sulfonamide resistance plasmid from *Actinobacillus pleuropneumoniae*. Antimicrob. Agents Chemother. 33, 235–238.

Yagi, J.M., Sims, D., Brettin, T., Bruce, D., Madsen, E.L., 2009. The genome of *Polaromonas naphthalenivorans* strain CJ2, isolated from coal tar-contaminated sediment, reveals physiological and metabolic versatility and evolution through extensive horizontal gene transfer. Environ. Microbiol. 11, 2253–2270.

Yau, S., Liu, X., Djordjevic, S.P., Hall, R.M., 2010. RSF1010-like plasmids in Australian *Salmonella enterica* serovar *Typhimurium* and origin of their sul2-strA-strB antibiotic resistance gene cluster. Microb. Drug Resist. 16, 249–252.

Zechner, E.L., de la Cruz, F., Eisenbrandt, R., Grahn, A.M., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C.M., Wilkins, B.M., Zatyka, M., 2000. Conjugative-DNA transfer processes. In: Thomas, C.M. (Ed.), The horizontal gene pool. bacterial plasmids and gene spread. Harwood Academic Publishers, Amsterdam, pp. 87–174.

Chapter 3. Conclusions and further research

3.1 Further research

Whilst working with IncQ plasmids, a number of matters have arisen that require further research and that were not discussed in the review of individual manuscripts presented in Chapter 1. Some of these will be rather challenging and are discussed below.

3.2 Integration of the regulation of replication and mobilization

The need for further research on the regulation of replication, the control of copy number and mobilization of IncQ plasmids was described on page 32 of Loftie-Eaton and Rawlings (2012). However, points that were not raised or adequately explained there are enlarged on here. As described previously, the main two promoters that control both replication and mobilization in the IncQ2 plasmids studied so far, lie within the *oriT* region with the major repressors being RepB in the direction of the replicon and MobC in the opposite direction (Figure 3.1). Although there is a strong promoter upstream of *pasABC*, the *pas* region together with its promoter can be deleted without affecting the PCN and as explained earlier, the *pas* is believed to play a regulatory role when the copy number of the plasmid falls below a certain threshold. A similar situation applies in the case of RSF1010, however, in this case it has not been tested whether ORFs E and F (two small ORFs that lie in the same position as the *pas* genes of pTF-FC2 or pTC-F14), can be deleted. We have demonstrated that ORFs E and F of RSF1010 are not part of a yet unrecognised TA system (unpublished).

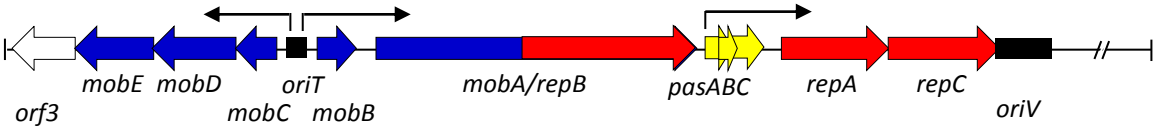


Figure 3.1 Plasmid pTF-FC2 indicating the main promoters that regulate transcription of the plasmid backbone and the gene product that serves as the negative regulator of each promoter. In the case of the *pas* operon, the PasA antitoxin is the main regulator with PasB serving as a co-repressor.

One can imagine that there will be times when the IncQ plasmids will be at the maximum copy number within a cell when an opportunity to be mobilized to a recipient cell arises. In that case, the plasmid will have a need for the induction of transcription from the *oriT* in both directions to ensure that the products of *mobBA* as well as *mobCDE* are available in sufficient quantities for mobilization to take place. What is unclear is how *mobBA* induction will take place in a situation where transcription in the direction of the replicon is repressed due to the maximum PCN having been attained. Presumably there will be a basic level of transcription from the relatively weak *mobBA* promoter even when repressed with the PCN being controlled primarily by the RepC monomer-dimer and iteron-binding dynamic. However, this has not been tested. Furthermore, whether RepC-bound iteron hand-cuffing (Chattoraj, 2000) occurs in the case of IncQ plasmids is uncertain as there is some evidence that may be interpreted as being against hand-cuffing (Meyer, 2009).

