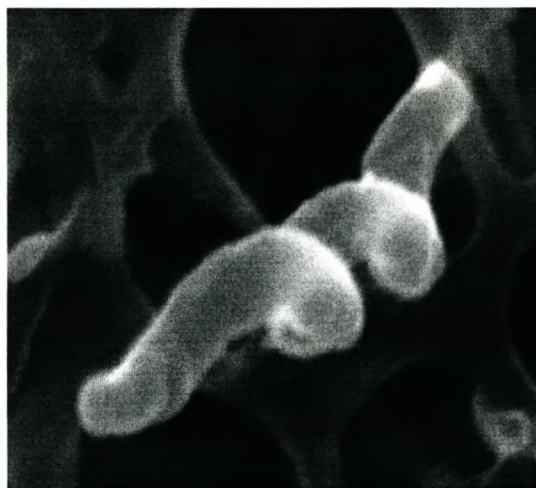


MOLECULAR
CHARACTERIZATION OF IRON-
OXIDIZING *Leptospirillum*
STRAINS FROM AROUND THE
WORLD

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Dissertation presented for the Degree of Doctor of
Philosophy at the University of Stellenbosch.

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December 2002

Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Nicolette J. Coram

Abstract

More than sixteen isolates of iron-oxidizing bacteria belonging to the genus *Leptospirillum* were included in this study, with the finding that they were clearly divisible into two major groups. Group I leptospirilla had mol% G+C ratios within the range 49-52%, three copies of *rrn* genes and based on 16S rRNA sequence data, clustered together with the *Leptospirillum ferrooxidans* type strain (DSM2705 or L15). Group II leptospirilla had mol% G+C ratios of 55-58%, two copies of *rrn* genes and based on 16S rRNA sequence form a separate cluster. Genome DNA-DNA hybridization experiments indicated that three similarity subgroups were present amongst the leptospirilla tested with two DNA-DNA hybridization similarity subgroups being found within group I. The two groups could also be distinguished based on the sizes of their 16S-23S rRNA gene spacer regions. We propose that the group II leptospirilla should be recognized as a new species with the name *Leptospirillum ferriphilum* sp. nov. Members of the two species can be rapidly distinguished from each other by amplification of their 16S rRNA genes and carrying out restriction enzyme digests of the products. Several but not all isolates of the group II leptospirilla, but none from group I (*L. ferrooxidans*) were capable of growth at 45°C.

Plasmid DNA was isolated from strain ATCC49879 (*L. ferrooxidans*). Restriction endonuclease mapping of what appeared to be about 60 kb of plasmid DNA, established that two plasmids of approximately 30.0 kb and 27.0 kb were present. These were named p49879.1 and p49879.2 respectively. Attempts to isolate the plasmids separately were not successful. Partial sequencing of the two plasmids was carried out and sequence analysis of p49879.1 and p49879.2 indicated that the plasmids shared regions of homology. Total plasmid DNA was DIG-labelled and used as a probe in Southern hybridization experiments with genomic DNA from all sixteen original leptospirilla isolates as the target DNA. All leptospirilla belonging to Group I gave a positive signal, little or no homology to Group II leptospirilla was obtained. The region of homology present in all *L. ferrooxidans* strains was localized to an area on plasmid p49879.2 showing high amino acid identity to a

transposase/putative transposase of *Methanosarcina acetivorans* and plasmid CP1 from *Deinococcus radiodurans* R1 respectively. Whether these regions of homology indicate that complete, functional transposons are present in all *L. ferrooxidans* isolates still remains to be determined. Preliminary sequence analysis of both plasmids resulted in the identification of regions with amino acid sequence identity to the TnpA and TnpR of the Tn21-like transposon family, and the mobilization regions of IncQ-like plasmids (particularly that of pTF1 from *At. ferrooxidans*). Another potentially interesting ORF was identified in p49879.2 with high amino acid sequence identity to an ArsR-like protein that belongs to a second atypical family of ArsR transcriptional regulators. Whether this protein is functional in the regulation of arsenic resistance genes has not yet been determined, nor have other arsenic resistance genes been identified. Future work includes further sequence analysis of these plasmids to better understand their contribution to the isolates in which they are found.

Opsomming

Meer as sestien isolate van die yster-oksiderende bakterieë, wat aan die genus *Leptospirillum* behoort, is in die studie ingesluit en die resultate het getoon dat dié groep verder in twee hoof groepe verdeel kan word. Groep I het 'n mol% G+C van tussen 49% en 52% gehad, sowel as drie kopieë van die ribosomale gene (*rrn*). Hiermeesaam het die 16SrRNA volgorde data getoon dat hierdie isolate groepeer saam met *Leptospirillum ferrooxidans* (DSM2705^T en L15). Groep II leptospirilla het 'n mol% G+C van tussen 55% en 58% gehad sowel as twee kopieë van die *rrn* gene en saam met die 16SrRNA volgorde data het hierdie isolate 'n aparte groep gevorm. Genoom DNA-DNA hibridisasie eksperimente het gewys dat daar drie subgroepe onder die *Leptospirillum* wat getoets was is, met twee naverwante groepe wat onder Groep I val. Daar kan ook tussen die twee hoof groepe onderskei word op grond van die grootte van hul 16S-23SrRNA intergeniese gebiede. Ons stel dus hier voor dat die Groep II leptospirilla as 'n nuwe spesie beskou word naamlik, *Leptospirillum ferriphilum* sp. nov. Die twee spesies kan maklik onderskei word deur die PKR amplifikasie produk van die 16SrRNA te verteer met restriksie ensieme. Vele, maar nie al van die Groep II isolate kan by 45°C groei nie, terwyl geen van die Groep I leptospirilla (*L. ferrooxidans*) kan nie.

Plasmied DNA was geïsoleer uit *Leptospirillum ferrooxidans* ATCC49879. Aanvanklike analise het gedui op die teenwoordigheid van een 60.0 kb plasmied. Verdere restriksie ensiem kartering het wel getoon dat hierdie, in teen deel, twee plasmiede van ongeveer 30.0 kb en 27.0 kb in grootte is: p49879.1 en p49879.2. Pogings om die twee plasmiede apart te isoleer was onsuksesvol. Totale plasmied DNA is gemerk met die Random primed DNA labelling kit (Roche diagnostics) en gebruik as peiler in Southern klad eksperimente met genoom DNA, van al sestien isolate, as teiken. Alle leptospirilla wat aan Groep I behoort het 'n positiewe sein gegee terwyl geen sein teen Groep II DNA opgemerk was nie. Die area wat, tussen die plasmiede en Groep I homologie getoon het, is gelokaliseer tot 'n area op plasmied p49879.2 wat hoë amino suur identiteit toon aan 'n transposase geen van *Methanosarcina acetivorans*, en 'n voorgestelde transposase

geen op plasmied CP1 van *Deinococcus radiodurans* R1. Dit moet nog vasgestel word of hierdie area van homologie dui op die teenwoordigheid van 'n volledige, funksionele transposon in alle *L. ferrooxidans* isolate. Gedeeltelike DNA volgorde bepalinge van beide plasmiede het gelei tot die identifikasie van areas met hoë amino suur volgorde identiteit aan die TnpA en TnpR gene van die Tn21-tipe transposon familie, sowel as aan die mobilisasie gene van IncQ-soortige plasmiede (veral die van pTF1 uit *Acidithiobacillus ferrooxidans*). 'n Oop lees raam van belang, wat op plasmied p49879.2 geïdentifiseer was, het hoë amino suur volgorde identiteit aan 'n ArsR-tipe geen getoon wat aan 'n tweede atepiese familie van ArsR transkripsionele reguleerders behoort. Op die stadium is dit nog onbekend of hierdie protein funksioneel is in die regulering van arseen weerstandbiedende gene.

Acknowledgments

Firstly I would like to thank my supervisor, Professor Douglas Rawlings, for his consistent support, guidance, and encouragement throughout the duration of this project. His enthusiasm in the quest for knowledge has been a priceless source of inspiration to me.

Many thanks to my colleagues in the laboratory, for their continual willingness to help in situations of calm and crisis. A special note of thanks to Lonnie van Zyl for helping with the abstract translation.

A warm word of thanks is extended to all academic and administrative staff, students and departmental assistants for their help throughout this study. In particular I would like to thank Dorette van der Merwe for her ability to “turn the impossible into possible”, at any time of the day or night.

I wish to acknowledge financial support from the National Research Foundation (NRF), the Harry Crossley Bursary Fund, and Stellenbosch University.

To the important people in my life, my family and friends. Without their prayers and unfailing belief in me, I would never have been able to pick up the pieces and continue to run the race.

Finally, I wish to thank my Father, not only for rewarding me generously with patience and perseverance, but also for His handiwork, without which this study would not have been possible.

*“Have you not known?
Have you not heard?
The everlasting God, the Lord,
The Creator of the ends of the earth,
Neither faints nor is weary.
His understanding is unsearchable.*

*He gives power to the weak,
And to those who have no might He increases strength.*

*Even youths shall faint and be weary,
And the young men shall utterly fall,*

*But those who wait on the Lord
Shall renew their strength;
They shall mount up with wings like eagles,
They shall run and not be weary,
They shall walk and not faint”.*

Isaiah 40: 28-31 (NKJV).

**In loving memory of my grandfather Matthew James McIver.
(1921-1999)**

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Abbreviations

A	adenosine
Amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine 5' -triphosphate
α	alpha
aa	amino acids
bp	base pairs
β	beta
C	cytosine
C-terminal	carboxyl-terminus
CsCl	cesium chloride
CSH	Cold Spring Harbour
CTP	cytosine 5' -triphosphate
ctRNA	countertranscribed ribonucleic acid
$^{\circ}\text{C}$	degrees Celsius
Da	daltons
DIG	dioxigenin- 11-dUTP (DIG-dUTP)
DNA	deoxyribonucleic acid
DNTP	deoxyribonucleotide triphosphate
dsDNA	double stranded deoxyribonucleic acid
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellculturen
dUTP	deoxyuridine triphosphate
EDTA	ethylenediaminetetraacetic acid
EtBr	ethidium bromide
G	guanine
g	gram(s)
G+C	guanine: cytosine ratio
GTP	guanine 5' -triphosphate
h	hour(s)
HCl	hydrochloric acid
H ₂ SO ₄	sulfuric acid
IR	intergenic region
IS	insertion sequence
kb	kilobase pair(s) or 1000bp
kDa	kilodaltons
K _i	bacterial inhibition constant
K _m	saturation constant
l	liter

mA	milliamp
mg	milligram
MgCl ₂	magnesium chloride
min	minute(s)
ml	milliliters
mM	millimolar
mm	millimeter
M	molar
mmol	millimole
mol%	mole percentage
µg	microgram
µl	microliter(s)
µM	micromolar
µm	micrometer
µmol	micromole
MS	mineral sulfide
NCBI	National Center for Biotechnology Information
N-terminal	amino-terminus
ng	nanogram
nm	nanometers
nt	nucleotide
OD ₆₀₀	optical density at 600 nanometers
ORF	open reading frame
<i>oriT</i>	origin of transfer
<i>oriV</i>	origin of vegetative replication
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
p	plasmid
pmol	picomole
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
s	second(s)
S	Svedberg unit
ssDNA	single stranded deoxyribonucleic acid
SDS	sodium dodecyl sulfate
SET	sucrose EDTA buffer
sp	species
sp. nov.	new species
SSC	saline-sodium citrate
T	thymine
TBE	Tris-borate EDTA buffer

TE	Tris EDTA buffer
Tris	Tris (hydroxymethyl) aminomethane
T-DNA	transfer deoxyribonucleic acid
T _m	melting temperature
ΔT _m	change in melting temperature
T _n	transposon
TTP	thymine 5' -triphosphate
U	unit (of enzyme activity)
UV	ultraviolet
V	volts
v/v	volume/volume
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-galactoside
Δ	delta
λ	lambda
μ	micro
<	less than
>	greater than
~	approximately
γ	gamma
%	percentage

Chapter 1

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1.1 Introduction

The use of microorganisms for the benefit of mankind has been applied throughout human history; from the production of various fermented foods and beverages to mining practices of the early Phoenicians and Romans. Ironically, metal leaching has been practiced in Spain, France, the United States, and other countries for many years without knowing of the microbial contribution to the process (Lundgren and Silver, 1980; Brierley, 1982; Brierley, 1978). In 1947, bacteria associated with acid rock drainage were discovered (Colmer and Hinkel, 1947). The bacterium isolated from this environment was initially named *Ferrobacillus ferrooxidans*, and in 1951, after further characterization (Colmer *et al.*, 1950), was renamed *Thiobacillus ferrooxidans*. More recently, the genus *Thiobacillus* was subdivided and a new genus, *Acidithiobacillus*, was created to accommodate the highly acidophilic members of the former genus. Hence, the bacterium previously referred to as *Thiobacillus ferrooxidans*, is now known as *Acidithiobacillus ferrooxidans* (Kelly and Wood, 2000).

The biotechnology of microbial mining began in 1963 when results of laboratory studies confirmed the involvement of bacteria in the solubilization of copper from sulfidic ores (Razzel and Trussel, 1963). Research and development between 1960-1980 yielded much information regarding bacterial involvement in the mining industry. For example: phenotypic characteristics of leaching bacteria were identified; metabolic pathways for iron- and sulfur- oxidizing bacteria were described along with microbial-mineral interactions and mineral metabolism; the influence of metal iron concentration and many environmental parameters on bioleaching were quantified; the ecology of copper dump leach operations was studied, resulting in the ability to employ large scale test facilities to evaluate copper bioleaching; and the use of stirred-tank reactors for bioleaching was also extensively tested, improving the efficiency of mineral sulfide oxidation. In the early 1990s, many commercial-scale bioleach/biooxidation plants were commissioned (Brierley, 1997). Currently the use of microbes in commercial mining practices is widely accepted, offering the mining industry cost-effective, simple, robust, high performance and environmentally friendly alternatives to conventional methods of mineral processing.

1.2 Microbial assisted- versus conventional- mining practices

Prior to the era of bacterially assisted mining, conventional methods such as the smelting of sulfide minerals and burning of sulfide-rich fossil fuels were practiced. Although the benefits of such practices were evident in the high mineral yields that were obtained, the cost was, extensive environmental pollution. For example, the large coal reserves in Illinois, having a high sulfur content, could not be burned without releasing unacceptable levels of sulfur dioxide (Rawlings and Silver, 1995). Mining and extraction also mobilizes $\sim 150 \times 10^{12}$ g of sulfur per year, contributing $\sim 50\%$ to the net river transport of sulfate into the ocean (Edwards *et al*, 2000). In addition to this, mining exposes large sulfide ore bodies to weathering by water and air, the combined activity of natural bacteria in this environment produces a noxious, metal-laden, often highly acidic effluent (acid mine drainage [AMD]), which is a serious and widespread form of stream and river pollution in many industrial and postindustrial areas. In order to control mining-generated pollution, the United States Surface Mining Control and Reclamation Act of 1977 set effluent quality standards for mine water discharges (Table 1) (Unz and Dietz, 1986). This resulted in escalating costs for the treatment of mine water generated by conventional mining practices. Not only was the cost of mine waste treatment increasing, but so was the need to mine at greater depths and work lower grade ore deposits, due to the depletion of rich surface ore deposits. Hence, mining companies have increasingly been looking for alternative methods to recover metals from lower grade ores, and the tailings or residual, “worked” rock, accumulated from mining operations without the associated economic and environmental burden.

Table 1.1: Effluent Limitations on Mine Water Pollutants as Mandated by the Surface Mining Control and Reclamation Act of 1977.

Pollutant	Maximum allowable value	Mean of daily values for 30 consecutive discharge days
Iron, total (mg/l)	7.0	3.5
Manganese, total (mg/l)	4.0	2.0
Total suspended solids (mg/l)	70.0	35.0
pH	6.0-9.0	6.0-9.0

Exploitation of microorganisms for the processing of ores has developed into one of the major areas of biotechnology, with an estimated market value of over 10 billion US dollars for 1998 (Johnson, 1998). Although the present commercial application for microbial leaching is mostly confined to copper, uranium, and gold, the potential of this technique may be applied to ores containing sulfides of zinc (Zn), lead (Pb), cobalt (Co), nickel (Ni), bismuth (Bi), and antimony (Sb), as well as some oxide minerals. A number of advantages in the use of microbial leaching over those of classical physicochemical techniques exist.

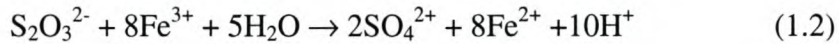
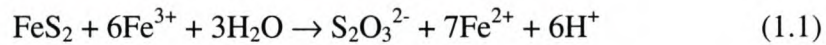
- i) The metal content of an ore is a major factor in the cost of metal recovery by conventional mining techniques, and therefore requires ores of a higher grade as a starting material. Bacterial activity is not as affected by ore grade and is applicable to a range of lower ore grades as well as waste material from traditional ore processing (Lundgren *et al.*, 1986; Brierley, 1978; Brierley, 1982).
- ii) Operating costs for bacterial leaching are frequently lower than conventional processes as most processes operate at ambient temperatures and have lower energy requirements.
- iii) Environmental aspects of microbial leaching are less hazardous. Mine tailings and wastes produced from physicochemical processes may be biologically leached when exposed to rain and air, resulting in the formation of acid mine drainage (Schippers *et al.*, 1996). Tailings from microbial leaching operations are less chemically active and the biological activity they can support is reduced by the extent to which they have already been bioleached. Sulfurous emissions do not occur, and the waste, produced in either liquid or solid form, can be contained (Lundgren *et al.*, 1986).
- iv) The treatment of recalcitrant ores with a microbial consortium prior to cyanidation has also been shown to improve cyanidation performance. Ores from which gold recovery was less than 50% with only conventional cyanide treatment, yielded greater than 90% gold recovery when pretreated with a microbial consortium (Hutchins *et al.*, 1986).

This data presents a strong case for the incorporation of microbial assisted mining in the suite of commercial operations available, although, the benefits of conventional practices, when applied to high-grade ores, cannot be disputed. The treatment of

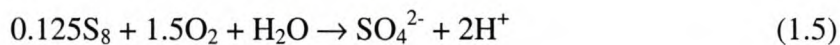
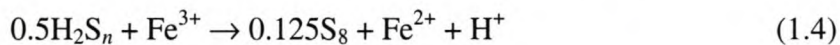
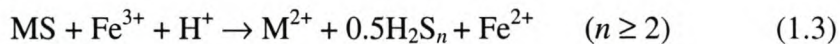
lower grade ores with microbes is both economically and environmentally superior, and will be reviewed here in detail.

1.3 Mechanisms of leaching

The oxidation of metal sulfides (MS) proceeds via different mechanisms depending on whether the mineral is acid-soluble or acid-insoluble. Sand and coworkers (Schippers and Sand, 1999; Schippers *et al*, 1996) have proposed two indirect mechanisms: the thiosulfate- and polysulfide- mechanisms (Figure 1.1). The thiosulfate mechanism is exclusively based on the oxidative attack by ferric iron (Fe^{3+}) on acid-insoluble metal sulfides such as FeS_2 (pyrite), MoS_2 (molybdenite), and WS_2 (tungstenite). Thiosulfate is the main intermediate, and sulfate the main end product. Using pyrite as an example the proposed reactions by Schippers and Sand (1999) are,



In the polysulfide mechanism, dissolution of acid-soluble metal sulfides such as ZnS (sphalerite), CuFeS_2 (chalcopyrite), and PbS (galena), occurs via a combination of ferric iron (Fe^{3+}) and protons (H^+), with elemental sulfur as the main intermediate. Elemental sulfur may subsequently be oxidized to sulfate by sulfur-oxidizing bacteria. For example,



This mechanism explains the ability of *At. thiooxidans* (a purely sulfur-oxidizing bacterium) to leach some metal sulfides, those susceptible to hydrolysis by proton attack, and not others (acid-insoluble).

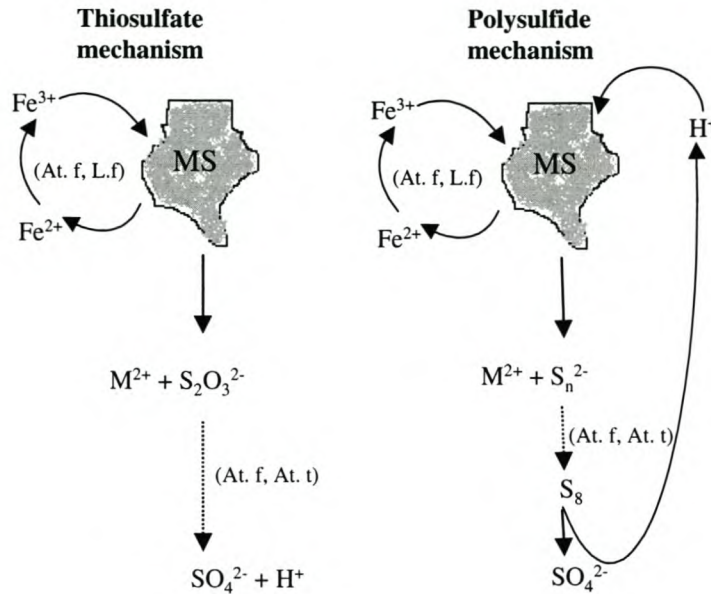
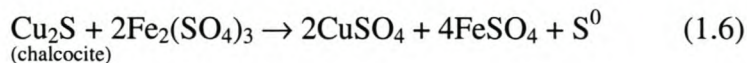
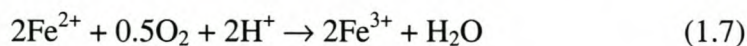


Figure 1.1: Bioleaching via the thiosulfate- and polysulfide- mechanisms. Dashed lines indicate the occurrence of intermediate compounds (Schippers and Sand, 1999).

Indirect mechanisms are based upon the abiotic chemical oxidation of metal sulfides and need not involve microorganisms, since ferric irons and protons may be physically added to a system. For example, ferric sulfate [Fe₂(SO₄)₃] is a strong oxidant capable of dissolving a wide variety of MS minerals in the absence of oxygen or viable bacteria (Hutchins *et al.*, 1986),



Although the reaction rate is fast, the abundance of Fe³⁺ irons is rate limiting. However, numerous authors have reported acceleration in mineral leaching on the addition of iron- and sulfur- oxidizing bacteria (Helle and Onken, 1988; Lacey and Lawson, 1970). Boon *et al.* (1995) proposed a sub-process to this oxidation reaction involving the reoxidation of ferrous iron to ferric iron by iron-oxidizing organisms.



In acid consuming reactions (polysulfide mechanism) the chemical addition of acid to a system can become costly. However, in the presence of sulfur-oxidizing organisms, proton formation is a by-product of reduced sulfur oxidation. Therefore, the role of microorganisms in indirect metal dissolution is to generate sulfuric acid (to ensure a constant supply of protons for hydrolysis attack), and to recycle ferrous to ferric iron (for an oxidative attack) (Schippers and Sand, 1999).

Since the reactions are purely chemical, with no direct contact/attachment of the bacteria to the ore being necessary for mineral dissolution to take place, the leaching mechanisms described here are referred to as non-contact (Figure 1.2) (Rawlings *et al.*, 1999b; Tributsch, 2001; Rawlings, 2002).

Whether the involvement of microorganisms in metal solubilization is solely chemical has been questioned, as not only has attachment of active-leaching bacteria to the mineral surface been shown to occur (Schippers *et al.* 1996), but it is also thought to enhance dissolution. Microbial attachment to sulfide surfaces has been shown to impact oxidative dissolution in the form of local, crystallographically controlled etching of the sulfide surface (Bennet and Tribusch, 1978; Edwards *et al.*, 1998; Edwards *et al.*, 1999); this does not occur in the presence of exclusively planktonic chemolithotrophs (Edwards *et al.*, 1998). Therefore another means of mineral sulfide leaching has been proposed, a direct mechanism, which is thought to be mediated by microorganisms attached to the mineral surface via enzymatic oxidation of the ferrous iron and sulfide moieties of the mineral at the point of contact.

There is considerable doubt on the validity of the direct mechanism of bacterial leaching due to inconsistencies obtained from experimental data. In an experiment with *At. ferrooxidans*, sub-culturing of cells in an iron-free salt solution for the analysis of sulfur metabolism, resulted in the complete loss of substrate degradation (reviewed in Sand *et al.*, 1995). This result does however depend on the mineral type and would occur only if the mineral was itself free of iron. The cells were therefore totally dependent on the presence of iron ions. A closer look at the mechanism of bacterial adhesion to MS (Gehrke *et al.*, 1998) identified extracellular polymeric substances (EPS) of *At. ferrooxidans* and *L. ferrooxidans*, which contained between 0.5% and 5.0% of tightly bound ferric iron. These compounds were not removable by washing procedures, but by centrifugation. Stripped cells needed several hours before

reattachment to pyrite became detectable, whereas untreated cells started to adhere within the first hour (Gehrke *et al.*, 1998). From these results it seems that:

- i) The attachment of bacteria to the MS surface is mediated by extracellular polymeric compounds;
- ii) The exopolymer contains a considerable supply of ferric iron, which seems to be obtained from the surrounding medium, even if the mineral is devoid of iron;
- iii) Iron-deficient cells are unable to degrade mineral sulfides due to the lack of iron compounds in the exopolymeric layer, preventing attachment to the mineral particles.

Work done by Blake *et al.* (1994), supported this theory by demonstrating that metal ions (Fe^{3+}) were needed to overcome the repellent effect that exists between the negatively charged sulfide minerals and the bacterial cells. The presence of metal cations in the exopolymeric layer causes a shift from negative to a slightly positive value, enabling attachment. After exopolymer-mediated attachment, ferric ions (within the EPS) oxidize the mineral sulfide; ferric iron is reduced to ferrous, which is reoxidized to ferric by the iron-oxidizing microbes. The oxidation reactions take place within the exopolymer layer between the cell and MS surface (Sand *et al.*, 1995). Evidence is available to suggest that the microbial mediated reoxidation of ferrous iron can result in a localized increase in pH within the EPS, which aids mineral dissolution (Fowler *et al.*, 1999).

Although contact between bacterial cell and mineral surface may occur, the nature of the dissolution reaction remains chemical, and is not enzymatic. The closest evidence available to suggest a direct interaction between the bacterial membrane and the metal and sulfide moieties in the mineral is from the work of Rojas-Chapana and Tributsch (2001). Unidentified carrier molecules containing reactive thiol groups provided by the amino acid cysteine were found to assist in pyrite oxidation within the EPS. Although cysteine on its own was shown to rapidly oxidize pyrite in the absence of bacteria, it is likely that cysteine found within the bacterial membrane could perform the same function. The free-SH groups from pyrite react with the sulfhydryl group of cysteine, resulting in cysteine being consumed by pyrite and an iron-sulfur species

being released. Tributsch and colleagues (2001) have presented strong evidence that close proximity between the cell and mineral surface is required for efficient oxidation of certain metals (pyrite). However, since direct contact between the cell surface (membrane) and the mineral does not seem essential, in the absence of firm evidence of direct mineral-cell membrane contact, the term “contact leaching” is preferred to that of direct leaching (Rawlings, 2002).

During contact leaching the EPS layers of bacteria feeding at the metal interface become loaded with colloidal sulfur, sulfur intermediates, and mineral fragments, much of which is released into the environment (Rojas-Chapana *et al.*, 1998). Released sulfur colloids, globules and particles feed other unattached/planktonic iron- and sulfur- oxidizing bacteria in a co-operative leaching interaction. Figure 1.2 illustrates how the three proposed mechanisms of leaching (contact, non-contact, and co-operative) work together in the biooxidation of pyrite.

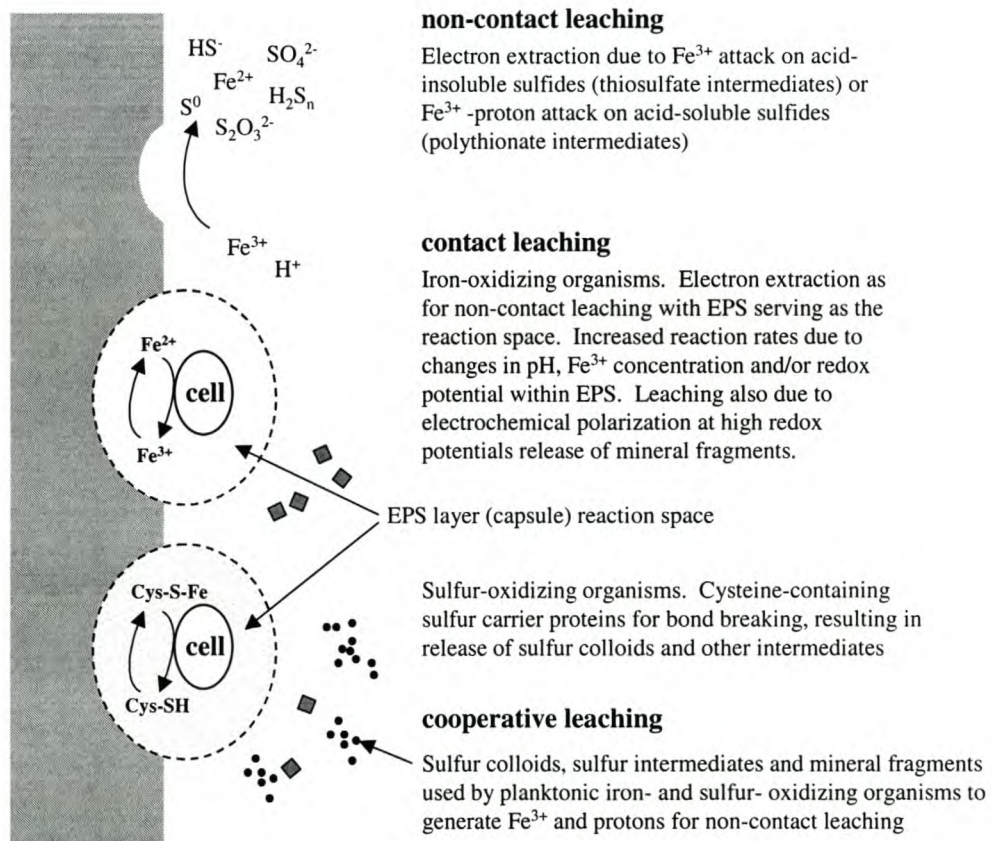


Figure 1.2: Schematic diagram illustrating the proposed mechanisms of pyrite biooxidation (Rawlings, 2002).

1.4 Microbial bioleaching/biooxidation

The use of microorganisms in the extraction of metals from sulfide ores may be referred to as either bioleaching or biooxidation depending on the phase of the final metal product. Both processes use the same consortia of bacteria, the mesophilic *At. ferrooxidans*, *Acidithiobacillus thiooxidans* (Kelly and Harrison, 1989), and *Leptospirillum ferrooxidans* (Markosyan, 1972), the moderately thermophilic *Acidithiobacillus caldus* (Hallberg and Lindström, 1994), *Sulfobacillus* species, and *Acidimicrobium ferrooxidans*, as well as the thermophilic *Acidianus* and *Sulfolobus* (Brierley, 1997). It is important to mention at this stage that although there is evidence that '*L. ferrooxidans*' comprises at least two groups, no attempt had been made to distinguish between the two and they are collectively referred to as '*L. ferrooxidans*'. Further mention regarding the genus *Leptospirillum* will be made later.

In bioleaching the bacteria leach metals such as copper, zinc, uranium, nickel and cobalt from their respective sulfide minerals; catalyzing the oxidation of an insoluble inorganic substrate to a soluble form (Sand *et al.*, 1995; Lundgren *et al.*, 1986). The metal of interest is placed in the solution phase, and handled for maximum metal recovery, whilst the solid residue is discarded. Biooxidation refers to the pretreatment of mineral sulfides, such as pyrite or arsenopyrite, which host/occlude the metal of interest (gold or silver). Sulfide oxidation breaks down the mineral matrix and exposes the entrapped metal (which remains in the solid phase), allowing greater access to the metal via metal solubilizing chemicals such as cyanide.

Biooxidation and bioleaching are collectively referred to as "biomining"; there are two main types of commercial biomining processes. The first type involves the percolation of leaching solutions through crushed ore or concentrates that have been stacked in columns, heaps, or dumps (Brierley, 1982). The second type makes use of continuously operating, highly aerated, stirred tank reactors (reviewed in Rawlings and Silver, 1995; Brierley, 1997). A large variety of metal containing ores (zinc, lead, cobalt, nickel, bismuth, uranium and antimony), may be obtained through bacterially-assisted oxidation of insoluble metal sulfides, however copper and gold-bearing arsenopyrite ores are currently leached in the greatest tonnage (Rawlings and Silver, 1995).

1.4.1 Dump, heap, and *in situ* leaching

In dump leaching, very large quantities of untreated, uncrushed, low-grade oxide and sulfide minerals normally from open-pit mining operations, which cannot be economically processed by any other means, are piled to depths of up to 350 meters (Brierley, 1997). The leach dumps are usually located in valleys to use natural slopes for stability and recovery of solutions (Figure 1.3) (Brierley, 1978). Leach solutions/raffinate (iron- and sulfate- rich recycled wastewater) is/are introduced by spraying, flooding, or injecting through vertical pipes (Lundgren and Silver, 1980), and are not inoculated with leaching bacteria. The microorganisms are ubiquitous, and when the correct conditions prevail, they proliferate. The fluid percolates through the dump where metal-solubilization takes place. The “pregnant” or metal-laden solution is collected for concentration/conditioning. The metal-free solution is then recycled to the top of the dump.

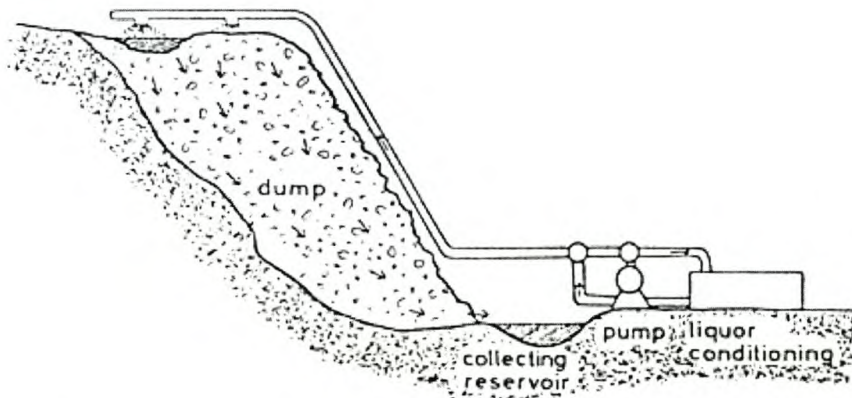


Figure 1.3: Illustration of dump leaching. (www.spaceship-earth.de/REM/Bergbau.html)

As a result of the construction methods used, dump leaching is a crude operation and from a biological viewpoint, remains an essentially uncontrolled process. Copper dump leaching was first initiated in the late 1960's (Brierley, 1978; Brierley, 1982) and remains an economically viable process for copper recovery; in fact the technology employed in dump leaching birthed the heap leaching technology commercially applied today (Brierley, 1997).

Heap leaching is more efficient than dump leaching, and is used to extract metals from sulfide and oxide minerals of a higher grade than those subjected to dump leaching. It also occurs on a smaller scale (2-10 meters in height), using finer, crushed, pretreated (agglomerated) ore, deposited in mounds on pads lined with high-density polyethylene to avoid solution loss, and on which aeration piping may be placed (Lundgren and Silver, 1980). The ore is acidified with sulfuric acid and the heaps are irrigated with solutions containing inorganic nutrients such as $(\text{NH}_4)_2\text{SO}_4$ and KH_2PO_4 . Since bioleaching bacteria are ubiquitous, inoculation is unnecessary, however the introduction of a bacterial inoculum could accelerate the leaching process (Morin, 1997). After percolation, the metal-rich solutions are collected and treated before recycling. Heap leaching is applied commercially to pre-treat low-grade refractory-sulfidic gold ores, and also to leach copper from chalcocite ores (Brierley, 1997).

In situ bioleaching is a promising technique for the recovery of metals from low-grade ores in inaccessible sites. It has been used for nearly 30 years to extract uranium and copper from not only new mines, but depleted underground operations in which up to 30% of the metal values still remain in the mine walls and pillars (Lundgren and Silver, 1980). The underground workings are blasted to fragment the ore and establish permeability. Mine shafts may be left intact and aid aeration and recovery of metal rich solutions (Sanmugasunderam, 1986). Haulage is not necessary, the ore remains in place surrounded by mostly impermeable rock (Sand *et al.*, 1993) where it is irrigated by acidified mine water seeded with leaching bacteria. Metal-rich solutions are pumped to the surface for metal recovery. Figure 1.4 is an example of an *in situ* mining operation modeled on an experimental site in Romania (Sand *et al.*, 1993).

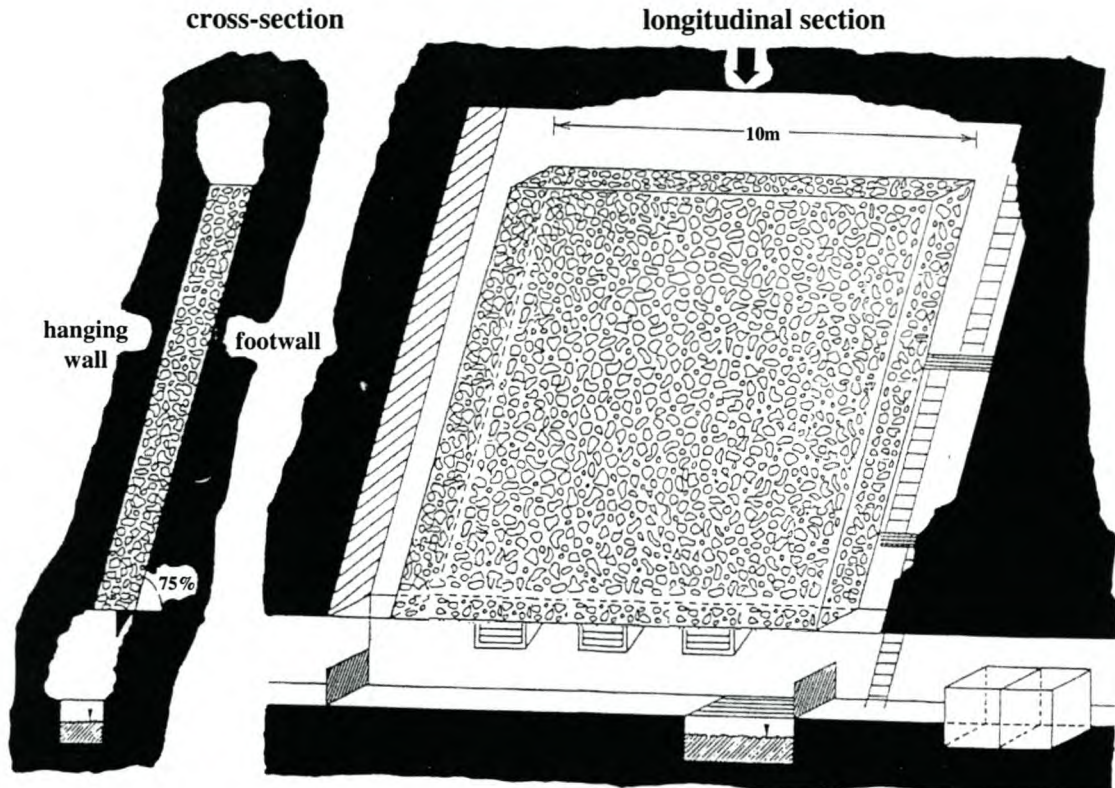


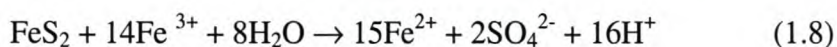
Figure 1.4: Diagram of the experimental ore body site of the *in situ* stope leaching at Ilba Mine (Romania). Ladders are present on both sides of the ore for access to the top tunnel. Basins for the collection of the leach liquor are blasted into the rock (Sand *et al.*, 1993).

1.4.1.1 Bioleaching of copper ores

Copper leaching consists of the conversion of insoluble copper sulfides to soluble CuSO_4 after oxidation, enhanced by acidophilic autotrophic bacteria (Rossi, 1990; Espejo and Romero, 1997). Studies on the microbes present reveal a number of bacterial species including *At. ferrooxidans*, *At. thiooxidans*, "*Leptospirillum*" species and heterotrophs belonging to the genus *Acidiphilium* (Goebel and Stackebrandt, 1994; Harrison, 1984; Pizarro *et al.*, 1996; Hutchins *et al.*, 1986). Bioleaching of copper ores is extensively practiced, and has been estimated to account for $\sim 15\%$ (1×10^6 tons of Cu) of the annual world production (Ehrlich and Brierley, 1990). The methods routinely employed to extract copper from copper-bearing ores are dump-, heap-, and *in situ*-leaching. Although use of stirred tank reactors is feasible, tank processes have not yet been applied to copper commercially.