What is even more unclear is the question of what regulates the MobC repressor in the case of transcription from the promoter in the direction of *mobCDE* so that expression of the three genes is derepressed prior to mobilisation. The issue as to what regulates a regulator is frequently a problem in molecular biology. One may understand how an operon is regulated at the first level (activator or repressor), but that is only part of the story, one needs to understand regulation at the next levels (regulation of the regulator and global regulation) as well. Is there a signal that is given to the IncQ plasmid that a potential recipient is available and that it needs to derepress its mobilization genes, or is the plasmid always ready to be mobilized whenever a mating bridge between a donor and recipient cell is available?

Work in the laboratory of Richard Meyer demonstrated that a 75-bp counter-transcribed RNA (ct-RNA) or antisense RNA is involved in the regulation of replication in plasmid R1162 (Kim and Meyer, 1986). This ct-RNA overlaps the ribosome binding site and initiation codon of *repA*. However, nobody has investigated the possibility of antisense RNA in any of the other IncQ-family plasmids. This is a glaring omission that needs to be addressed.

It would therefore appear that a substantial amount of work remains to be done on the regulation of replication in the IncQ plasmids. Even more research on the regulation of mobilization and the integration of the regulation of mobilization and replication is required so as to understand how either process may take place when the other is not required.

3.2.1 The discovery of pLAtc1 and the questions it raises

The fairly recent report on the sequence of plasmid pLAtc1 (9778 bp) by You et al. (2011) (GenBank CP002575.1) has raised some additional questions regarding the regulation of the IncQ plasmids that may complicate the rather simple model shown in Figure 3.1. This plasmid was isolated from *Acidithiobacillus caldus* SM1, the same species of bacterium as from which pTC-F14 was isolated, although from a different strain. Plasmid pLAtc1 is clearly of the IncQ2 type with a mobilization region consisting of five genes, a *mobA-repB* fusion and a *repAC* gene pair as indicated in Figure 3.2.

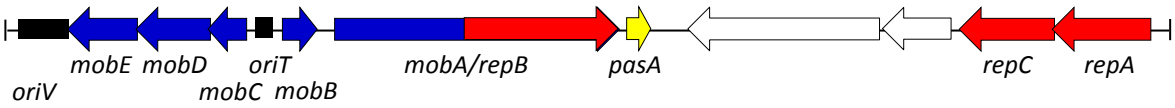


Figure 3.2 The genetic map of the backbone of plasmid pLAtc1.

Examination of the predicted amino acid (aa) sequences of the pLAtc1 ORFs, indicates that this plasmid is clearly related to the pTF-FC2, pTC-F14 and the pRAS3 plasmids (see Table 3.1). The relatedness varies depending on which ORFs are compared with maximum relatedness being to the predicted MobD of pTF-FC2 and pRAS3.2 (both 95% aa identity) and to the RepA of pTC-F14 (93% aa identity). The *repC* gene of pRAS3.2 and the *mobCDE* genes of pTC-F14 were predicted to have been exchanged with those of as yet unidentified plasmids (Loftie-Eaton and Rawlings, 2102) and it can clearly be seen that in the case of these gene products, pLAtc1 is less closely related to pRAS3.2 and pTC-F14 than to the other plasmids (marked with asterisks in Table 3.1). Plasmid pLAtc1 appears to possess an ORF that encodes a PasA-like antitoxin, but no PasB-like toxin. Since pRAS3.2 has a different TA system from the other IncQ2-like plasmids there is no product related to PasA to be

compared. It appears that a transposon (or IS element) has become inserted into the replicon of pLAtc1 and that this has disrupted its TA structure. The putative product of TnpA is most closely related to transposase A (approx. 79% aa identity) and that of TnpB to transposase B (approx. 46% aa identity) of the ISChy9 insertion sequence.

Table 3.1. Relatedness of the predicted products of the ORFs from pLAtc1 that have homologues with other IncQ2 plasmids (aa sequence identity).