Over 350 copper minerals exist; the most commonly investigated are chalcopyrite (CuFeS_2), chalcocite (Cu_2S), and covellite (CuS) (Lundgren and Silver, 1980; reviewed in Rawlings and Silver, 1995). Chalcopyrite is the major primary sulfide and is considered economically important, but bioleaching is nonviable due to the long leach times at ambient temperatures. It has been reported that some chalcopyrite waste dump operations, irrigated over a 4-6 year period, recovered a mere 15% of the available copper (Schnell, 1997). More recently, attempts are being made to bioleach chalcopyrite at temperatures of 65°C or greater. Because of the difficulties in leaching chalcopyrite, the application of microbial assisted metal recovery to secondary copper sulfides (chalcocite and covellite) has been more extensively explored.

Copper is most often found in close association with the sulfide mineral pyrite (FeS_2), and it is the oxidation of pyrite which drives the changes in mineral composition within the heap and dump operations. The overall reaction takes place at $\text{pH} < 4$ in heaps and dumps, releasing large quantities of heat energy (Schnell, 1997), and involves ferric leaching of the mineral sulfide to form ferrous iron and sulfate in solution.



Ferrous iron is reoxidized to ferric iron in the bacterial catalyzed reaction (Boon *et al.*, 1995).



Chemolithotrophic bacteria are involved in the dissolution of both primary and secondary copper sulfide ores via the biological generation of sulfuric acid to supply protons for hydrolysis attack and/or the maintenance of iron ions in an oxidized state (Fe^{3+}) for an oxidative attack (Schippers and Sand, 1999). The mechanisms differ depending on whether the ores are acid soluble (chalcopyrite and chalcocite), or acid insoluble (covellite). Acid insoluble MS are degradable only by an oxidizing attack (Fe^{3+}) (reaction 1.16), whereas acid soluble MS may be dissolved by Fe^{3+} ions and protons (chalcopyrite), or protons alone (chalcocite) (Schippers and Sand, 1999). In the case where both Fe^{3+} and protons are involved, the dissolution of the MS is started

by a proton attack (reaction 1.10), and followed by a consecutive Fe^{3+} mediated oxidation reaction (reaction 1.11).

Acid soluble copper sulfide ores

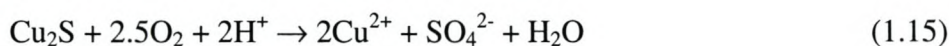
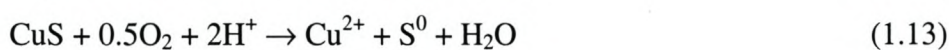
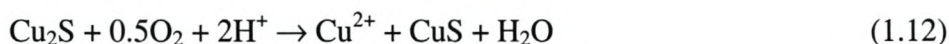
Primary:

Chalcopyrite (CuFeS_2)



Secondary:

Chalcocite (Cu_2S)



Acid insoluble copper sulfide ores

Secondary:

Covellite (CuS)

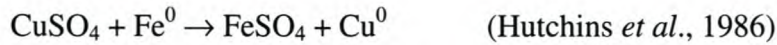


Reactions 1.14, 1.16, and 1.18 are all bacterially catalyzed, involving iron- and sulfur-oxidizing bacteria. Although not shown, reactions 1.10 and 1.11 also include bacterial catalyzed reactions as indicated by reactions 1.14 and 1.16.

1.4.1.1.1 Copper recovery

Solubilized copper may be recovered from the metal-rich leach liquor by cementation or solvent extraction and electrowinning.

Cementation involves the precipitation of copper from solution when the copper-acid solution is allowed to make contact with iron scraps/fillings at a 3Fe: 1Cu ratio. The following reaction occurs:



The “cement copper” is recovered and refined, while the “barren-solution” is recycled to leaching.

Solvent extraction (SX) and **electrowinning (EW)** take place in a closed triple-loop-circuit (Figure 1.5), which in three steps extracts, strips and purifies the copper from the initial leach-solution. The initial leach solution, as it is harvested from a heap or dump, often contains low copper concentrations. SX is a means of sufficiently concentrating the copper in solution to warrant the expense of recovery by electrowinning. EW occurs by the electrical reduction of copper ions to copper metal, which is deposited onto suitable electrodes. The process takes place in acid resistant cells through which electrolyte is circulated. The cells contain a lead alloy anode and either a stainless steel cathode or a copper starter sheet. Copper is harvested from the cathode after a 7-day deposition cycle (Schnell, 1997), and is sold for the manufacture of products, without the need for additional refining.

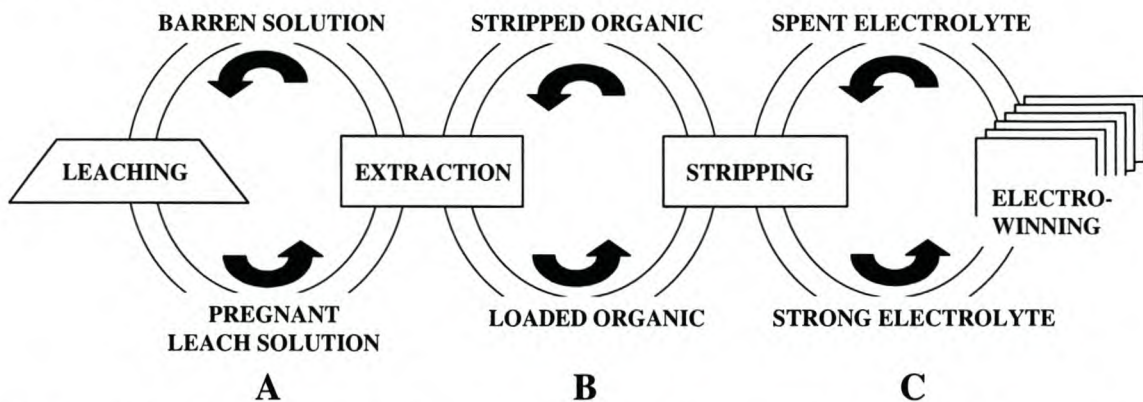
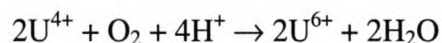


Figure 1.5: Solvent extraction via a closed triple-loop-circuit. (A) Pregnant leach solution is introduced to an organic mixture where copper is extracted to the organic solution. The barren solution is returned for further leaching. (B) Copper laden organic solution is brought into contact with a highly acid spent electrolyte, stripping the copper from the organic solution. Barren organic solution exits stripping cell and re-enters extraction circuit. (C) The strong electrolyte is sent to the electrowinning plant. High purity copper is deposited by electrolysis and the electrolyte is returned to the SX stripping cell (Schnell, 1997).

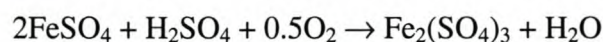
1.4.1.2 Bioleaching of uranium ores

Microbial leaching of uranium has been used since the middle 1960's (reviewed in Hutchins *et al.*, 1986). In 1988 approximately 300 tons of uranium was recovered from the Dennison mine in the Elliot Lake district of Canada, with a value of over \$25 million (US) (M^cCready, 1988). Uranium occurs in the tetravalent (U⁴⁺) and hexavalent (U⁶⁺) states in the natural environment; U⁴⁺ is insoluble, and when oxidized to U⁶⁺, becomes soluble (Brierley, 1978; Lovley, 1993). *At. ferrooxidans* was shown to directly associate with the uranium ore during oxidation (DiSpirito and Touvinen, 1982), in a reaction that requires oxygen. The association between cell and mineral may simply be close contact, and does not necessarily imply a direct-enzymatically induced leaching mechanism.

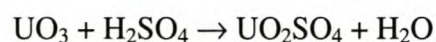
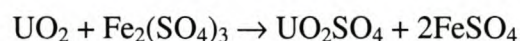


This does not however explain uranium leaching in oxygen-depleted environments (*in situ*), where most commercial uranium leaching takes place (reviewed by Rawlings and Silver, 1995). Heap leaching from low-grade uranium ores has however also been reported in Russia (reviewed in Brierley, 1978).

In situ uranium leaching may be explained in a two-part process. Firstly, the biological production of ferric iron- and sulfuric acid- containing solutions (lixiviants), which require oxygen and is, therefore carried out on the surface.



Secondly the chemical solubilization of uranium by the lixiviant, which does not require oxygen and is carried out underground.



The ability to separate the biological- (leachate preparation) and chemical- oxidation reactions (actual leaching reactions) is ideal for *in situ* leaching. Therefore, once the uranium ore has been prepared (worked out stopes, or an unworked ore body, fragmented via explosives), the leaching solution is applied through periodic spraying or flooding. Uranium bearing solution drains to the lower portions of the mine and accumulates in sumps. The solution is then pumped to the surface for uranium recovery. It was estimated that the operation at the Denison mine made an additional 4×10^6 tons of ore available that would have been unavailable to conventional technologies. Due to a reduction in the world demand, uranium is however no longer commercially bioleached in Canada. The Denison mine, which was the largest mine that recovered uranium via bioleaching, ceased operating in 1993.

1.4.1.3 Examples of current commercial scale irrigation-type processes

A number of irrigation-type metal recovery processes are in operation, and have been for many years. Although mainly applied to the bioleaching of copper-containing ores, heap reactors have also been used for treating refractory gold-bearing ores. The Newmont Gold Company built a plant to demonstrate gold-ore pretreatment technology in Carlin, Nevada. In this process the heap is flooded with an acidic-ferric iron solution containing bacteria, and then with recycled heap reactor fluid (Brierley, 1997). The process allows low-grade ore with as little as 1 g of gold per ton of ore to be processed, the cost of gold extraction being in the range of U.S. \$ 4-6 per ton of processed ore. An example of a current, successful copper dump leaching operation is the Baja Ley Plant at the Chuquicamata Division of Codelco, Chile. The plant started in 1993 and was designed to produce 15 000 tons of copper from a 0.35% copper run-of-mine finger (ore bodies) operation. The plant produces copper at less than U.S. \$ 0.18/kg, with an overall estimated recovery of 20% (reviewed by Schnell, 1997). The best-known copper dump leaching operation is located at the Kennecott Copper mine in Bingham Canyon, Utah (Brierley, 1978 and 1982), with some of the dumps at this site consisting of up to 4 billion tons of low-grade copper ore waste. The Quebrada Blanca plant in northern Chile, which produces 75 000 tons Cu/ annum from chalcocite ore containing 1.3% copper, is an example of a modern heap bioleaching operation (Schnell, 1997). The plant started in 1994, and today produces a high quality Grade A copper at approximately U.S. \$ 0.27/kg.

Several *in situ* metal extraction processes are in operation. The San Manuel operation of BHP Copper is one example (Schnell, 1997). San Manuel is located in the southern western United States about 60 km northeast of Tucson, Arizona. The operation came into being as an after-thought due to difficulty in mining the remaining ore from an open pit operation. Initial *in situ* mining began in 1988 where an array of injection wells were used to introduce acidified leaching solution into the mineral deposit. The leach solution was gravity driven through the ore, and collected in the abandoned underground workings where it was pumped to the surface. Although fluid loss at San Manuel is about 13.5%, production of 20 000 tons of copper cathodes per year was maintained. Gunpowder's Mammoth mine in Queensland, Australia is another example of an irrigation-type *in situ* mining operation (Brierley, 1997).

1.4.2 Stirred tank biooxidation

The use of aerated stirred tank bioreactors is generally reserved for the leaching/oxidation of high-grade ores due to high capital and operating costs. Although all of the commissioned commercial aerated, stirred tank reactor plants are technically biooxidation facilities, as they operate with refractory-sulfidic gold flotation concentrates as feed stocks; stirred-tank reactors can also be applied to bioleach base metal concentrates (Brierley, 1997). The system comprises a number of reaction vessels, primary reactors (typically two or three), operating in parallel, and secondary reactors operating in series (Figure 1.6) (Lindström *et al*, 1992; van Aswegen *et al*, 1991). This arrangement achieves a longer retention time for the ore (feed) in the primary reactors, allowing a stable bacterial population to be established in order to prevent washout. The feed comprises a mineral concentrate suspended in water, to which small amounts of nutrients $[(\text{NH}_4)_2\text{SO}_4$ and $\text{KH}_2\text{PO}_4]$ have been added. Between 50% and 70% of the sulfide sulfur is oxidized in the primary reactors. Overall residence time for biooxidation typically varies between four and six days.

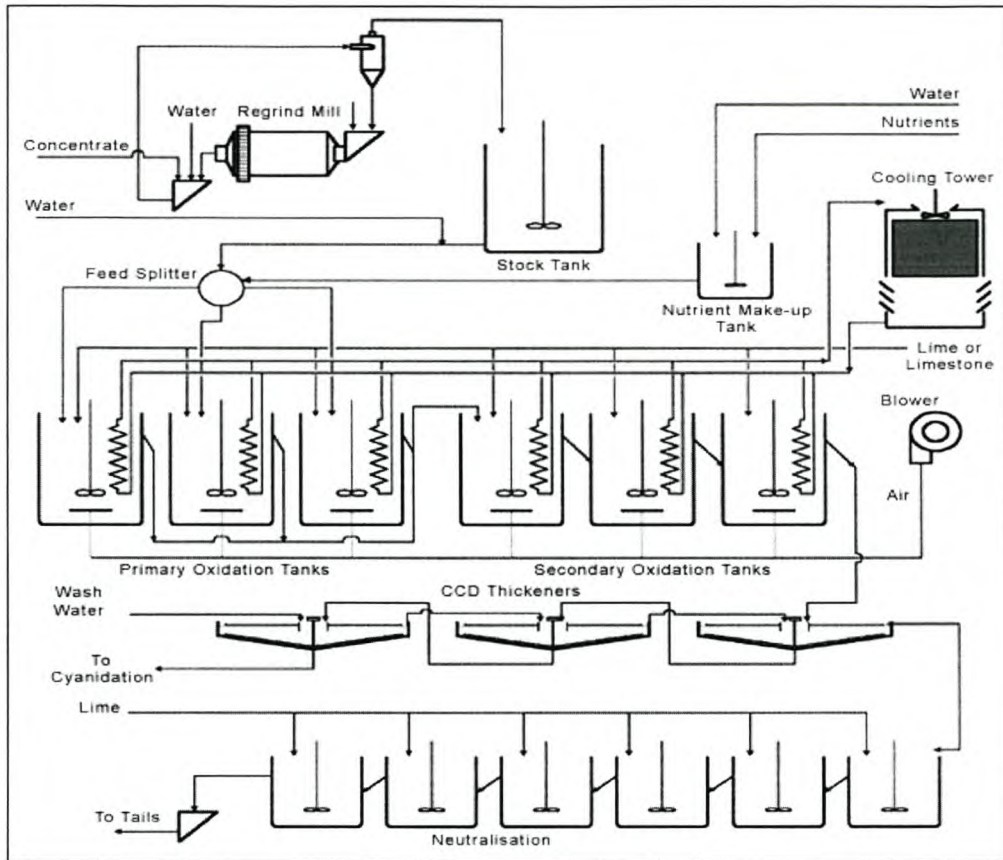
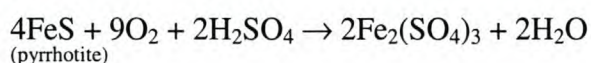
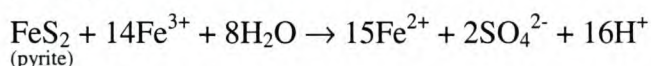
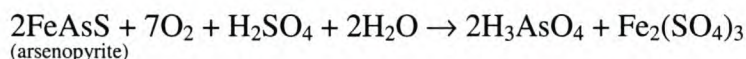


Figure 1.6: A typical biooxidation process flowsheet (www.goldfields.co.za).

Owing to the exothermic nature of the oxidation of mineral sulfides, the tanks are equipped with cooling coils/water jackets, to maintain an optimum temperature to facilitate microbial activity. Temperatures of 40°C and 50°C are maintained in the commercial Biox[®] (Billiton S.A. Ltd) and BacTech (Australia) processes respectively. Cooling water is circulated through stainless steel coils and the heat is removed in an evaporative cooling tower. Oxygen is required as a terminal electron acceptor for sulfide compound oxidation, and to support bacterial growth. Agitation and aeration are produced by means of air-injection at the base of the reactors and mechanical agitators/impellers provide acceptable oxygen transfer and utilization rates. The impellers also serve to maintain the solids/ore in suspension, for maximum bacterial exposure. Mineral composition determines whether the process is acid generating or acid consuming; pyrite oxidation produces sulfuric acid, whereas arsenopyrite and pyrrhotite oxidation consumes acid. A constant pH is maintained between 1.2-1.8 by the addition of concentrated sulfuric acid, lime, or limestone. Depending on the metal, different recovery procedures are carried out.

1.4.2.1 Biooxidation of gold-bearing arsenopyrite ores

The principle sulfide minerals associated with refractory gold ores are arsenopyrite (FeAsS), pyrite (FeS₂), and pyrrhotite (FeS). Microorganisms are able to degrade the surrounding sulfide matrix to achieve gold liberation via oxidation reactions, which may be summarized as follows:



These reactions require large quantities of oxygen, careful pH management, and are highly exothermic. Gold-bearing concentrates are valuable substrates compared with copper and uranium ores; therefore leaching is carried out in a more efficient and controlled manner, via vat or stirred tank operations (refer to Figure 1.6). Within these tanks, temperature, pH and oxygen supply are carefully monitored to satisfy the requirements of the bacterial cultures. Typical operating conditions include temperatures of 35°C-50°C, pH 1.2-2.0 and approximately 2.2 kilograms (kg) oxygen per kg of sulfide oxidized. Failure to meet these requirements results in reduction of sulfide oxidation and subsequent decrease in gold recovery. For example a high pH influences sulfide oxidation through metal salt precipitation, resulting in the occlusion of gold particles. Finally, the biooxidized ore is washed and neutralized (pH 7.0-8.0) with lime or limestone in a two-stage neutralization plant to produce a stable solid precipitate of iron and arsenic, which is deposited on a tailings dam. The biooxidation product is washed in a counter-current circuit, removing excess iron that could inhibit gold recovery. The circuit is designed to give an iron concentration of less than 1g/l in the underflow from the final thickener, which is then pumped to the cyanidation circuit.

1.4.2.1.1 Gold recovery

Biooxidation of gold-bearing ores is merely a pretreatment of the ore in preparation for gold recovery by cyanidation. For the last 100 years cyanidation has been the main process for extracting gold from ores (Morin, 1997). It involves the oxidation

and dissolution of gold in a cyanide solution at ambient temperatures, forming a highly stable chemical gold-cyanide complex. Gold is then extracted from solution by adsorption on activated carbon, cementation on zinc dust or ion exchange on synthetic resins, and ultimately refined. This process is renowned for its simplicity and economic benefits when treating high-grade, accessible ores. However, the cost of treating more inaccessible ores is high as grinding needs to be applied to make the ore amenable to cyanidation. Even then not all ore responds well to cyanidation, regardless of grinding; these ores are said to be refractory or recalcitrant. Gold ores are refractory when iron sulfide minerals encapsulate the precious metal and hence are unavailable for contact with cyanide (Hutchins *et al.*, 1986).

Prior to the development of biooxidation, refractory ores were simply discarded with the tailings of the milling circuit, or subjected to either roasting or pressure leaching. However, due to the depletion of high-grade accessible ores, and the increasing environmental pollution, for example the release of sulfur dioxide and arsenic into the atmosphere during roasting, the need for new methods of refractory ore processing arose. The use of microorganisms as a pretreatment of recalcitrant ores permits cyanide access to the mineral and improves metal recovery. Gold recovery percentages from less than 50% prior to bacterial ore pretreatment, to greater than 95% after biooxidation have been reported (Hutchins *et al.*, 1986; Rawlings and Silver, 1995); for this reason biooxidation and cyanidation are used in conjunction with one another (Livesey-Goldblatt *et al.*, 1983).

Additional bacterial assistance in the gold-extraction process occurs in the treatment of cyanide waste. Cyanide presents a major source of pollution, in some mines (Homestake Mine in Lead, South Dakota), approximately 21 000 m³ of wastewater containing millimolar amounts of cyanide, is discharged daily (Whitlock and Smith, 1989). At this particular mine however, cyanide waste is passed over rotating biological contactors (plastic disks containing up to 18 tons of bacterial biomass), where it is metabolized to carbon dioxide and urea, and the urea ultimately to nitrate.

1.4.2.2 Commercial stirred tank processes

The first commercial stirred tank operation was commissioned in 1986 at the Fairview mine in Barberton, South Africa. Since then similar plants have been built at Sao

Bento (Brazil), Harbour Lights, Wiluna and Youanmi (Australia), Ashanti, Sansu (Ghana), and Tamboraque (Peru) (Rawlings *et al.*, 2002). The largest plant is at Sansu, Ghana, which consists of 24 tanks of 1 000 000 liters each, processing approximately 1000 tons of gold per day, earning half of Ghana's foreign exchange. The Biox[®] (40°C) process designed by Gold Fields is used at all plants except Youanmi, which uses BacTech (50°C) technology. Stirred tank processes are not exclusively used for the processing of gold-bearing ores, but have been applied to cobalt- (Kasese, Uganda), and nickel- containing ores (pilot scale BioNIC plant, Billiton, S.A.) (Dew, 1997). With the exception of the Kasese plant, all other commercial scale biooxidation tanks work on gold-bearing concentrates. The extension of this technology to the recovery of copper, nickel and zinc is currently at a pilot scale level.

The use of microorganisms in the treatment of mineral ores and mine wastewater is more efficient both economically and environmentally when compared to conventional mining practices. It is predicted that, in the future, "biomining" will compete favorably with or in fact replace the more costly physical processes entirely (Rawlings and Silver, 1995). In order to improve the process of microbial ore oxidation, a more complete understanding of the microbes involved is needed.

1.5 Microbes in Biomining

Microbes, which are capable of oxidizing iron- and/or sulfur- containing minerals, may be readily isolated from sites of natural mineral oxidation. They are ubiquitous and may be divided according to preferential growth temperatures, mesophiles (25-35°C), moderate thermophiles (45-60°C), and extreme thermophiles (60-80°C). Although mineral oxidation can take place at a variety of temperatures, current commercial processes do not operate at average temperatures above 55°C. In heap and dump leaching a proposed average temperature range at which biooxidation occurs is 20-35°C, while the temperature in commercial biooxidation tanks is controlled at either 40°C or 50°C (Miller, 1997; van Aswegen, 1991). Processes that operate at elevated temperatures (75-80°C) are under development. For this reason the most commonly encountered microbes in mineral ore oxidation are the mesophilic *At. ferrooxidans*, *At. thiooxidans*, and '*L. ferrooxidans*', and the moderately

thermophilic *At. caldus* and sulfobacilli (Norris *et al.*, 1996; Golovacheva and Karavaiko, 1979). Associated with these autotrophs are acidophilic, heterotrophic bacteria belonging to the genus *Acidiphilium* (Harrison, 1984); which are not primarily involved in mineral decomposition, but grow in a close commensal relationship with the chemolithotrophic bacteria. Archaea have also been isolated from commercial bioreactors and sites of acid mine drainage (*Ferroplasma acidophilum*, Golyshina *et al.*, 2000; *Ferroplasma acidarmanus*, Edwards *et al.*, 2000; *Sulfolobus*, Norris *et al.*, 2000; *Metallosphaera*, Norris, 1997; and *Acidianus*, Segerer *et al.*, 1986). With the exception of the *Ferroplasma* these archaea oxidize mineral sulfides at elevated temperatures (65-80°C), which are not accommodated by the current commercial processes, therefore these microorganisms will not be addressed in further detail.

1.5.1 Microbial dominance in biomining

For many years *At. ferrooxidans* was considered to be the most important microorganism in biomining processes that operate at 40°C or less (Rawlings and Silver, 1995; Brierley, 1982). This was however the result of an incomplete understanding of the biochemistry of biomining organisms as well as the limited detection ability of the available techniques. Direct molecular analysis of DNA has enhanced the ability to access microbial diversity, and rRNA gene analyses has confirmed the view that conventional identification methods involving culturing, overlooks many of the bacteria originally present in the system. Microbial diversity in commercial bioleaching and biooxidation operations has been re-examined over the last 10 years with the finding that *At. ferrooxidans* does not in fact play the dominant role in some processes, which are dominated by *At. caldus* and '*Leptospirillum*' (Rawlings *et al.*, 1999a; Espejo and Romero, 1997; Vásquez and Espejo, 1997; Pizarro *et al.*, 1996; Goebel and Stackebrandt, 1994; and Rawlings, 1995).

1.5.1.1 Bacterial dominance in irrigation-type processes

In 1995 Goebel and Stackebrandt conducted a study analyzing the microbial biodiversity in an acidic water sample collected from a shallow pond fed by the runoff water from a chalcocite overburden heap. Primers were designed by comparison of the 16S rRNA sequences from isolates and clones previously recovered from acidic

mine water environments (Goebel and Stackebrandt, 1994; Lane *et al.*, 1992) and 16S rRNA sequences available from the Ribosomal Database Project (Larsen *et al.*, 1993), in order to screen genomic DNA recovered from bacterial cells. Of the 120 clones analyzed 70 clustered with the known sequences from "*Leptospirillum*" isolates. Only 37 clones were identified as being related to *At. ferrooxidans*.

Bacterial populations from a commercial scale copper leaching plant were analyzed by PCR of the spacer regions between the 16S and 23S rRNA genes (Espejo and Romero, 1997; Vásquez and Espejo, 1997; Pizarro *et al.*, 1996). Amplification products were compared with those from the main species isolated from bioleaching systems. Although the relative abundance of each amplification product is not directly proportional to the amount of bacteria present, it gives an indication of population shifts during leaching. '*L. ferrooxidans*' and *At. thiooxidans* were found to dominate the process, whereas *At. ferrooxidans* was only detected at high ferrous iron concentrations (> 5 g/l). 16S-23S product profiles at conditions of high acidity (pH 0.7) were also analyzed and were consistent with '*L. ferrooxidans*' and *At. thiooxidans* dominance, no product corresponding to *At. ferrooxidans* was present (Vásquez and Espejo, 1997).

De Wulf-Durand *et al.* (1997) analyzed the diversity of acidophilic, bioleaching-associated bacteria in the silver-catalyzed bioleaching of chalcopyrite at 37°C. Primers derived from the 16S rRNA sequences of six groups of microorganisms (*Acidiphilium cryptum*, '*L. ferrooxidans*', *Sulfobacillus thermosulfidooxidans*, *At. caldus*, *At. ferrooxidans* and *At. thiooxidans*) all involved in commercial bioleaching of mineral ores were used as a selection tool. The primers used for identifying *Leptospirillum* were designed both in this study (LEPTO679R), and in an earlier study by Goebel and Stackebrandt (1995) (LEPTO176F). *At. ferrooxidans* and *At. thiooxidans* were not detected. Although the moderately thermophilic *Sulfobacillus* and *At. caldus* were present along with '*L. ferrooxidans*', after passage of the batch culture through the column and recirculation for a 30-day period; only '*L. ferrooxidans*' could be detected with a single PCR. '*L. ferrooxidans*' therefore seems to be the principal species responsible for iron oxidation in the column environment studied.

1.5.1.2 Bacterial dominance in stirred tank processes

PCR-based technology has also been applied in the detection of bacteria in continuous flow bioreactors. In a study conducted to assess the bioleachability of zinc sulfide ore concentrates, strains identified as *At. ferrooxidans*, *At. thiooxidans*, '*L. ferrooxidans*' and *A. cryptum* on analysis of the 16S rRNA gene sequences, were isolated from both the natural site and a batch bioreactor (Goebel and Stackebrandt, 1994). However, only '*L. ferrooxidans*' and a moderately thermophilic strain of *At. thiooxidans* (now known to be *At. caldus*) could be recovered from a continuous bioreactor running under steady-state conditions (pH 1-2, 35-40°C).

Previously 16S rDNA from isolates of *At. ferrooxidans*, *At. thiooxidans*, *At. caldus* and '*L. ferrooxidans*' were cloned and mapped (Rawlings *et al.*, 1999a; Rawlings, 1995). From a comparison of the restriction maps, restriction sites allowing rapid identification of each species were discovered. Using this technology, bacteria in the biooxidation plant at the Fairview mine (Barberton, South Africa), which operates at 40°C and pH 1.6, were examined. A restriction pattern corresponding to *At. ferrooxidans* was undetectable and the population was reported to be dominated by '*L. ferrooxidans*' and *At. thiooxidans* (Rawlings, 1995). Subsequent studies by this group have shown that the restriction enzyme patterns of *At. thiooxidans* and *At. caldus* are similar, and that the predominant sulfur oxidizing bacterium was almost certainly the more thermotolerant *At. caldus* (Gardner and Rawlings, 2000), originally known as the *At. thiooxidans* physiological group II (Rawlings *et al.*, 1999a).

The bacterial population in commercial biooxidation tanks has also been investigated with the microscopic immunofluorescence technique (Schloter *et al.*, 1995). Results differed to those of the PCR-based techniques, as *At. ferrooxidans* was detected in most samples, albeit that these bacteria were in the minority (reviewed in Rawlings *et al.*, 1999b). Bacterial proportions from the São Bento (Brazil) and Fairview (South Africa) biooxidation plants were 48-57% '*L. ferrooxidans*', 26-34% *At. thiooxidans*, and 10-17% *At. ferrooxidans* (Dew *et al.*, 1997).

It is currently understood that *At. ferrooxidans* is not favored in processes where the concentration of ferric iron greatly exceeds that of ferrous iron (high redox potential),

as is found in continuously operating stirred tank reactors operating under steady-state conditions (Rawlings *et al.*, 1999b). Under these conditions '*L. ferrooxidans*' is found to be the primary iron oxidizer, in pyrite enrichments incubated at 40°C (Norris, 1983), and *At. caldus* the primary sulfur oxidizer at 40°C and above (Goebel and Stackebrandt, 1994). This is not to say that *At. ferrooxidans* cannot be the dominant bacterium in dump/heap leaching operations where the ferrous iron concentration in solution remains high (≥ 5 g/l) (Pizarro *et al.*, 1996). *Leptospirillum* spp. however seem to be widely distributed in highly acidic, metal-rich, natural and industrial environments associated with sulfide mineral oxidation, which include mines (both metal and copper), mine tailings, and the liquid waste (run off) that drains from them. In the study of Edwards *et al.* (1999) the distribution of '*L. ferrooxidans*' in acidic drainage waters at a conventional mine site (Iron Mountain) in California using fluorescent *in situ* hybridization was examined. Included in the probes were LC206 and LF581, specific to '*L. ferrooxidans*', which allowed '*L. ferrooxidans*' to be identified as the dominant iron-oxidizing bacterium present in extremely low pH (0-1) and higher temperature ($> 40^\circ\text{C}$) sites within the mine. Studies by Bond *et al.*, (2000a and 2000b), performed at the same site, but using 16S rRNA analysis, confirmed the abundance of *Leptospirillum* within this environment.

This study focuses on '*Leptospirillum*'. Although each of the primary biomining organisms will be addressed in short, a more extensive account of the understanding to date of the genus *Leptospirillum* will be given.

1.5.2 *Acidithiobacillus ferrooxidans*

At. ferrooxidans is a Gram-negative, acidophilic (pH 1.5-2), obligate autotroph, whose cells comprise short, straight rods approximately 1.0 μm in length and 0.5 μm in diameter. *At. ferrooxidans* was the first organism isolated from an acidic bioleaching environment (Colmer *et al.*, 1950), and is capable of oxidizing both ferrous iron and inorganic sulfur compounds. Although it is preferentially aerobic, oxygen may be replaced by ferric iron as the electron acceptor in the presence of a reduced inorganic sulfur compound (the electron donor) (Pronk, 1991). However, in situations of high redox potential, *At. ferrooxidans* is inhibited by ferric iron ($K_i = 3.1 \text{ mM Fe}^+$) (Norris *et al.*, 1988). A study on a large number of *At. ferrooxidans* isolates was performed

by Harrison (1982), in which at least four different DNA-DNA hybridization similarity groups were convincingly determined. Hybridization percentages (10-50%) were sufficiently low to warrant separation at the species level, Rosselló- Mora and Amann (2001) suggested that genomes having hybridization percentages of less than 50-70% could be considered as belonging to a different species. Although DNA-DNA hybridization percentages seemed convincing for a species level separation, the values for mol% G+C were not as persuasive. Mol% G+C over all isolates was in the range of 56-59%, with one strain (m1) at 65%. Subsequent sequencing of the 16S rRNA of m1 has confirmed that it belongs to a different, as yet unnamed genus (personal communication). Since mol% G+C values that differ by 5% or less generally indicate a single species (Stackebrandt and Goebel, 1994), a species level separation suggested by the hybridization data is not supported.

1.5.3 *Acidithiobacillus thiooxidans*

At. thiooxidans was discovered and named by Waksman and Joffe (1921). It is a Gram-negative, obligately autotrophic, acidophile, and is restricted to using reduced sulfur compounds as an electron donor (Kelly and Harrison, 1989). *At. thiooxidans* is highly acid-tolerant (pH 0.5-5.5) and has an upper growth temperature limit of 35°C. *At. thiooxidans* may also consist of more than one similarity group, with most strains having a mol% G+C of 52-53%, strain DSM612 is an exception at 62% (Harrison, 1984). DNA-DNA similarity between *At. thiooxidans* and *At. ferrooxidans* is approximately $\leq 20\%$ (Harrison, 1982).

1.5.4 *Acidithiobacillus caldus*

For many years *At. caldus* was mistaken for *At. thiooxidans*, however in 1994 Hallberg and Lindström described it as a separate species. *At. caldus* cells are short, motile, Gram-negative rods, capable of chemolithoautotrophic growth on thiosulfate, tetrathionate, sulfide, sulfur and molecular hydrogen (Hallberg and Lindström, 1994). Unlike *At. thiooxidans*, *At. caldus* is also able to grow mixotrophically using yeast extract or glucose. Growth does not occur on ferrous iron. *At. caldus* is a moderately thermophilic acidophile with an optimum growth temperature of 45°C and pH range of 2.0-2.5. A G+C content of 63.9% has been determined for the type strain

DSM8584. No significant DNA-DNA hybridization has been detected with other thiobacilli.

1.5.5 *Sulfobacillus*

Sulfobacilli are Gram-positive, moderately thermophilic, acidophilic, endospore-forming bacteria, with rod shaped cells (Norris, 1996). Three species have been formally described: *Sulfobacillus thermosulfidooxidans* (48-50 mol% G+C, 20-60°C, pH 1.1-5.0) (Golovacheva, 1980), *Sulfobacillus acidophilus* (55-57 mol% G+C, 45-50°C, pH 2) (Norris *et al.*, 1996), and *Sulfobacillus disulfidooxidans* (53±1 mol% G+C, 4-40°C with 35°C optimum, pH 0.5-6.0) (Dufresne *et al.*, 1996). They are capable of oxidizing ferrous iron and reduced sulfur compounds, growing autotrophically and mixotrophically on ferrous iron, on elemental sulfur in the presence of yeast extract, and heterotrophically on yeast extract. Autotrophic growth on sulfur was obtained consistently for *S. acidophilus* alone.

1.5.6 *Acidiphilium*

Bacteria belonging to the genus *Acidiphilium* are acid-tolerant, Gram-negative heterotrophs (Harrison, 1984). They are not primarily involved in mineral decomposition, but grow in a close commensal relationship with the chemolithoautotrophic bacteria (Hallberg and Johnson, 2001). As they are unable to oxidize iron or sulfur, they probably grow on organic carbon excreted by the chemolithotrophs. Removal of organic materials from the leachate, which would otherwise inhibit the iron oxidizers, is a possible means of heterotrophic assistance in sulfide mineral oxidation (Wichlacz and Thompson, 1988). Members of the genus *Acidiphilium* are also able to use ferric iron as an electron acceptor during heterotrophic growth at low oxygen levels. The regeneration of ferrous iron, which serves as an electron donor for the iron oxidizers, is another possible contribution that *Acidiphilium* make to biomining. Strains of *Acidiphilium* have often been found in close, inseparable association with *At. ferrooxidans* (Goebel and Stackebrandt, 1994). In 1995 the genus *Acidiphilium* was reclassified into *Acidiphilium* and *Adidocella* (Kishimoto *et al.*, 1995).

1.6 *Leptospirillum*

The genus *Leptospirillum* was first proposed over 25 years ago but has only recently been validated by Hippe (2000). The information presented here describes only what was known of *Leptospirillum* spp. prior to this study and will be supplemented in Chapters 2 and 4.

1.6.1 Phylogeny

Two species of *Leptospirillum* have been described. The type strain *L. ferrooxidans* (DSM2705), which was first isolated in 1972 from a copper deposit in Armenia (Markosyan, 1972), and *Leptospirillum thermoferrooxidans*, isolated from acidic iron-containing hydrothermal springs (pH ~2, 45°C) on the island of Kunashir (Kuril Islands) (Golovacheva *et al.*, 1993). Only a limited amount of work has been carried out on *L. thermoferrooxidans*, the culture of which has subsequently been lost. A study on relationships among sulfur- and iron- oxidizing eubacteria by Lane *et al.* (1992), discovered that, based on partial 16S rRNA sequence comparisons, *L. ferrooxidans* and two *L. ferrooxidans*-like isolates were closely related to one another (94%), but were not significantly related (80%) to any other bacterium whose 16S rRNA sequence was available at the time (some 350 partial/complete sequences). However, more recent information has placed '*L. ferrooxidans*' within the proposed *Nitrospira* phylum (Ehrlich, 1995). One of the closest bacterial relatives to members of the genus *Leptospirillum* that has been reported so far is the magnetotactic bacterium *Magnetobacterium bavaricum* (Goebel and Stackebrandt, 1995). '*L. ferrooxidans*' is only distantly related to the other important bacteria involved in biomining, the acidithiobacilli (Figure 1.7). Phylogenetically, the genus *Acidithiobacillus* is situated very close to the branch point between the β - and γ -subdivisions of the *Proteobacteria* (Lane *et al.*, 1992; Rawlings, 2001).

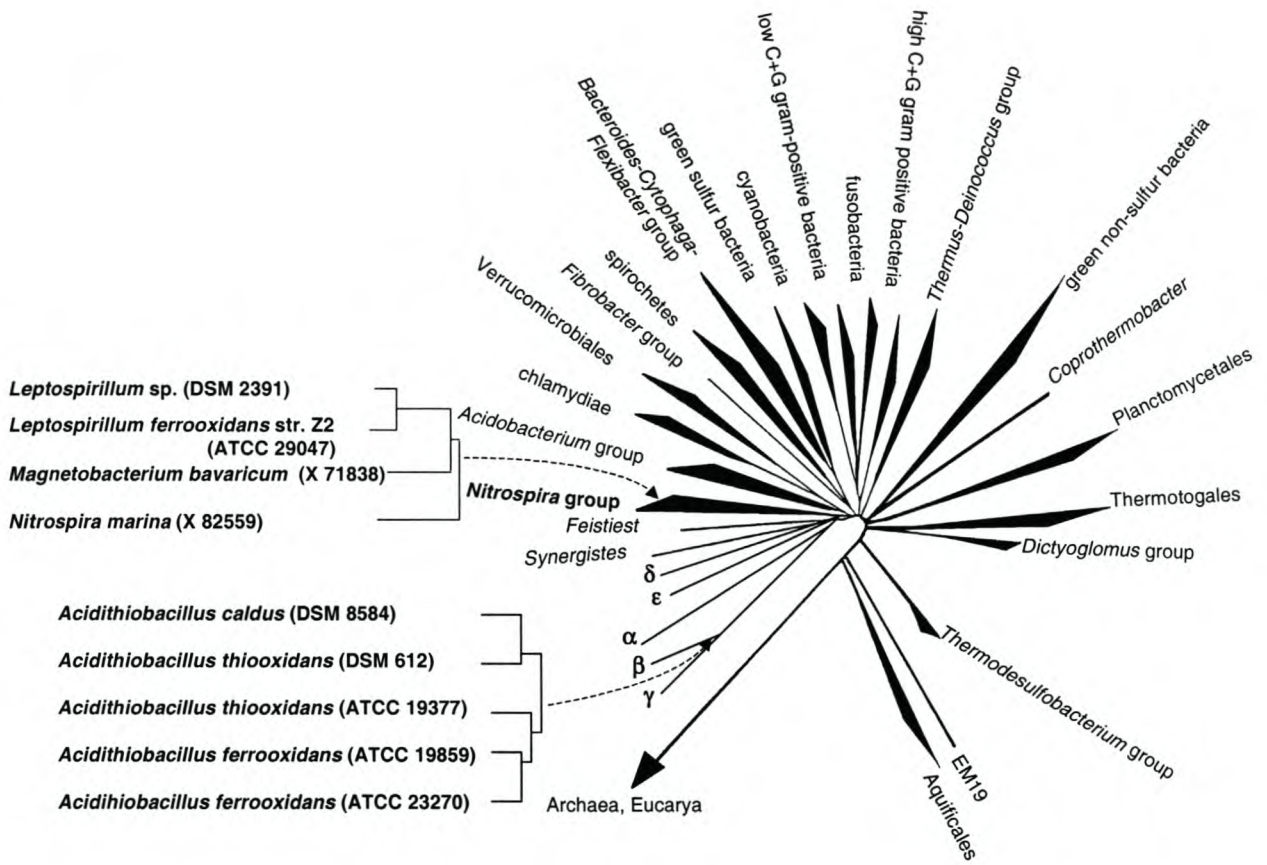


Figure 1.7: Phylogenetic relationship of *Leptospirillum* (with other *Nitrospira* group members) and the acidithiobacilli involved in biominerogenesis to known bacteria based on 16S rRNA sequence data. Greek symbols α , β , γ , δ and ϵ indicate subgroups within the division *Proteobacteria* (modified from Rawlings, 1999c).