Predicted aa sequence of ORF	pTF-FC2 ex *At. ferrooxidans* strain FC	pTC-F14 ex *At. caldus* strain F	pRAS3.2 ex *Aeromonas salmonicida*
RepA	85	93	85
RepB	67	65	61
RepC	44	44	19*
MobA	41	65	83
MobB	57	49	83
MobC	83	20*	83
MobD	96	41*	95
MobE	35	17*	35
PasA	65	77	-

* it has been proposed that the genes encoding these proteins have been exchanged during plasmid evolution.

What makes pLAtc1 unique among IncQ plasmids so far reported is that the *repA* and *repC* genes are transcribed in a different direction to the *mobA/repB* genes. This means that regulation of the expression of *repAC* cannot be by reading through from a promoter immediately upstream of *mobB* as for the other IncQ plasmids. Furthermore, jumpstarting *repAC* expression from strong, self-regulated promoter of a toxin-antitoxin system cannot occur. Although a potentially autoregulated gene related to *pasA* is present, the *repAC* genes are facing in the wrong direction to be regulated by the *pasA* promoter.

This means that part of what appeared to be a common theme for the regulation of the IncQ plasmids cannot apply in all aspects to pLAtc1. Unfortunately, no studies on the biology of pLAtc1 have been published and therefore how competitive the 'disrupted replicon' of this plasmid is in comparison with other IncQ plasmids is unknown. This would be an interesting line of enquiry.

3.3 Host-range studies

With rare exceptions, such as our studying the copy number of pTC-F14 in *At. caldus* as well as in *E. coli* and the stability of pTF-FC2 in *Pseudomonas putida*, very little work on IncQ plasmids has been carried out in bacteria other than *E. coli*. There are relatively few broad host-range plasmids that provide the opportunity for such studies and the opportunity provided by the IncQ plasmids should not be missed. It has long been argued by Kornelia Smalla (personal communication) that the true test for a plasmid being broad host-range is not the ability of the plasmid to replicate in the host being tested under conditions of plasmid selection, but the ability of a plasmid to be stably maintained in a variety of hosts in the absence of selection. Insufficient studies of this type have

been carried out to test whether the IncQ plasmids are broad host-range using this more stringent test as the standard.

Another example of host variation that was referred to in Chapter 1 is that we found that the *pas* does not function equally well in stabilizing pTF-FC2 in all strains of *E. coli*. Furthermore, the extent to which the *pas* was effective could be correlated with the sensitivity of the strain to the toxin with the *pas* working best in the strains that were most toxin sensitive. This raises the question of whether the *pas* functions as a post-segregation TA stability system in some species of bacteria but not in others? This is what one might predict if differential toxicity holds in other hosts. Then there is the question of whether the strongly autoregulated promoter of the *pas* serves to transiently kick-start plasmid replication should the copy number fall below a threshold even in those bacteria in which the *pas* may not function as a TA system. One may predict that this replication 'kick-start' function is a characteristic of a strong, autoregulated promoter and should be independent of whether the TA system is toxic to a given host. However, whether this is indeed the case needs to be tested.

The effect of host range on other aspects of plasmid biology such as copy number, the regulation of replication and mobilization should all be studied to see to what extent these may differ in different types of bacterial hosts.

3.4 Plasmid incompatibility

Plasmid incompatibility has been defined as 'the failure of two coresident plasmids to be stably inherited in the absence of external selection' (Novick, 1987). It is generally understood that incompatibility arises as a result of the sharing of one or more elements of the plasmid replication or partition systems. Where elements of replication are shared, plasmid loss is typically a result of interference with the plasmids ability to correct fluctuations in its copy number as both plasmids are 'counted' as belonging to the same plasmid pool. Variations in individual PCN are not corrected, with the result that the contribution of one of the plasmids to the pool may fall below the level required for stable inheritance. In the case of partitioning systems, current understanding is that incompatibility arises because the system does not adequately distinguish between the two plasmids resulting in the total number of plasmids being segregated into progeny cells in approximately equal numbers but randomly. The effect of this is that although each progeny cell may receive an equal number of plasmids, there is a statistical chance that all of the plasmids received by one progeny cell may be of one type and those by the other progeny cell of the other type. In both of these mechanisms, plasmid incompatibility occurs because of shared or related systems. In other words, incompatibility is an indication of plasmid relatedness such that incompatible plasmids are considered to be more closely related than compatible plasmids.