There is increasing evidence that '*L. ferrooxidans*' comprises more than one species (Harrison and Norris, 1985; Lane *et al.*, 1992; Hallman *et al.*, 1992; Sand *et al.*, 1992; Battaglia *et al.*, 1994; Bond *et al.*, 2000a). In the study by Harrison and Norris (1985), six isolates comprising *L. ferrooxidans* and *L. ferrooxidans*-like bacteria were examined. Analysis of DNA base composition grouped isolates with relatively low (51 to 52%) and relatively high (55 to 56%) mol% G+C contents. This result was further supported by DNA-DNA hybridization studies, in which two isolates had DNA sequence similarity of 71 to 73% while all other isolates had > 6 to 31% DNA-DNA similarity. Unfortunately, all but the *L. ferrooxidans* type strain (DSM2705) from this early study were lost (P.R. Norris, personal communication). Hallman *et al.* (1992) carried out DNA-DNA hybridization studies with six isolates of leptospirilla.

Two pairs of strains were 100% related to each other, and there was 38 to 50% relatedness between these pairs and 31 to 50% relatedness among all other isolates. Although evidence for the separation into two different species seemed apparent, these studies were not taxonomically complete and were applied to only a small number of members of the genus *Leptospirillum*.

Recently, 16S ribosomal DNA (rDNA) belonging to a third group of leptospirilla was amplified from DNA isolated directly from slime streamers of an acid mine drainage site (Bond *et al.*, 2000a). This third group was found to have low 16S rDNA sequence similarity to the existing sequences (*L. ferrooxidans* and *L. ferrooxidans*-like bacteria), and is thought to represent a new species within the genus *Leptospirillum*. Since bacteria belonging to the third group have not been isolated in pure culture, no further analysis has been conducted.

1.6.2 *Leptospirillum thermoferrooxidans* (Hippe 2000, 503^{VP})

Description is based on the type strain L-88^T, the only strain to be isolated, which has since been lost. *L. thermoferrooxidans* is a Gram-negative, moderately thermophilic, aerobic, chemolithoautotrophic bacterium with an optimum temperature of 45 to 50°C (maximum, 55 to 60°C), pH optima of 1.65 to 1.9 (minimum pH 1.3) for growth and a mol% G+C of 56% (Golovacheva *et al.*, 1993). Utilizes ferrous iron as sole energy source, but not iron-containing sulfide minerals (Johnson, 2002). The cells are polymorphic, displaying vibriion-like (1.5-2.0 × 0.2-0.5 µm), and spiral (2.0-3.0 × 0.2-0.5 µm) forms. Motile by means of a single polar flagellum, 25 nm in diameter. When compared to the mesophilic '*L. ferrooxidans*', *L. thermoferrooxidans* (strain L-88) displayed a greater resistance to Fe²⁺. DNA-DNA relatedness to the type strain of *L. ferrooxidans* is 26.7% (Golovacheva *et al.*, 1993). The phylogenetic position of this moderate thermophile remains unknown since it was lost prior to being deposited in any culture collection.

1.6.3 *Leptospirillum ferrooxidans* (Hippe 2000, 503^{VP})

In this description, most of the data obtained was from the *L. ferrooxidans* type strain (L15^T ex Markosyan 1972, also known as Z-2, ATCC29047, DSM2705), and may therefore not apply to subsequently described species.

1.6.3.1 Phenotypic characteristics

Morphology: This organism constitutes small, Gram-negative, vibrioid or spiral-shaped cells (0.9-2.0 μm in length and 0.2-0.5 μm in diameter) (Figure 1.8A and B) (Johnson, 2001). Helical forms comprising 2-5 turns (Figure 1.8C) as well as helices of over 20 turns have been observed depending on the growth conditions (Pivovarova *et al.*, 1981). Under conditions of extremely low pH coccoid and pseudococcoid (highly coiled vibrios) forms have been observed. Cells are motile by means of a single polar flagellum (18-22 nm in diameter) (Figure 1.8A). Polarity of the flagellum has been shown to differ in some isolates, being sub-polar rather than polar, some cells may also have two flagella (Goebel, 1997). Colonies are small (1-2 mm), round, entire, orange to light brown colored, iron-encrusted forms (Johnson, 2001). Variation in colony size is apparent between different strains of *L. ferrooxidans*. The cell wall is similar to that of other Gram-negative bacteria. The cell membrane consists of two electron dense layers (0.6-1.0 and 0.35-0.6 nm thick) with an intervening electron-translucent layer (0.7-0.8 μm). Nuclear structures are visible in the cells center, intracellular membrane structures are less apparent (Pivovarova *et al.*, 1981). Polyribosomes may occur in the cytoplasm, no β -hydroxybutyrate reserves were found. Cells are often submerged in a slime layer (100-450 nm thick), in the absence of which abundant slime rivulets coming from the cells surface may be observed.

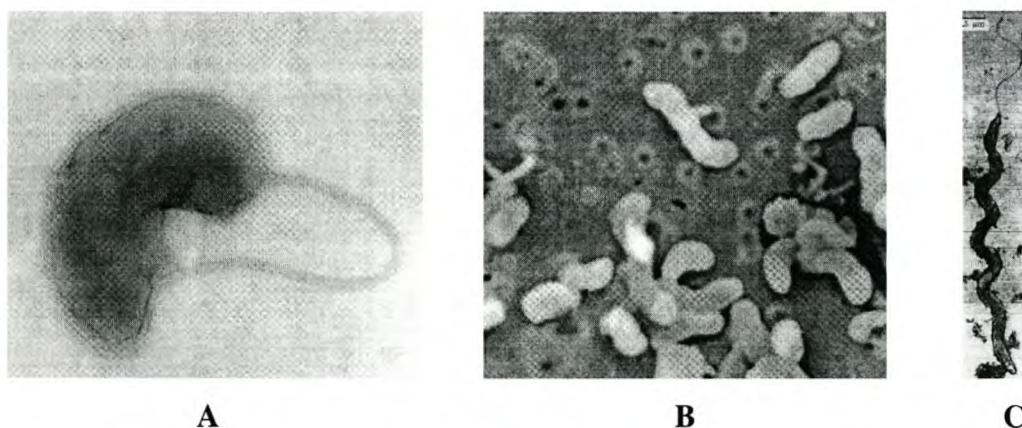


Figure 1.8: Electron micrographs showing the different morphological states of *L. ferrooxidans*. (A) Vibrioid-shaped cell with polar flagellum, (B) Spiral-shaped cells, and (C) helical form with 2-5 turns (Johnson, 2001; Pivovarova *et al.*, 1981).

Physiology and biochemistry: Growth of *L. ferrooxidans* occurs on ferrous iron or iron-containing sulfide minerals such as pyrite (FeS_2) as sole energy source. Possibly as a result of this substrate specificity, they have a high affinity for ferrous iron ($K_m = 0.25 \text{ mM Fe}^{2+}$) relative to *At. ferrooxidans* ($K_m = 1.34 \text{ mM Fe}^{2+}$) (Norris *et al.*, 1988). Optimum leaching efficiency is obtained at lower substrate concentrations than have been reported for *At. ferrooxidans* (Sand *et al.*, 1992); and *L. ferrooxidans* has also been shown to be less inhibited by ferric iron ($K_i = 42 \text{ mM Fe}^{3+}$) than *At. ferrooxidans* ($K_i = 3.1 \text{ mM Fe}^{3+}$) (Rawlings *et al.*, 1999b). Owing to the redox potential (E°) of the $\text{Fe}^{2+}/\text{Fe}^{3+}$ couple being very positive (+ 770 mV at pH 2), leptospirilla are forced to use the $\text{O}_2/\text{H}_2\text{O}$ redox couple (+ 820 mV) as their electron acceptor, and are therefore obligately aerobic organisms (Rawlings, 2002). Mineral sulfide oxidation occurs via the indirect/non-contact mechanism, where ferric iron acts to chemically oxidize the mineral, in turn being reduced to ferrous iron. Ferric iron is kept in abundance by bacterial reoxidation of ferrous iron. Direct attachment to the surface of pyrite has also been reported (Schippers *et al.*, 1996), close proximity may accelerate mineral oxidation, which still occurs via an indirect/chemical interaction, mediated through the exopolymeric slime layer (Sand *et al.*, 1995). The exopolymeric layer is rich in polysaccharides, has a significant ferric iron content, and apparently differs in composition between ferrous sulfate- and pyrite- grown cells (Gehrke *et al.*, 1995).

Leptospirillum spp. are obligate autotrophs, fixing carbon by the Benson-Calvin cycle, active ribulose biphosphate carboxylase has been found in cell-free extracts of *L. ferrooxidans* (Norris *et al.*, 1995). Blake and Shute (1997) characterized a novel acid-stable, acid-soluble, red cytochrome from *L. ferrooxidans*, which is rapidly oxidized by ferrous iron in cell-free extracts, indicating that this cytochrome is a principal component of the iron respiratory chain. *L. ferrooxidans* is highly acidophilic, growing optimally at pH 1.3-2.0, with a lower pH limit of 1.1 (Battaglia *et al.*, 1994). However, Vásquez and Espejo (1997) showed *L. ferrooxidans* to be more acid-tolerant, when reported to be the dominant iron oxidizer in a copper leaching study at pH 0.7. Acidity is the result of ferric iron hydrolysis, and in the presence of sulfur-oxidizing bacteria, the production of sulfuric acid. *L. ferrooxidans* is mesophilic, growing optimally at 30-37°C, it is however tolerant of lower and higher temperatures growing, albeit slowly, within a 10-45°C temperature range (Johnson, 2001). *L. ferrooxidans* grows very poorly on solid media, probably due to the presence of

organic materials within the gelling agent, to which it is highly sensitive. This has however been overcome by the development of a specific bi-layered growth medium (Johnson, 1995). This medium incorporates the use of acidophilic heterotrophic bacteria, present in the lower half of the bi-layered medium, to utilize the organic materials and facilitate *L. ferrooxidans* growth. Growth on solid media is slow, and only visible after incubation for 7-14 days at 30°C. Doubling times in liquid ferrous iron media is strain dependant and varies from 10 to over 20 hours (Norris *et al.*, 1988).

1.6.3.2 Genomic characteristics

As previously mentioned there is mounting evidence suggesting that mesophilic iron-oxidizing bacteria identified, as '*L. ferrooxidans*' comprise more than a single species. Since members of the genus *Leptospirillum* have a limited range of physiological characteristics that can be used in their identification (Johnson, 2001), genomic characteristics are key to discovering suitable differences in order to determine whether more than one species is represented. Genomic characteristics that are necessary for this level of taxonomic classification are rRNA sequence analysis (the 16S rRNA gene in particular), determination of mol% G+C and DNA-DNA hybridization/relatedness between isolates. However, since *L. ferrooxidans* and *L. ferrooxidans*-like isolates have not yet been classified into two different species, results from studies such as that by Harrison and Norris (1985) are represented as a range and assigned to '*L. ferrooxidans*'. Therefore the mol% G+C for '*L. ferrooxidans*' is 51-56% (Harrison and Norris, 1985). DNA-DNA hybridization results with a range from > 6-100%, seem meaningless if used to describe a single species. The 16S rRNA sequence of the *L. ferrooxidans* type strain L15^T has been determined and deposited in GenBank with the accession number X86776.

1.7 Aims of this project

The first aim of this study was to determine the diversity of *Leptospirillum* isolates from different geographical locations using a variety of molecular techniques to establish whether there were sufficient differences to warrant subdivision at a species level (Chapter 2). These studies provide an extended description of a number of characteristics that can be used in the identification of the more commonly encountered leptospirilla. From these findings, we propose that two distinct *Leptospirillum* species are represented among these isolates. These results are in agreement with the current evidence suggesting that the bacteria previously referred to as *L. ferrooxidans*, represent more than one species.

The second aim was to determine which *Leptospirillum* type dominated industrial biooxidation tanks. This would help to identify which species should be the focus of long term molecular biology research.

The third aim of this study was to screen *Leptospirillum* isolates for plasmids (Chapter 3), with the long-term goal of further development of *Leptospirillum* for industrial purposes. Exploration of the molecular biology of *Leptospirillum* is in its infancy, as much of the attention to biomining organisms has been given to *At. ferrooxidans*. The development of improved leaching bacteria is a growing field where biotechnologists are challenged to improve the rate of cell growth and ore oxidation. Improvement of the bacterium may occur by genetic engineering where new genetic material, carrying a favorable characteristic, is gained by the bacteria. New genes are introduced into foreign bacteria by plasmid (shuttle) vectors, which can replicate in genetically well-characterized strains (*E. coli*) as well as the target bacterium (i.e. *At. ferrooxidans* and *L. ferrooxidans*). These vectors also carry selectable genetic markers that are expressed in both organisms. The strategy adopted in the genetic manipulation of *At. ferrooxidans*, was to utilize the well-characterized *E. coli* vectors by cloning into them the required *At. ferrooxidans* origins of replication and selectable markers (Rawlings and Woods, 1995). An important step in this process was isolating plasmids, found in a large number of *At. ferrooxidans* strains, which are an obvious source of replication origins. No plasmids have yet been reported in *Leptospirillum*, but these could similarly serve as sources of replicons and selectable markers for the development of a genetic system for the leptospirilla.

Chapter 2

Molecular relationship between Two Groups of the Genus *Leptospirillum*

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2.0 Summary

The genetic diversity of sixteen *Leptospirillum* isolates from different geographical locations was analysed by means of phylogenetic (16S rRNA, 16S-23S gene spacer region), genomic (DNA-DNA hybridization, mol% G+C), and phenotypic classification. In addition, partial analysis was carried out on three other isolates. Clear separation into two major groups was evident. Group I leptospirilla had mol% G+C within the range 49-52%, three copies of *rrn* genes and based on 16S rRNA sequence data these isolates clustered together with the *Leptospirillum ferrooxidans* type strain (DSM2705 or L15). Group II leptospirilla had mol% G+C of 55-58%, two copies of *rrn* genes and based on 16S rDNA sequence form a separate cluster. Genome DNA-DNA hybridization experiments indicated that two similarity subgroups were present amongst the leptospirilla tested within group I whilst all leptospirilla tested from group II belonged to a single DNA-DNA hybridization group. The two groups could also be distinguished based on the sizes of their 16S-23S rRNA gene spacer regions. We propose that the group II leptospirilla should be recognized as a separate species with the name *Leptospirillum ferriphilum* sp. nov.

2.1 Introduction

The species concept is a controversial issue, and from as early as the 17th century biologists have tried to develop a universally acceptable means of classification. The concept of species interests both microbiologists and eukaryote taxonomists alike, therefore the introduction of a more universal concept that covers all major groups of organisms, making the species unit comparable is indeed tempting (Claridge *et al.*, 1997). At present, at least twenty-two different concepts have been developed to accommodate species, all of which can be grouped (more or less) into three categories, each with different theoretical commitment (Mayden, 1997; Hull, 1997). A theoretical foundation has traditionally been regarded as a valuable characteristic of a species concept, although its significance is disputed between philosopher and scientist. In general scientists see less need to include a theoretical foundation in their concepts; adding to this, the more theoretically significant a concept is, the more difficult it is to apply (Hull, 1997).

The Phenetic or Polythetic Species concept (PhSc): *'a similarity concept based on statistically co-varying characteristics, which are not necessarily universal among the members of the taxa'* (Hull, 1997; Sokal and Crovello, 1970). This concept is considered theory-free, and has been adopted to describe the prokaryotic species in part.

The Evolutionary species concept (ESC): *'an entity composed of organisms which maintains its identity from other such entities through time and over space, and which has its own independent evolutionary fate and historical tendencies'* (Mayden, 1997). This is the most theoretically committed of all the species concepts and is regarded as the only one that can act as a primary concept due to its ability to accommodate all known species types. However, this concept has little significance for the prokaryotes, as due to the limited knowledge (fossil record) available for this group of organisms, no evolutionary fate or historical tendency can be predicted.

The Phylogenetic species concept (PSC): *'the smallest diagnosable monophyletic unit with a parenteral pattern of ancestry and descent'* (Hull, 1997). Regardless of the lack of fossil record, the establishment of genealogical trees through the analysis of gene sequences (e.g.: 16S rRNA) has been made possible among the prokaryotes. However, phylogenetic reconstruction is then based on the analysis of a single gene, which falls short in the representation of the whole organism.

These last two concepts are generally applicable among the different eukaryotic lineages (Ereshefsky, 1992; Templeton, 1989).

The current approach adopted for the speciation of prokaryotes includes both a phenetic and phylogenetic evaluation, combining as many different techniques as possible (polyphasic), and is referred to as the phylo-phenetic species concept.

2.1.1 The phylo-phenetic species concept

This concept combines the strengths of the PhSc and PSC's, adapting the guidelines to suit prokaryotic capabilities. Phenetic classifications include both phenotypic and genomic characters, both of which need to be included in an investigation for taxonomic purposes; the lack of either could result in the rejection of a classification proposal.

2.1.1.1 Phenotypic analysis

The phenotype is the visible or otherwise measurable physical and biochemical characteristics of an organism, resulting from the interaction of genotype and environment. Phenotypic classification is therefore based on morphology, physiology and growth conditions of the organism (biochemistry). The morphology of a bacterium includes cellular (shape, endospore, flagella, inclusion bodies, Gram staining etc.) and colonial characteristics (colour, dimensions, form etc.). Physiological and biochemical features include data on growth at different temperatures, pH values, salt concentrations, atmospheric conditions (e.g. aerobic/anaerobic) etc. These characteristics have been used in Bergey's Manual of Determinative Bacteriology to reorganised taxa and genera divisions into 33 different sections based on single or combinations of phenotypic traits. Since prokaryotes lack complex morphological features, phenotypic analysis involves techniques that test (directly or indirectly) different properties such as enzyme activities, substrate utilization profiles and growth conditions. Although genomic data alone is sufficient to allocate taxa in a phylogenetic tree, the consistency of phenotypic and genomic characters is required to generate useful classification systems (Vandamme, 1996). However, should two organisms be void of distinguishable phenotypic traits, taxonomy is based on phylogenetic and genomic relationships (Lee *et al.*, 1998).

2.1.1.2 Genomic analysis

Genomic information is obtained from the data retrieved from nucleic acids (both DNA and RNA molecules). This may occur directly through sequencing, or indirectly via DNA-DNA similarity percentages or mol% G+C values. As nucleic acids are universally distributed, and their composition is largely independent of environmental changes, these are excellent tools to be used as standards for wide-ranging comparisons. Obviously the most complete source of genomic information is

the entire bacterial genome, however this is not feasible as a quick means of analysis, therefore other approaches have been developed.

2.1.1.2.1 mol% G+C

The primary structure of DNA is universal; in so much as it is comprised of four nucleotide bases (A, T, G, and C). However, the relative ratio $[G+C]/[A+T]$ varies from genome to genome. The G+C content is calculated as a percentage of the sum of the four nucleotide bases, and is usually one of the genomic characteristics recommended for the descriptions of species and genera. G+C contents among prokaryotes are known to vary between 20-80% (Tamaoka, 1994), and differences in DNA molecules greater than 20-30 mol% are almost void of common sequences (Logan, 1994). A difference of 5 mol% is the common/acceptable range found within a species (Rosselló-Mora and Amann, 2001). Although mol% differences are taxonomically useful in group separation, the similarities in base compositions do not necessarily indicate a close relationship, as the linear sequence of the bases in the DNA molecules is not accounted for.

2.1.1.2.2 DNA-DNA relatedness

The use of whole genome DNA-DNA similarity is the standard technique for prokaryotic species delineation (Lee *et al.*, 1998). Data to support using this parameter in setting the borders of speciation has been obtained from numerous studies, where a correlation between genomic DNA- and phenotypic –similarity was found (Stackebrandt and Goebel, 1994). There are several methodologies to measure DNA-DNA relatedness, all of which rely on the same principle (Figure 2.1). Ultimately, the overall pairing of the nucleic acid fragments is dependent upon similar linear arrangement of the nucleotide bases along the DNA.