While it has been recognised that incompatibility is not always a reflection of plasmid relatedness, the studies on several of the IncQ family plasmids provide among the best examples of this. Some plasmids that were members of same IncQ subgroup were compatible while those belonging to different subgroups were incompatible. An example of this was the observation that the related IncQ2 plasmids pTF-FC2 (IncQ2α) and pTC-F14 (IncQ2β) were compatible but both were unidirectionally displaced by the less related pIE1108 (IncQ1γ) replicon (Gardner *et al*., 2001). That

is, when either pTF-FC2 or pTC-F14 was the selected plasmid, coresident pIE1108 was not displaced from the *E. coli* host, however if pIE1108 was the selected plasmid, both pTF-FC2 and pTC-F14 were lost. Similarly, when a R300B replicon-based plasmid (also IncQα and believed to be identical to RSF1010) was selected, a coresident pTC-F14 was lost but not vice versa. In this case, the R300B replicon was compatible with pTF-FC2. In a subsequent study (Gardner and Rawlings, 2004), this incompatibility was found to be associated with the RepC DNA-binding protein of the IncQ1α plasmid rather than the iterons which were compatible when tested on their own. This incompatibility was attributed to the non-productive binding of the heterologous RepC to the iterons of pTC-F14 that prevented the RepC of pTC-F14 from binding to the iterons of its own *oriV*.

The point of this discussion is not that the model for incompatibility as described in the review by Novick (1987), and also by others, is invalid. Rather that the assumption that the occurrence of incompatibility between plasmids is an indication that the plasmids have related replicons or partitioning systems may not be true in all cases.

Since IncQ plasmids do not have an active partitioning system (as opposed to a post-segregational killing TA system identified in only the IncQ2 subfamily), incompatibility would be expected to be due to interference between replication systems. The type of incompatibility observed between the IncQ2 and the IncQ1 plasmids did not appear to be due to the inability to correct the copy number of individual plasmids as typically associated with replication incompatibility but rather binding interference. Nevertheless, the incompatibility was of a 'gentle' type, that is, it was possible for the two plasmids to coexist in the same cell provided there was continuous selection for the growth of cells that contained both types of plasmid otherwise one was lost.

In contrast, the type of incompatibility observed between the pRAS3 plasmids (IncQ2γ group) and pTF-FC2 (IncQ2α group) or pTC-F14 (IncQ2β) group was 'violent' (Loftie-Eaton and Rawlings, 2009). That is, one could never detect both plasmids in the same *E. coli* host cell. If either of the pRAS3 plasmids were present in an *E. coli* host, one could not transform the cells with either pTF-FC2 or pTC-F14 although one could readily transform these same cells with other plasmids (e.g. pUC19 or pACYC177). If either pTF-FC2 or pTC-F14 were present in an *E. coli* host it was possible to transform the cells with either pRAS3 plasmid, but pTF-FC2 or pTC-F14 was immediately lost and the transformed cells would not grow on media containing selectable markers for both plasmids. The ORF3 that was identified as the source of the incompatibility (Loftie-Eaton and Rawlings, 2009) was immediately downstream of, and transcribed together with, *mobCDE* but appeared to have no effect on plasmid replication and a minor effect (2-fold enhancement) on mobilization. Although there was an ORF in a similar position in pTF-FC2, the two ORFs were not sequence related. When the pRAS3.1 ORF3 was inactivated by insertion mutagenesis, the mutant plasmid and pTF-FC2 were perfectly compatible. However when ORF3 was expressed from a different coresident plasmid, incompatibility was not restored and therefore incompatibility appeared to be a cis rather than a trans effect that one would expect if the product of ORF3 was involved. Why this is so remains a mystery and we did not pursue this further. This 'violent' incompatibility was clearly distinguishable from the 'gentle' type of incompatibility that arises from control problems that may occur between two related plasmids.

There was yet a third type of displacement of one plasmid by another that did not have to do with either of the more commonly recognised replication or active partitioning incompatibilities. This was

the displacement of pTF-FC2 by pTC-F14 due to interference between their TA post-segregational killing systems. To detect this type of displacement an *E. coli* host that was sensitive to the PasA toxin had to be used as well as a more sensitive method for determining plasmid displacement. The typical method that we had used for determining plasmid compatibility associated with the replicons was to transform *E. coli* host cells containing a resident plasmid with an incoming plasmid and then select for the incoming plasmid only. Since these new transformants would have been grown on a lawn of untransformed cells that contained the original resident plasmid, the new transformants were restreaked so as to obtain colonies derived from single cells on solid medium that again selected for only the incoming plasmid. Individual colonies from this restreaking would be free of the background lawn of cells. These individual colonies were then tested to find out how many had retained the original resident plasmid. These host cells would have gone through two rounds of growth from a single cell to a colony on solid medium in the absence of selection. In contrast, displacement of pTC-F14 by pTF-FC2 due to the interference of their *pas* post-segregational stability systems was tested by a method similar to when determining plasmid stability. To detect this type of displacement, a liquid plasmid stability assay was carried out beginning with an *E. coli* host that contained the two coresident plasmids in a single cell rather than in separate hosts as was typical for a stability comparison assay. The assay was conducted by growing host cells in the absence of selection for either plasmid for 100 generations and then testing which of the plasmids had been retained. In the presence of pTF-FC2, pTC-F14 behaved like a plasmid that had lost its ability to be stably inherited. This result was consistent with that predicted from a loss of its post-segregational stability system as we had shown that the PasA of pTF-FC2 was able to repress the expression of the *pas* of pTC-F14. Incompatibility was due to a loss of stability due to the non-expression of the TA system of pTC-F14 rather than due to an interference with replication or a sharing of copy number with the accompanying inability to correct copy number fluctuations. Although this loss-of-stability type displacement is slow compared with replication incompatibility and enough time must be allowed for the pTC-F14 to be lost, it may nevertheless be viewed as being a type of plasmid incompatibility as active interference would have taken place that would not have been present if pTF-FC2 had not been coresident with pTC-F14. In other words, in the absence of pTF-FC2, pTC-F14 would have been stable.

Taken together these findings indicate that the causes of plasmid incompatibility and the interpretation of plasmid incompatibility is more complicated than is generally understood.

Plasmid incompatibility is undoubtedly an important biological phenomenon to be considered when doing plasmid research or working with cloning vectors, but not very useful in plasmid classification.

3.5 IncQ3 and IncQ4 family plasmids

The IncQ3 and IncQ4 subfamilies of IncQ plasmids (Figure 3.3) were identified by sequence analysis only. No biological research on either subfamily has been reported and there is clearly much to be done. Both sub-families appear to have mobilization genes that are transcribed in the direction of the replicon only with no mobilization genes in the opposite direction. Whether this is indeed the case requires confirmation. Neither subfamily has an equivalent of the TA system present in the IncQ2 plasmids or other genes such as the *repEF* genes of RSF1010 between *repB* and *repAC*. In the case of the IncQ3 plasmids there is no space for such genes and in the case of the only IncQ4 plasmid

so far identified, no gene could be identified. Exactly how the mobilization and replication functions of these plasmids are regulated and whether the plasmids have a mechanism for the regulation of these two functions independently of each other needs to be investigated.