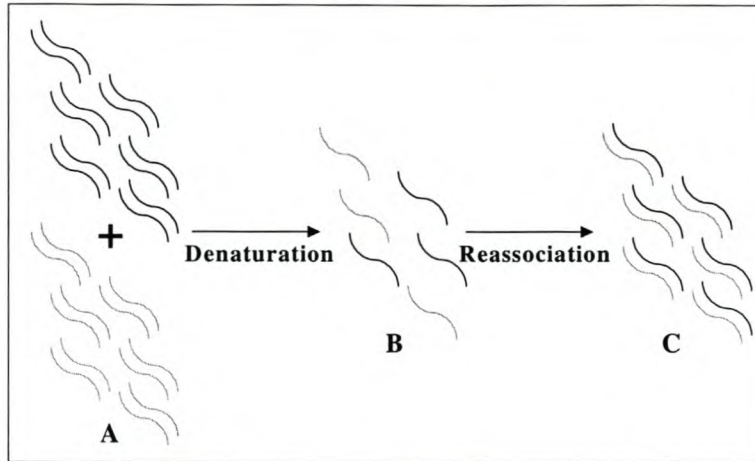


Figure 2.1: The principle of DNA-DNA reassociation. (A) DNA's of two different organisms are mixed and denatured to give a mixture of single-stranded DNA molecules (B). Reassociation occurs under controlled conditions, resulting in the formation of hybrid molecules (C). Comparison of (C) with (A) yields a degree/percentage of similarity.

The degree of relatedness between two DNA molecules is measured by two parameters: (i) the relative binding ratio (RBR), and (ii) the difference in thermal denaturation midpoints (ΔT_m) (Johnson, 1989). RBR represents the relative amount of heterologous DNA duplex formation in comparison to the homoduplex DNA, which is considered to represent 100% reassociation. A number of different techniques are available to determine RBR and can take place both in solution or on a solid surface.

- (1) DNA reassociation may be measured spectrophotometrically by determining the initial rate of reassociation (De Ley *et al.*, 1970), this procedure compares the rates of reassociation of preparations of DNA from organism A (V_A) and from organism B (V_B) with that of an equal mixture of the two DNA preparations (V_M).
- (2) The reassociation of DNA is measured by determining the time and concentrations at which one-half ($C_{0t_{1/2}}$) of the DNA has reassociated (Britten and Kohne, 1968; Seidler and Mandel, 1971).

- (3) By use of a probe, either radioactively or biotin labeled, the reassociation of the probe with excess unlabelled DNA can be followed by resistance to S1 nuclease or by adsorption to hydroxylapatite (Crosa *et al.*, 1973; Johnson *et al.*, 1980). Alternatively, the use of a DIG-labeled probe in conjunction with immobilized DNA on a membrane (nitrocellulose or nylon); DNA reassociation can be determined via densitometry analysis of the final autoradiograph (Sohier *et al.*, 1999).

RBR results are therefore subject to differences that are related to the hybridization technique used and should always include a known standard (i.e. *Escherichia coli*), against which results may be measured (Grimont *et al.*, 1980).

ΔT_m is measured once the heterologous DNA duplexes have formed, and reflects thermal stability of the dsDNA. The melting temperature (T_m), or thermal denaturation midpoint, is the temperature at which 50% of the dsDNA is denatured. Since heteroduplex DNA's would account for a lower number of paired bases than those from the homoduplex (due to the formation of fewer hydrogen bonds), the expected T_m of heteroduplex DNA's would be lower than the homoduplex T_m . DNA-DNA relatedness in this regard, ΔT_m , is the difference between the homoduplex DNA T_m and the heteroduplex DNA T_m , as illustrated in Figure 2.2.

It is currently recommended that values of 70% or higher RBR, and 5°C or lower ΔT_m , are reasonable borders to define a given species (Wayne *et al.*, 1987). DNA reassociation represents the best approach for the deduction of genotypic relationship amongst closely related prokaryotes, followed by the G+C content. In fact, both parameters are necessary for an adequate species classification (Rosselló-Mora and Amann, 2001).

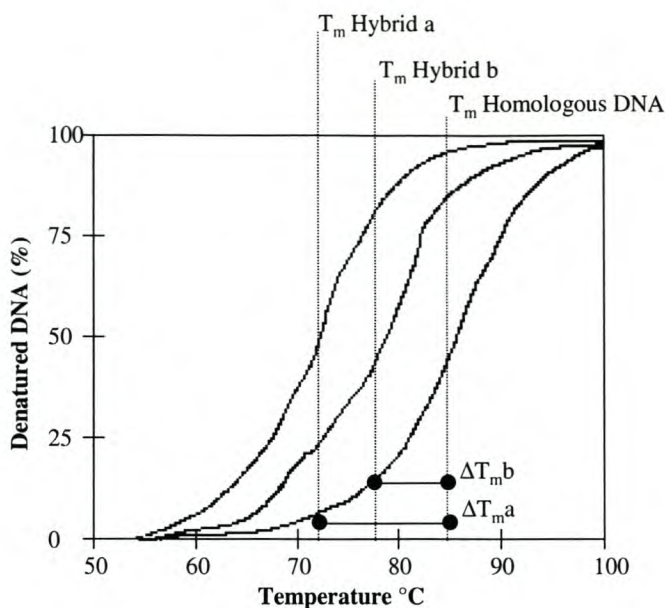


Figure 2.2: Thermal denaturation curves of a homoduplex DNA and two heteroduplex DNA's (Reproduced from Rosselló-Mora and Amann, 2001).

2.1.1.3 Phylogenetic analysis

Ribosomal DNA (rDNA) has been used extensively in determining phylogenetic relationships among microorganisms (Woese, 1987; Woese *et al.*, 1990). rRNA's, especially 16S rRNA, due to their high information content, conservative nature, and universal distribution, allow most relationships (including the most distant) to be measured (Rogall *et al.*, 1990). However, two assumptions are basic for the validity of this approach, namely that horizontal gene transfer has not occurred between rRNA genes, and that the amount of evolution or dissimilarity between rRNA sequences of a given pair of organisms is representative of the variation shown by the corresponding genomes (Goodfellow *et al.*, 1997).

Within a single bacterial genome, there are frequently multiple rRNA loci [seven copies in *E.coli* (Morgan *et al.*, 1977), ten in *Bacillus subtilis* (Loughney *et al.*, 1982), ten in *Clostridium perfringens* (Garnier *et al.*, 1991), one in *Mycobacterium* sp. (Bercovier *et al.*, 1986), and one to two in *Mycoplasma* sp. (Amikam *et al.*, 1984)]. Although rRNA sequences are, in general, believed to show low if any variability within the same genome, heterogeneities between copies of 16S rRNA genes have been reported (Pettersson *et al.*, 1998). Microheterogeneities (nucleotide differences) are probably far more common than macroheterogeneities (insertions ranging from 50 to several hundred nucleotides), and have been reported in the form of nucleotide

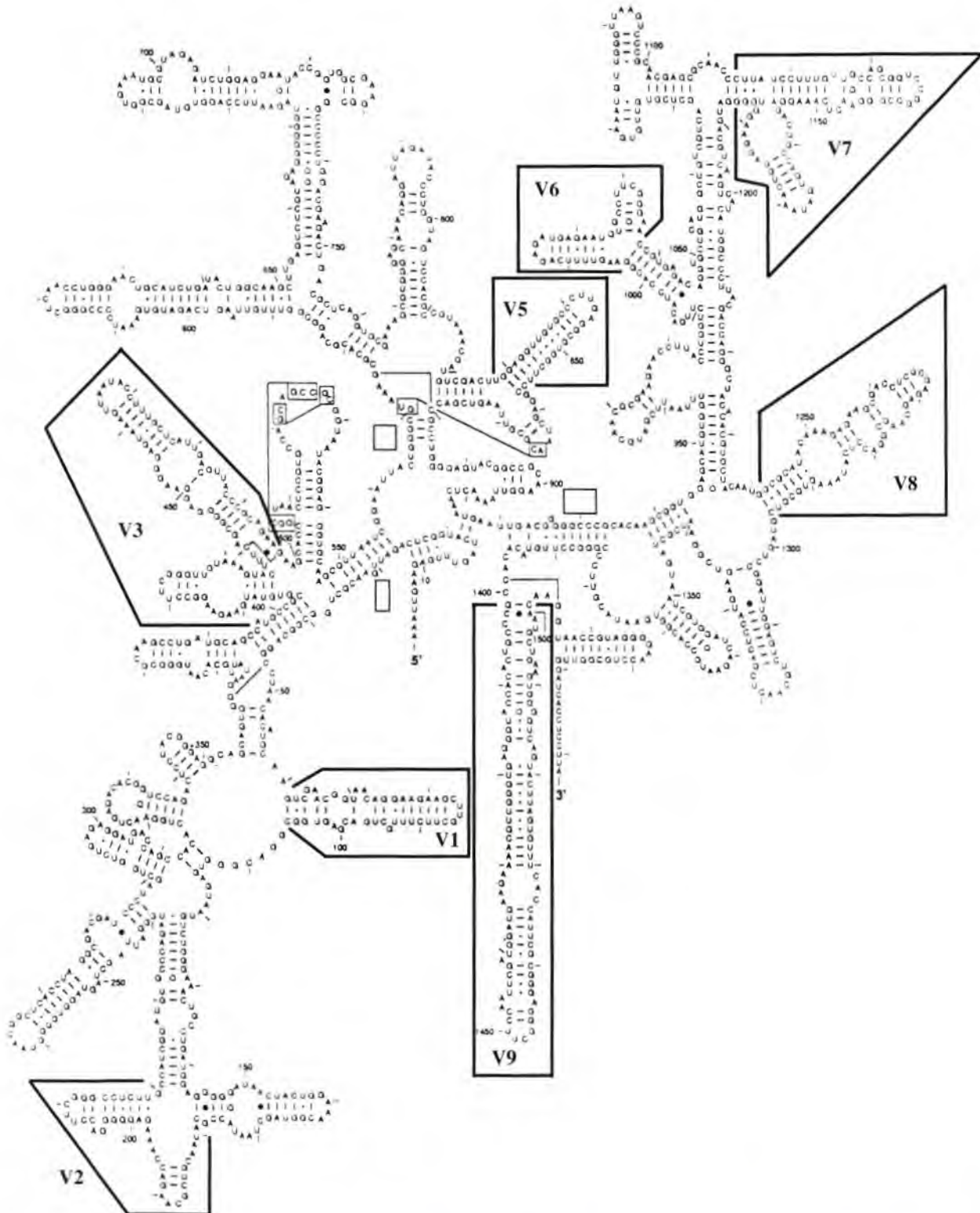


Figure 2.3: Secondary structure model for *E. coli* 16S rRNA displaying hyper-variable regions, numbered V1-V9, respectively, V4 is absent in prokaryotic ssRNAs. Canonical base pairs are connected by lines, G:U pairs are connected by dots, A:G-type pairs are connected by open circles, and other noncanonical pairings are connected by solid circles. Thicker, longer solid lines connect “Tertiary” interactions. Every 10th position is marked with a tick mark, and every 50th is numbered. Primary structure was determined by Brosius *et al.*, 1978 and 1981.

differences between multiple *rrn* operons within a genome (polymorphisms), although such polymorphisms are rare. Examples of species where polymorphisms have been identified include *Haloarcula marismortui* (Mevarech *et al.*, 1989), *Bacillus sporothermodurans* (Pettersson *et al.*, 1996), and *Mycoplasma capricolum* (Pettersson *et al.*, 1998). The occurrence of variability in 16S rRNA genes between species and subspecies has also been observed, at the micro- and macro-heterogeneity level. An intervening sequence (IVS) of 148 bp was identified in the single 16S rRNA gene copy of five out of twelve *Campylobacter helveticus* isolates on analysis of the 16S rDNA PCR products. This was the first report of an IVS in the 16S rRNA gene of a eubacterium (Linton *et al.*, 1994). A larger IVS was identified in the (lone) 16S rRNA gene of *Pyrobaculum aerophilum*, a 713 bp intron, which upon excision is circularised (Burggraf *et al.*, 1993).

Through the analysis of available 16S rRNA gene sequences of many different microorganisms submitted to the EMBL or GenBank nucleotide sequence libraries, the occurrence of heterogeneities were localised to eight hyper-variable regions present in prokaryotic 16S rRNA's (Neefs *et al.*, 1990). Variable regions are illustrated in Figure 2.3 (opposite page) based on the 16S rRNA secondary structure model of *E.coli*.

Owing to their highly conserved nature, closely related organisms are often found to have nearly identical 16S rRNA gene sequences, therefore as evolutionary distances decrease a point is reached where insufficient base sequence diversity exists to differentiate strains of a given species. Although comparative analysis of 16S rRNA is a good method to describe an initial phylogenetic affiliation between organisms, it is necessary to use additional variable genetic markers to clarify interspecific and intraspecific phylogenetic relationships (LeBlond-Bourget *et al.*, 1996).

One solution to this problem is to use other evolutionary conserved genes/regions, such as the 23S rRNA genes and the spacer regions between 16S- and 23S- rRNA genes. At ~2500 bp the 23S rRNA molecule is a larger information unit than the 16S (~1550 bp), and in many cases has higher resolving power for phylogenetic reconstructions (Ludwig *et al.*, 1998; Ludwig and Schleifer, 1999). However, due to its length, 23S rDNA sequencing has not been as popular as 16S rDNA sequencing,

and the number of 23S rRNA sequences in the database is much smaller. In most prokaryotes the ribosomal genes are arranged in the order 16S-23S-5S, with few exceptions (Yoon *et al.*, 1997). The rRNA genes are separated by intergenic spacer regions (ISR) or intergenic regions (IR), which display large degrees of sequence and length variation at both the genus and species levels. The ISR between the 16S and 23S genes contain regions with secondary structure and sometimes transfer RNA (tRNA) genes (Perez Luz *et al.*, 1998). In fact, spacer regions found within bacterial genomes containing multiple rRNA loci, may also display a significant degree of variation (intercistronic heterogeneity) (Jensen *et al.*, 1993). For example in *E. coli* K-12 there are seven ribosomal operons, in three of them the ISR contains two tRNA genes (ISR2) for isoleucine and alanine (operons A, D, and H) with an average size of about 450 bp. The remaining four ISR's (operons B, C, E, and G) have a single tRNA gene for glutamic acid (ISR1) and are smaller in size (350 bp) (Condon *et al.*, 1995). It has been shown that due to the extreme divergence in length and sequence polymorphisms of the spacers within the *rrn* loci, together with their location between highly conserved rRNA genes, they can be used to successfully discriminate between different species of prokaryotes (Zhang *et al.*, 2001; Mehta and Rosato, 2001; Abraham *et al.*, 1999; Perez Luz *et al.*, 1998; Gurtler and Stanisich, 1996).

Studies making use of the PCR-mediated amplification of all copies of the ISR's of the 16S-23S rDNA of one or more bacteria are highly dependent on the availability of suitable sequence present in the flanking termini of the 16S and 23S rRNA genes. Full length sequences for the 16S rRNA gene have been reported for about 2500 different bacterial species (including over 18 000 entries in 1999) (Olsen *et al.*, 1991; Roselló-Mora and Amann, 2001), while full length sequences from only 21 species that represent 18 genera of 23S rRNA genes are available (Gurtler and Stanisich, 1996). From this sequence data, conserved regions within the 16S and 23S genes were identified as being suitable for the amplification of adjacent DNA's (Figure 2.4).

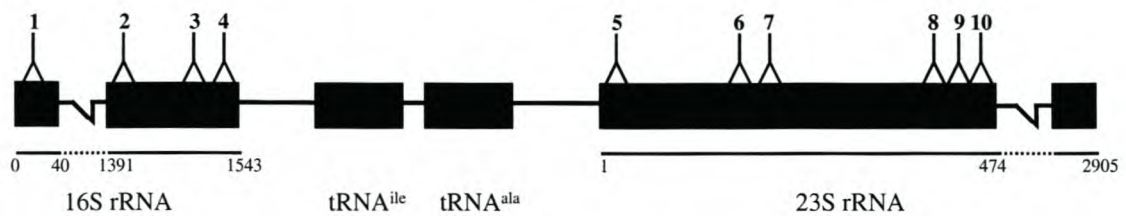


Figure 2.4: Conserved regions within the *E. coli* rRNA operon. The boxed areas represent the various genes of the bacterial rRNA operon. Some bacteria have two tRNA genes as shown; others have either one (either tRNA glu, tRNA ala or tRNA ile) or none. The dark, bold lines represent the intergenic spacer regions. Dark jagged lines represent breaks in the 16S and 23S genes, which have not been shown fully. The 5S rRNA gene is not shown. The thin numbered line shows the nucleotide numbering of the 16S and 23S rRNA genes of *E. coli* with breaks shown as dashed lines. The numbers 1-10 represent the following nucleotide sequence blocks. 16S rRNA gene; 1, 8-27; 2, 1390-1407; 3, 1491-1506; 4, 1525-1541. 23S rRNA gene: 5, 21-38; 6, 115-132; 7, 188-208; 8, 422-437; 9, 441-460; 10, 456-474 (Gurtler and Stanisich, 1996).

In order to amplify the spacer DNA, sequences 2, 3, and 4 are suitable; region 2 is however the most highly conserved sequence present in eubacteria, archaea and eukaryotes (Lane *et al.*, 1985). Region 3 and 4 are present in most or many eubacteria. Region 10 of the 23S rRNA gene is the most highly conserved; next, in order of identity, are regions 6, 9, 8, 7, and 5. Therefore isolate analysis by means of amplification of the 16S-23S rDNA ISR's is most likely to be successful using regions 2 and 10 of the 16S rRNA gene and 23S rRNA gene respectively, for primer construction (Gurtler and Stanisich, 1996).

The evolutionary rate of the 16S-23S rDNA spacer region is ten times greater than the evolutionary rate of the 16S rDNA (LeBlond-Bourget *et al.*, 1996). Hence these two molecules provide different complementary phylogenetic information. The 16S rDNA sequence is a good tool for inferring inter- and intragenic relationships (Fox *et al.*, 1992), while the 16S-23S spacer comparisons provide information concerning intraspecific links and allows for the detection of recently diverged species.

Ribosomal operons have acquired paramount relevance for the study of bacterial evolution and phylogeny. The rRNA 16S and 23S genes are the most widely used molecular chronometers and have been instrumental in developing a comprehensive view of microbial phylogeny and systematics (Gutell *et al.*, 1994). As the rapid and reliable identification of strains remains the most important task in taxonomy, 16S rRNA analysis is a most valuable tool in bacterial classification above the species level. Due to the highly conserved nature of the 16S rRNA, there is no linear correlation between DNA-DNA similarity % and 16S rRNA similarity for closely related organisms (Figure 2.5) (Stackebrandt and Goebel, 1994; Grimont, 1988), therefore the bacterial species definition can never be solely based on sequence similarity of rRNA's. However, for distinction at the species level 16S rDNA analysis is extremely helpful in deciding whether sufficient relationship exists between microorganisms to warrant the application of laborious DNA hybridization /reassociation experiments. The resolution power of DNA hybridization still remains significantly higher than that of sequence analysis, as DNA reassociation values may be as low as 25% at rRNA similarity values of 99.8% (Amann, *et al.*, 1992).

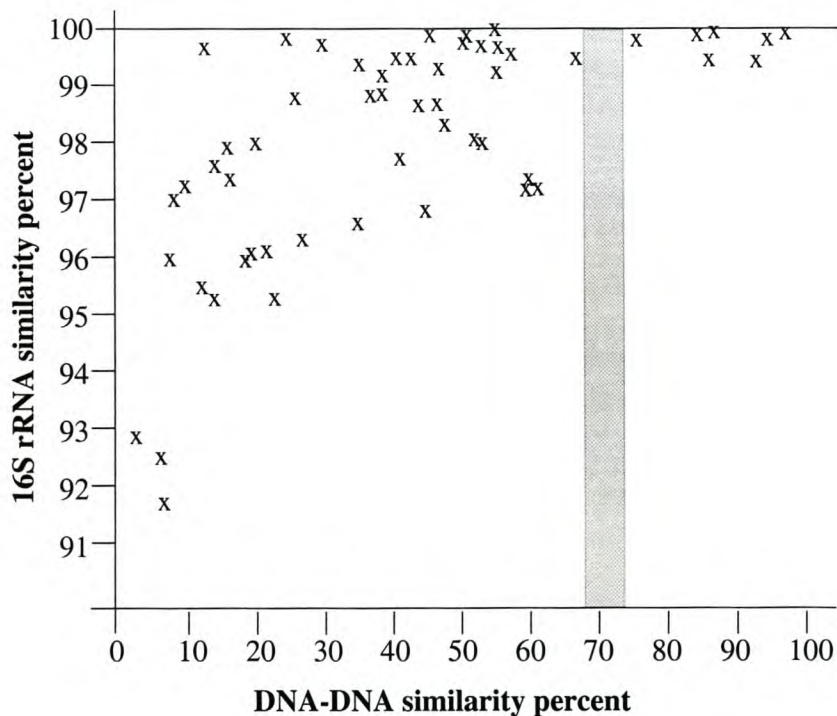


Figure 2.5: 16S rRNA homology versus DNA-DNA reassociation values. The bar indicates the DNA threshold value for species delineation (reproduced from Stackebrandt and Goebel, 1994).