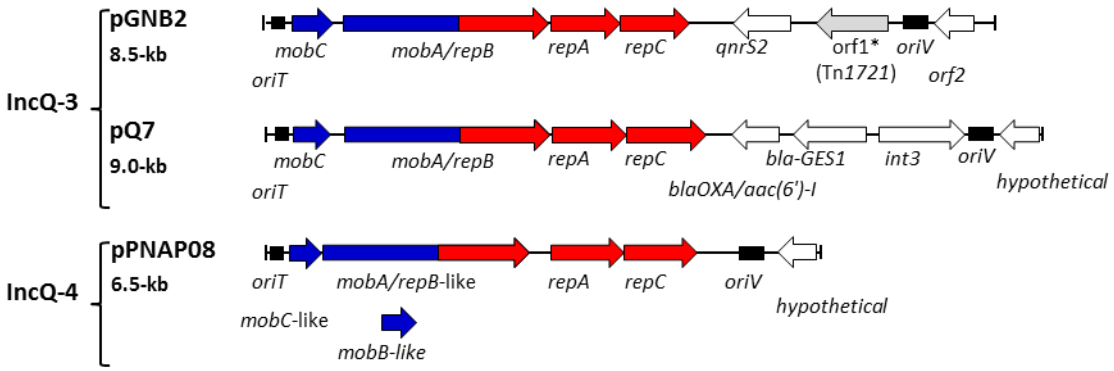


Figure 3.3 Genetic maps of the IncQ plasmids that have been assigned to IncQ3 and IncQ4 sub-familes (Loftie-Eaton and Rawlings 2012).

3.6 The future of research on IncQ plasmids

The future of research on IncQ plasmids faces a double challenge, that is, a lack of funding and also people to work on the topic. I was never specifically funded for working on IncQ plasmids and this work had to be 'piggy-backed' onto the biomining research for which I did receive funding. Nevertheless, work with plasmids and especially the IncQ plasmids became a passion, whereas the biomining research was interesting and 'paid the bills'. For the past decade or so, the only laboratories that carried out research on the biology of IncQ-family plasmids were led by Richard Meyer (University of Texas, Austin) and myself. Richard Meyer's research on IncQ plasmids had focussed on a single IncQ1 plasmid, R1162, while my own laboratory has worked with the IncQ2 plasmids (pTF-FC2, pTC-F14 and pRAS3.1 and pRAS3.2). We have worked with other IncQ plasmids only when carrying comparative, complementation or incompatibility studies. Given the small number of researchers, manuscripts in this field tend to be seldom cited and this affects funding. For example only one of the manuscripts that form part of this dissertation has been cited more than 50 times as yet, whereas in biomining research, seven of my own manuscripts have been cited more than 100 times each. For the past few years Richard Meyer has ceased to receive funding for his work on IncQ plasmids and as a result his work has stalled. Similarly, I have recently temporarily filled a number of senior administration positions at the University of Stellenbosch and have not been able to continue with research on IncQ plasmids. Hopefully others will become interested in this group of highly promiscuous plasmids and people with an in-depth knowledge of the IncQ plasmids will not totally disappear.

As IncQ plasmids have a broad host-range and all IncQ plasmids tested are readily mobilizable (those not yet tested also contain mobilization genes), this makes them highly promiscuous. As they are exposed to many potential hosts that are able to grow in a wide a variety of conditions, they are likely to be subject to recombination with a large number of genetic elements including other plasmids and transposons. As has been explained earlier, the result of this is that several evolutionary lineages exist within the IncQ-like plasmid family and both micro- and macro-

evolutionary events can be identified. They are therefore prime candidates for studies on the adaptability and evolution of plasmids and it will be a pity if further research on these plasmids is not taken up without much delay.

3.7 Additional references

Chattoraj D.K. 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. Mol. Microbiol. **37**: 467-476.

Kim, K. and Meyer R.J. 1986. Copy-number of broad host-range plasmid R1162 is regulated by a small RNA. Nucl. Acids Res. **14**: 8027-8046.

Meyer, R. 2009. Replication and conjugative mobilization of broad host-range IncQ plasmids. Plasmid **62**: 57-70.

Novick R. 1987. Plasmid incompatibility. Microbiol. Rev. **51**: 381-395.

You XY, Guo X, Zheng HJ, Zhang MJ, Liu LJ, et al. (2011) Unraveling the *Acidithiobacillus caldus* complete genome and its central metabolisms for carbon assimilation. J. Genet. Genomics **38**: 243-252.