From Figure 2.5 it is evident that each method is strong in those areas of relationship in which the other method fails to reliably depict relationships (Stackebrandt and Goebel, 1994). 16S rDNA sequence analysis is the superior of the two methods from the level of domains (starting at about 55%) to moderately related species (below 97.5%). Above this value DNA-DNA hybridization is the more accurate method for determining relationship and can either be low or as high as 100%. The combined use of these techniques, along with phenotypic confirmation, enables the confident separation of strains at the species level.

This chapter describes the application of a combination of phylogenetic, genomic and phenotypic techniques, to sixteen *Leptospirillum* isolates to determine the level of relationship that exists within this sample group. Furthermore, partial analysis of a further 3 strains was carried out. From these findings, we propose that two distinct *Leptospirillum* species, *Leptospirillum ferrooxidans* and *Leptospirillum ferriphilum* sp.nov., are represented among these isolates.

2.2 Materials and Methods

Materials and methods used in more than one chapter are presented in Appendix C and D.

2.2.1 Bacterial strains, media and growth.

Strains used in this study are listed in Table 2.1. Strains were routinely grown at 30°C in 800 ml mineral salts medium (% w/v): (NH₄)₂SO₄, 0.2; K₂HPO₄, 0.05; MgSO₄·7H₂O, 0.05; KCl, 0.01; Na₂SO₄, 0.1; and Ca(NO₃)₂, 0.001, supplemented with FeSO₄·7H₂O (500 mM) and 1000× trace elements solution (1 µl/10 ml), and adjusted to pH 1.6 with concentrated H₂SO₄. Media used throughout this study is described in detail in Appendix C. Strain purity was checked using the overlay technique of Johnson (1995). Experiments at 45°C were carried out using the same media. Ferrous iron concentration was determined by volumetric titration with potassium dichromate using diphenylamine 4-sulfonic acid indicator (Vogel, 1961).

Table 2.1: Strains of *Leptospirillum*

Strain	Type/ Group	Source	Origin	GenBank Accession no ^a .
P _{3a}	I	Barrie Johnson	Coal mine, North Wales, UK	AF356837
ATCC49879	I	Wolfgang Sand	Romania	AF356832
SY	I	Barrie Johnson	Sygun Cu mine, North Wales, UK	AF356839
N ₂₅	I	Barrie Johnson	New Zealand	ND
Crys13	I	Barrie Johnson	Ag mine, Montana	ND
BCT2	I	Barrie Johnson	Birch Coppice, UK	AF356833
Parys	I	Barrie Johnson	Parys Mountain, Anglesey Cu mine, Wales	AF356838
CF12	I	Frank Roberto	Idaho Co mine, USA	AF356834
Chil-Lf2	I	Barrie Johnson	Cu mine Chile	AF356835
DSM2705	I	DSMZ, Braunschweig, Germany ^b	Markosyan strain (1972), Cu mine, Armenia	X86776
Fairview	II	Ellen Lawson	South Africa	AF356830
Warwick	II	Paul Norris	Warwick, UK	AF356831
ATCC49880	II	Wolfgang Sand	Romania	ND
ATCC49881	II	Wolfgang Sand	Peru	AF356829
Bionic 3.1	II	Shelly Deane	Nickel pilot plant, Billiton, South Africa	ND
Mont. 4	II	Peggy Arps	Pyrite column, Montana, USA	ND
Adapt	II	Shelly Deane	Nickel pilot plant, Billiton, South Africa	ND
BN Mod	II	Shelly Deane	Nickel pilot plant, Billiton, South Africa	ND
617	II	Shelly Deane	Copper concentrate, Zaire (1998)	ND

^a ND, not determined.

^b DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

2.2.2 DNA preparation

Bacterial cells were harvested by centrifugation at 15 000 x g for 35 min and washed with acid water (pH 1.2) to remove ferric ion precipitate. Cells were either used immediately or stored frozen at -20°C in SET Buffer (25% sucrose, 2 mM EDTA, 50 mM Tris; pH 8.0). Prior to lysis, cells were treated with proteinase K (20 ng/μl), at 37°C for 30 min. Cell lysis was achieved by the addition of 10% sodium dodecyl sulfate (SDS). DNA was extracted via spooling and resuspended in TE buffer by shaking overnight at 30°C.

2.2.3 16S rRNA copy number determination

For Southern hybridization experiments used in ribotyping, 5 μg chromosomal DNA was digested with *Bam*HI and the restriction nuclease fragments separated by agarose gel electrophoresis. DNA was denatured in 0.25 M HCl, neutralised in 0.4 M NaOH and transferred to nylon, Hybond N⁺, membrane (Amersham) by capillary blotting overnight. The 1.5 kb 16S rDNA PCR product of isolate P_{3a} (chosen randomly from the 16 isolates) was labelled with digoxigenin using the DIG oligonucleotide 3'-end labelling and detection kit (Roche Biochemicals) and used as the hybridization probe. Hybridization temperature was 40°C. Washing was for 20 min at room temperature followed by 20 min at 65°C. Membrane detection performed as per manufacturer's instruction (Roche Biochemicals).

2.2.4 PCR amplification for restriction enzyme mapping

PCR amplifications of the 16S rRNA gene were routinely carried out to generate a 1.5 kb band on electrophoresis using primers pfDD₂ (5'-CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG-3') (8 to 27 *E. coli* numbering), which contains cloning sites *Bam*HI and *Sal*I towards the 5' end, and primer prDD₂ (5'-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3') (1512 to 1492 *E. coli* numbering), which has *Hind*III and *Xba*I cloning sites. Approximately 100 ng of chromosomal DNA was subject to amplification in a total volume of 50 μl containing 20 mM (NH₄)₂SO₄; 75 mM Tris-HCl, (pH 8.8 at 25°C); 0.1% (v/v) Tween 20; 3 mM MgCl₂; 2.5 μM (each) deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 0.2 μM of each primer; and 2U Redhot polymerase (Advanced Biotechnologies). Denaturation was at 94°C for 60 s followed by 25 amplification

cycles of 30 s at 94°C, 30 s at 52°C, and 90 s at 72°C. An additional 120 s at 72°C and a cooling step at 4°C for 60 s completed the reaction. Reactions were carried out in a Biometra® Personal Cycler. PCR product restriction enzyme analysis was performed using *EcoRV*, *StuI*, *KpnI*, *AvaI*, *SmaI*, *AgeI*, *MroI*, *NcoI*, *AvrII*, *BfrI*, *SspI*, *SacII* and *HindIII* in order to generate a discriminatory banding pattern on gel electrophoresis.

2.2.5 PCR of the 16S rDNA for sequencing

Three different sets of prokaryotic specific primers targeting internal regions to the 16S rRNA gene were used. Forward and reverse sequencing primers from conserved 16S rRNA gene regions were made based on nucleotides 8 to 27, 517 to 536 and 1053 to 1074 in the forward direction, and nucleotides 1512 to 1492, 1074-1053 and 536-515 in the reverse direction (*E.coli* numbering). A maximum of 50 ng of template DNA was used per reaction in a 50 μ l volume combined with: 20 mM (NH₄)₂SO₄; 75 mM Tris-HCl, (pH 8.8 at 25°C); 0.1% (v/v) Tween 20; 0.5 mM MgCl₂; 2.5 μ M (each) deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 10 μ M of each primer; and 2.5 U Redhot polymerase (Advanced Biotechnologies). Amplification protocol was as follows: one cycle of 2 min at 96°C, followed by 25 cycles of; 45 s at 96°C, 30 s at 51°C, 90 s at 72°C, and finally one cycle of 45 s at 96°C, 30 s at 51°C, 3 min at 72°C. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen), following manufacturer's recommendations. Concentrations were determined by a single wavelength reading at 260 nm in an UV spectrophotometer.

2.2.6 Sequencing and analysis of the 16SrRNA gene

The 16S rDNA was sequenced using the dideoxy chain termination method. Cycle sequencing reactions (maximum of 40 ng template DNA), using fluorescent labelled Cy5-Far Red primers; were performed with a ThermosequenaseTM Cycle Sequencing Kit (Amersham Pharmacia Biotech UK Ltd.). Sequencing reactions were run on an Alfexpress automated DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). Each isolate was sequenced in both the forward and reverse directions. PILEUP and CLUSTALW were used for multiple sequence alignments and phylogenetic dendrogram (Figure 2.7) construction was done using the DNAMAN for Windows program, Version 4.13 (1994-99). A secondary-structure model of the 16S rRNA

molecule transcribed from the primary sequence of isolate Fairview was constructed by Robin Gutell (Gutell *et al.*, 1994) and the file interpreted using the Aladdin Ghostscript version 5.1 graphical interface software. *Leptospirillum* sequences determined in this study were assigned GenBank accession numbers, listed in Table 2.1.

2.2.7 PCR amplification and analysis of the 16S-23S intergenic regions

The conditions used for 16S-23S amplification were the same as those used for 16S rRNA gene amplification, except the annealing step took place at 45°C. Primers used in amplification were G1.2 (5'-GTCGTAACAAGGTAICCG-3') and L1.2 (5'-GCCAAGGCATCCACC-3') modelled on primers designed by Jensen *et al.* (1993).

2.2.8 mol% G+C

Genomic DNA was treated with ribonuclease A, at a final concentration of 50 µg/ml, for 30 min at 37°C. DNA was then phenol extracted, followed by ethanol precipitation. Purified DNA was dissolved in 0.1×SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate, pH 7), at concentrations between 10-40 µg/ml and dialyzed against 0.1× SSC overnight. The DNA solutions were stored in 0.1× SSC at 4°C. The guanine plus cytosine (GC) content of the DNA was determined as described by Harrison (1982).

2.2.9 DNA-DNA hybridization

Genomic DNA was prepared as for mol% G+C determinations, with the exception of 0.1× SSC dialysis. DNA was resuspended in TE buffer. Three two-fold dilutions, 1 µg starting concentration, of all genomic DNA were prepared in a denaturing solution (final concentration 0.4 M NaOH/10 mM EDTA). Samples were boiled for 10 min, flash cooled, and loaded onto a positively charged nylon membrane using a slot blot manifold as described in Sambrook *et al.* (1989). The membrane was rinsed briefly in 2× SSC and air-dried. Genomic DNA probes were sonicated for seven 10 s periods by a Biosonik III (Bronwill Scientific Inc., Rochester, N.Y) instrument at an energy setting of 60% before labeling with digoxigenin using the DIG oligonucleotide 3'-end labeling and detection kit (Roche Biochemicals). Hybridization was in DIG-Easy Hyb at 40°C, followed by washing in 1x SSC at 25°C and a second washing in 0.1

XSSC at 65°C. Quantification of hybridization signals was carried out on a Uvidoc gel documentation system using the Alphaimager 2000 software. The method chosen for DNA-DNA hybridization is not the technique most used in taxonomic classification. The method of Crosa *et al* (1973) is widely accepted, however when applied to a large sample number the number of hybridizations required (13×16 in this study) becomes unwieldy. The current method is beneficial for two reasons: (i) all isolates may be incorporated on to one blot, (ii) the use on a non-radioactive probe enables the same blot to be stripped and reused for different probes, maintaining consistency between experiments. Although blots could be reused, final hybridization percentages were obtained as an average from blots prepared in triplicate.

2.3 Results

2.3.1 Number of *rrn* genes and ribotyping

Genomic DNA from nineteen different *Leptospirillum* isolates (Table 2.1) was analysed in Southern hybridization experiments using 16S rDNA from strain P_{3a} as a probe (Figure 2.6A). Each band represented a single copy of an *rrn* operon, as genomic DNA was digested with *Bam*HI and it had been established that none of the *Leptospirillum*-derived 16S rDNA PCR products had an internal *Bam*HI cleavage site. Two main groups of *Leptospirillum* could be distinguished from each other, one with two *rrn* operon copies and the other with three *rrn* copies. This result was confirmed by digestion of *Leptospirillum* genomic DNA with *Sal*I, which also has no internal 16S rDNA cleavage site (Figure 2.6B). Where band numbers were unclear, the blot was repeated (data not shown).

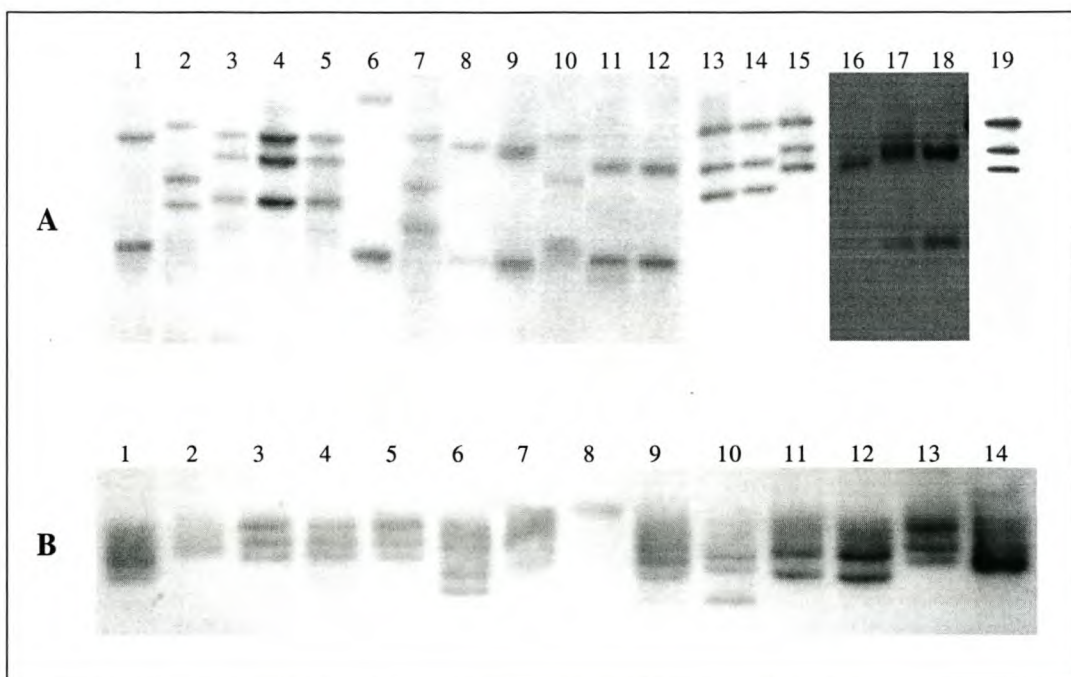


Figure 2.6: Number of *rrn* genes illustrated by Southern blotting of all nineteen *Leptospirillum* isolates, probed with DIG-labeled 16S rDNA from strain P_{3a}. Genomic DNA cut with (A) *Bam*HI and (B) *Sal*I. Part (A) is comprised of four different blots which were obtained at different stages of this study, the band sizes between blots are not to scale, banding pattern are however comparable. Part (B) confirms results obtained in (A) for most isolates tested, however isolates in lanes 2, 7, 8, and 14 were not completely digested. (A) Lanes: 1, Fairview; 2, P_{3a}; 3, Parys; 4, CF12; 5, Chil-Lf2; 6, Warwick; 7, ATCC49879; 8, ATCC49880; 9, ATCC49881; 10, SY; 11, Bionic 3.1; 12, Mont 4; 13, Crys13; 14, BCT2; 15, N₂5; 16, 617; 17, Adapt; 18, BN Mod; 19, DSM2705. (B) Lanes 1-13 same as in (A), lane 14, N₂5.

A further subdivision of the two main groups into ribotype subgroups can be made from a comparison of hybridization fragment sizes (Table 2.2). These subgroups provide an indication of the positioning of *Bam*HI restriction endonuclease sites flanking the 16S rRNA genes. Four subgroups within each *rrn* group were identified. Interestingly, some members that belonged to the same subgroup were isolated from very different geographical locations. For example the group with three *rrn* gene copies has a 5.08, 2.8, 2.1 kb ribotype subgroup containing leptospirilla isolated from Romania, Montana and England, while the 5.0, 4.5 and 2.7 kb ribotype subgroup has leptospirilla isolated from Wales, Idaho and Chile.

Table 2.2: Ribotyping fragment sizes

Isolate	Fragments (kb) ^a	Isolate	Fragments (kb) ^a
3 <i>rrn</i> subgroup		2 <i>rrn</i> subgroup	
P ₃ a	5.10, 2.9, 2.6	Fairview	4.75, 1.97
N ₂ 5	5.10, 2.9, 2.6	Adapt ^b	4.75, 1.97
DSM2705	5.10, 2.9, 2.6	BN Mod ^b	4.75, 1.97
ATCC49879	5.08, 2.8, 2.1	Warwick	10.5, 1.97
BCT2	5.08, 2.8, 2.1	ATCC49880	4.6, 1.97
Crys13	5.08, 2.8, 2.1	ATCC49881	4.6, 1.97
SY	5.08, 2.6, 2.4	617 ^b	4.6, 1.97
Parys	5.0, 4.5, 2.7	Bionic 3.1	4.4, 1.97
CF12	5.0, 4.5, 2.7	Mont 4	4.4, 1.97
Chil-Lf2	5.0, 4.5, 2.7		

^a Sizes of bands of genomic DNA following digestion with *Bam*HI, separation of fragments on an agarose gel and Southern hybridization with labelled 16 S rDNA.

^b isolates did not form part of the original study but were added during the course of the investigation.

2.3.2 Sequence analysis of the 16S rDNA PCR products

The 16S rRNA genes of ten of the sixteen *Leptospirillum* isolates were sequenced directly from the PCR amplified products in both forward and reverse directions. A homology matrix (Table 2.3) between these sequences and seven other *Leptospirillum* sequences previously deposited in GenBank/EMBL/RDP databases was constructed. Although the number of *rrn* copies was not known for the isolates obtained from the database, all seven previously deposited sequences grouped together with the 2 *rrn*

Table 2.3: Homology values derived from 16S rDNA gene sequences

Isolate	% Homology with:																
	ATCC49879	P _{3a}	DSM2705	Parys	Chil-Lf2	CF12	SY	BCT2	ATCC49881	Fairview	OS7	OS4	Lf30-A	LA	DSM2391(Bu-1)	Warwick	OS17
ATCC49879	100																
P _{3a}	99.9	100				3 rrr											
DSM2705	99.8	99.9	100														
Parys	99.2	99.3	99.1	100													
Chil-Lf2	99.2	99.3	99.1	99.7	100												
CF12	99.3	99.3	99.2	99.8	99.8	100											
SY	99.4	99.5	99.5	99.2	99.2	99.4	100										
BCT2	98.2	98.2	98.8	98.3	98.3	98.5	98.4	100									
ATCC49881	93.4	93.5	93.4	93.1	93.2	93.3	93.3	92.3	100								
Fairview	93.5	93.6	93.4	93.2	93.3	93.3	93.3	92.4	100	100					2 rrr		
OS7	93.1	93.2	93.2	92.8	92.9	92.9	93.2	92.7	99.8	99.8	100						
OS4	93.2	93.3	93.3	93.0	93.0	93.1	93.3	92.8	99.6	99.6	99.5	100					
Lf30-A	93.0	93.0	93.0	92.7	92.7	92.7	92.9	92.3	99.1	99.1	99.4	99.2	100				
LA	92.6	92.8	92.8	92.4	92.4	92.5	92.7	92.3	98.7	98.7	98.8	98.5	98.6	100			
DSM2391(Bu-1)	93.1	93.2	93.2	92.7	92.8	92.8	93.0	92.6	98.5	98.5	98.2	98.5	98.7	99.0	100		
Warwick	92.0	92.1	92.9	91.6	91.6	91.8	91.7	91.1	97.2	97.2	98.1	97.9	97.4	99.1	99.9	100	
OS17	92.6	92.7	92.9	92.2	92.3	92.3	92.5	92.1	98.0	98.0	98.1	97.9	98.3	98.9	99.7	99.7	100

copy subgroup. Isolates within the group with 2 *rrn* gene copies had 16S rDNA sequences, which were 97.2-100% identical whereas those within the group with three *rrn* gene copies were 98.2-99.9% identical. Sequence identity between the members of the two groups was 91.0-93.4% (Table 2.3, opposite page).

A dendrogram of all strains of *Leptospirillum* for which sequences are available illustrates the clustering of the two-*rrn* groups (Figure 2.7). Two additional 16S rRNA sequences were included in the dendrogram, Snottite clone SC07 and Slime clone BA29 (Bond *et al.*, 2000). These isolates do not fall into the two *Leptospirillum* groups represented in this study and appear to form a third group. The snottite and slime clone 16S rRNA sequences were obtained by PCR from a mixed, crude, cellular extract using universal- and Bacteria- specific primer sets respectively. Although these results indicate further diversity within the *Leptospirillum* genus, pure cultures of these isolates have not been obtained and were therefore not available to be included in this study as a way of comparison. The extent to which they differ physically or physiologically from other leptospirilla has not yet been determined.

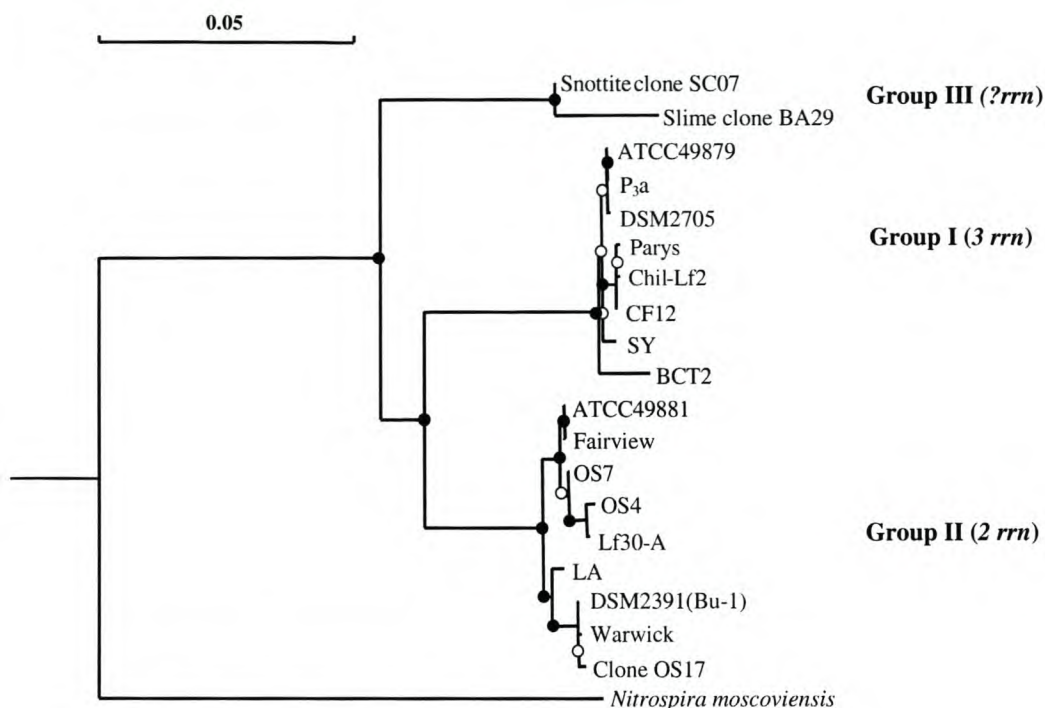


Figure 2.7: Evolutionary-distance dendrogram of leptospirilla based on approximately 1450 bp of 16S rDNA sequence. Branch points supported by bootstrap values of > 75% are shown by solid circles, and those supported by bootstrap values between 50 and 75% are shown by open circles. The scale bar represents changes per nucleotide. Based on 16S rDNA sequence data, the genus *Leptospirillum* has been placed within the division *Nitrospira*, and *Nitrospira moscoviensis* has been used as the outgroup. Database accession numbers are as follows: snottite clone SC07, AF225453; slime clone BA29, AF225448; ATCC49879, AF356832; P_{3a}, AF356837; DSM2705, X86776; Parys, AF356838; Chil-Lf2, AF356835; CF12, AF356834; SY, AF356839; BCT2, AF356833; ATCC49881, AF356829; Fairview, AF356830; OS7, X86773; OS4, X86770; Lf30-A, X72852; LA, AJ237902; DSM2391 (Bu-1), M79383; Warwick, AF356831; clone OS17, X86772; and *N. moscoviensis*, X82558.

Further comparison of the 16S rDNA may be conducted at the level of secondary structure. When compared across the entire phylogenetic spectrum, about 40% of the 16S rRNA molecule is not completely conserved in its secondary structure (Gutell *et al.*, 1985). Within these variable regions are sections that are conserved within phylogenetic groups (defining kingdom-specific motifs), and other sections that show variation even within phylogenetic groups. From analysis of all the prokaryotic 16S rRNA sequences available in the database eight-hypervariable regions were identified (Figure 2.3) (Neefs *et al.*, 1990). Differences in the region of 16S rRNA between

nucleotide positions 180 and 220 (variable region 2) have been shown to distinguish between the various subdivisions of the purple bacteria (Figure 2.8) (Woese, 1987). Examples are also available where a portion of the secondary structure has been conserved, although not at the sequence level, i.e. the penultimate stem of *E. coli* 16S rRNA, between bases 1409 and 1491 (Firpo and Dahlberg, 1998). This section is characterized by a base helix (positions 1409-1416/ 1484-1491), which when mutated caused either increased or decreased translational fidelity (Gregory and Dahlberg, 1995) and conferred resistance to aminoglycoside antibiotics (De Stasio *et al.*, 1989). This suggests the importance of helix preservation for its role in translation.

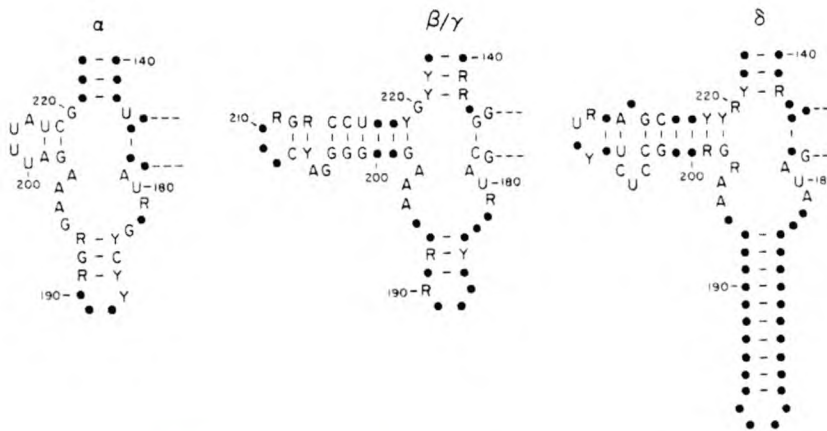


Figure 2.8: Differences in higher-order structural detail among the various subdivisions of the purple bacteria for the region of 16S rRNA between positions 180 and 220. Composition of a position is given when it is invariant or highly conserved within a subdivision, but it is shown as a dot otherwise. Base pairs are indicated by connecting lines (Woese, 1987).

With the assistance of Robin Gutell (Gutell *et al.*, 1994) a secondary structure diagram of the *Leptospirillum* strain Fairview 16S rRNA was drawn (Figure 2.9). Secondary structure comparison over the entire 16S rRNA sequence was only performed for the ten isolates sequenced in this study. Nucleotide (nt) insertions and deletions along the 16S rRNA secondary structure are the result of sequence changes in the following isolates: nt 4 -ATCC49879, CF12, P_{3a}; nt 86 -P_{3a}; nt 112 -Warwick; nt 541 -P_{3a}; nt 602 -SY; nt 664, nt 794, and nt 969 -BCT2; nt 1469 and 1470 -BCT2, CF12, SY, P_{3a}, ATCC49879, Parys, and Chil-Lf2; nt 1516 -CF12; nt 1520 and 1523 -BCT2; nt 1526 -all isolates but Fairview; and nt1527 -CF12.

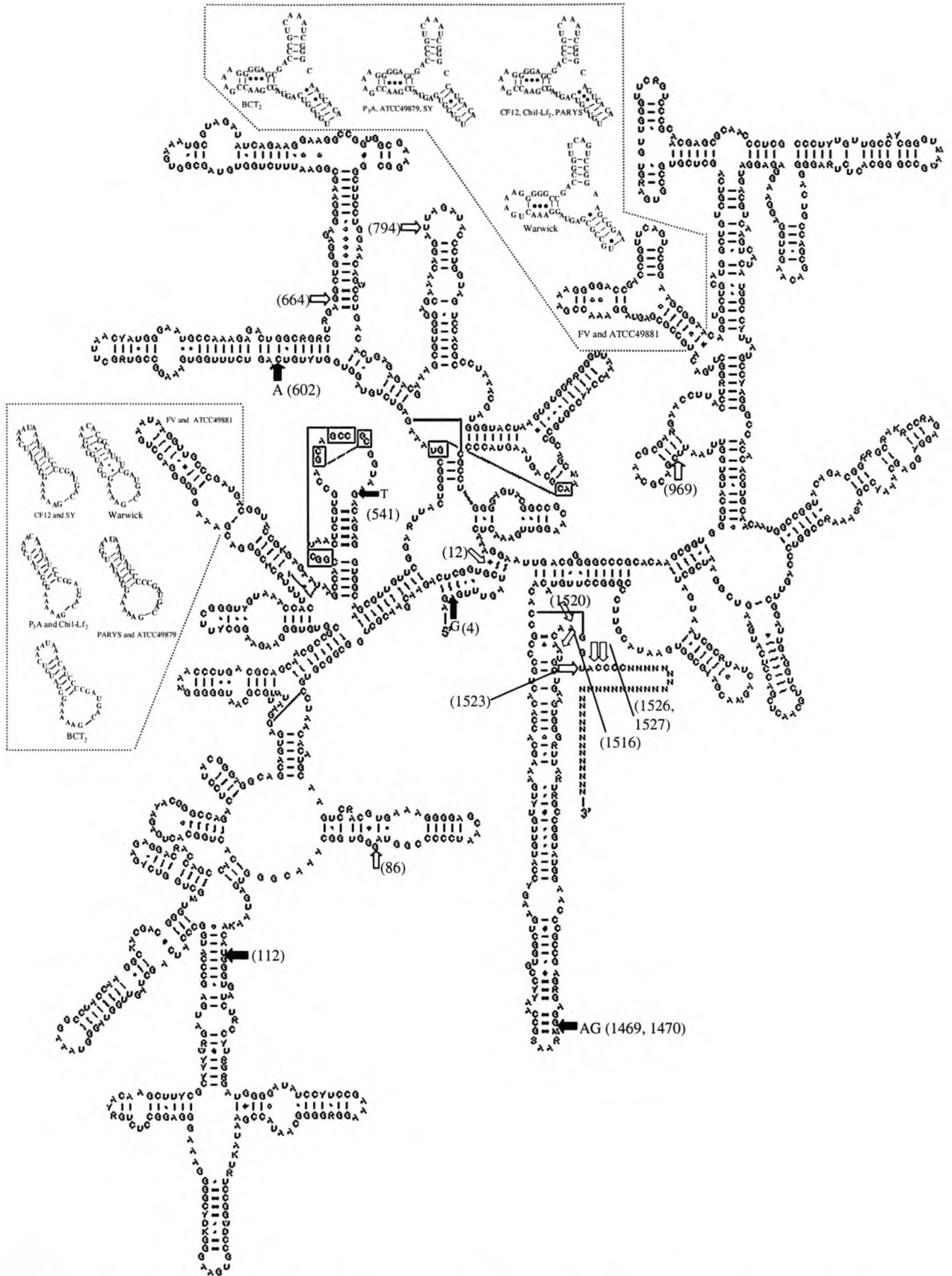


Figure 2. 9: Secondary structure model of the 16S rRNA sequence from *Leptospirillum* isolate Fairview. Comparative secondary structures, in the regions of greatest variability, are represented in the insets. Single base changes are shown by replacement of the respective nucleotide with one of the following: M=A/C, R=A/G, W=A/T, S=G/C, Y=C/T, H=A/C/T, D=A/G/T, and B=C/G/T. Filled arrows represent insertions; open arrows indicate deletions, (nucleotide positions are indicated in brackets), further information provided in the text. 16S rDNA model drawn with assistance of Robin Gutell.

Although variations in sequence between groups with 2 and 3 *rrn* gene copies occurred in many regions of the 16S rRNA, most variation occurred within variable regions 3 and 6. This was consistent with the *Leptospirillum*-like 16S rRNA sequences present in the database (Figure 2.10).

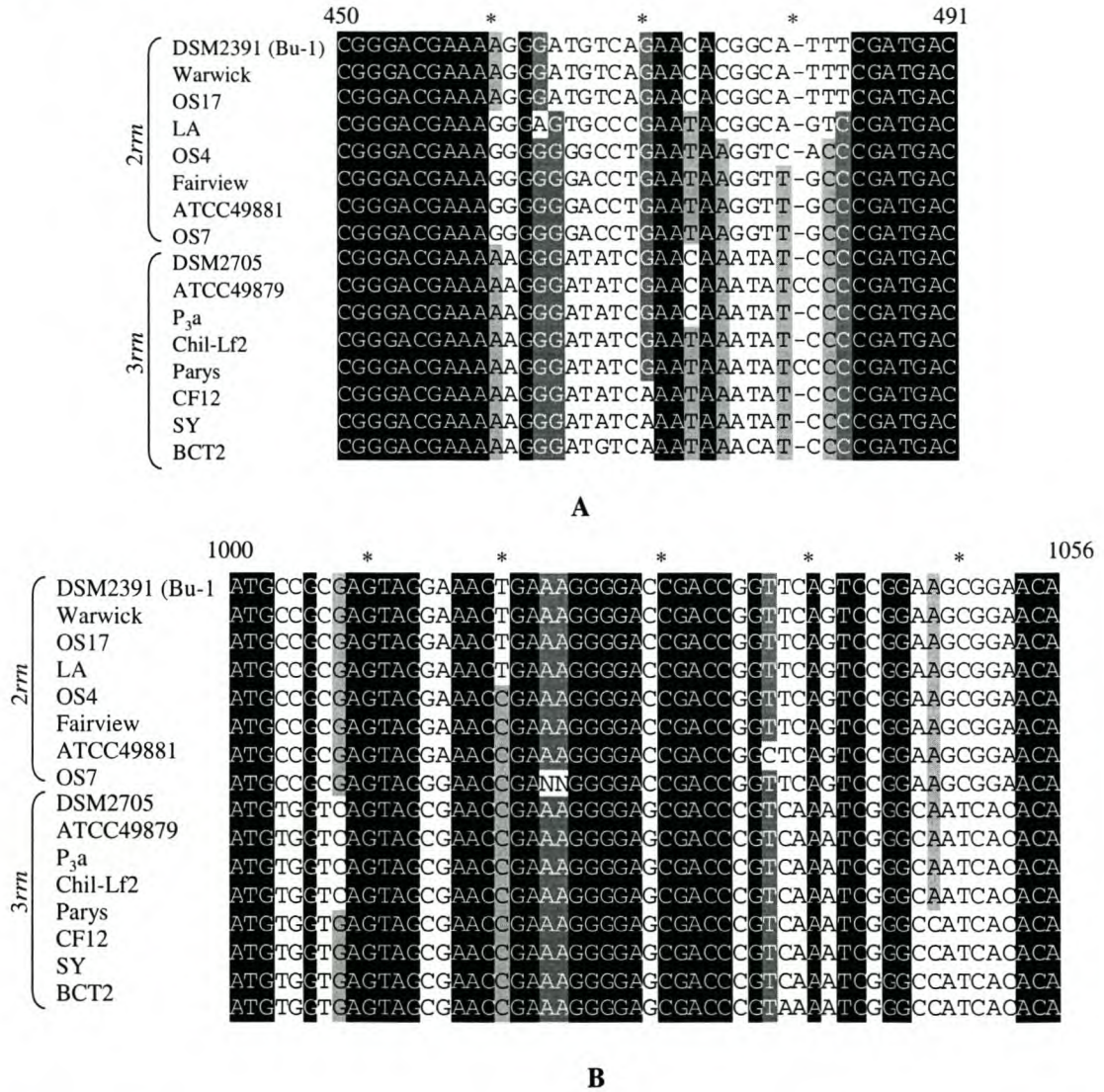


Figure 2.10: Multiple alignments of two 16S rDNA variable regions. (A) Variable region 3 (nucleotide positions 450-491), and (B) variable region 6 (nucleotide positions 1000-1056). Every 10th nucleotide is marked with an asterisk. Separation into groups with 2 *rrn* and 3 *rrn* copies is indicated by means of brackets. Shading: 100% homology = black, 75% homology, grey. Accession numbers as in Figure 2. 7.

There have been reports of polymorphisms within multiple copies of 16S rRNA genes within the same organism. For example, *Mycoplasma capripneumoniae* subsp. *capripneumoniae* has two copies of 16S rRNA genes and between 11 and 24 differences in nucleotide sequence between the copies were found in 20 isolates examined (Pettersson *et al.*, 1998). Sequence determinations for the 16S rRNA genes of the leptospirilla sequenced in this study were carried out directly from the PCR amplified product. Assuming that all copies of the 16S rRNA genes were amplified with equal efficiency, then polymorphisms between gene copies would have resulted in a mixed population of non-identical amplification products and ambiguous sequence data in certain positions. No positions of sequence ambiguity were found and all copies of 16S rRNA genes therefore appeared to be identical.

2.3.3 PCR amplification and restriction enzyme mapping of the 16S rDNA

We have routinely used restriction enzyme mapping of amplified 16S rDNA as a convenient method for rapidly identifying isolates of previously isolated iron- and sulfur-oxidizing microorganisms present in biooxidation tanks (Rawlings, 1995; Rawlings *et al.*, 1999). We wished to determine whether this simple technique could be used as quick screening method to distinguish between the major groups of *Leptospirillum*. Comparison of the 16S rDNA sequence data from this study and from previously sequenced leptospirilla deposited in the GenBank and RDP databases enabled us to identify several 6 bp recognition sequence restriction endonucleases which would give different digestion patterns that could be used for this purpose. Based on the view that the presence of a cutting site has more value than the absence of a site, four endonucleases (*AgeI*, *MroI*, *NcoI* and *SmaI*) were identified that allow for specific identification of the group of leptospirilla with two *rrn* gene copies and six endonucleases (*AgeI*, *AvrII*, *BfrI*, *EcoRV*, *SspI* and *StuI*) for specific identification of the group with three *rrn* gene copies (Figure 2.11). The *AgeI* cutting site was present with the 16S rDNA of both groups but in sufficiently different positions to allow specific identification.

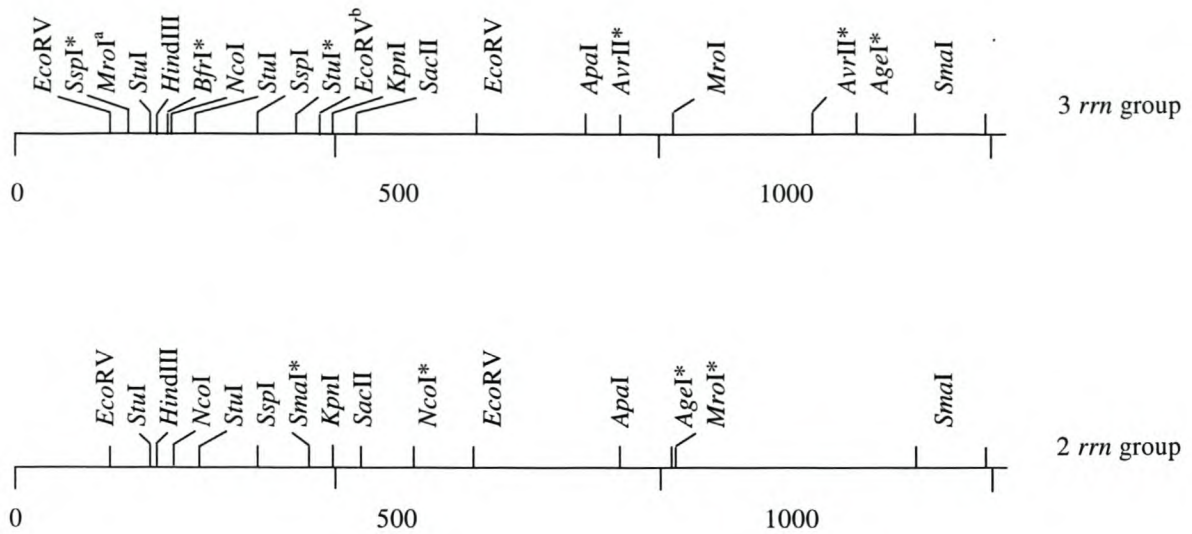


Figure 2.11: Map of 6 bp restriction endonuclease cutting sites within the 16S rRNA genes of *Leptospirillum* isolates with 2 *rrn* and 3 *rrn* copies. Sites which enable the 3 *rrn* group to be distinguished from the 2 *rrn* group and which were consistent among all isolates used in this study, or for which sequence information is available are marked with asterisks. Restriction endonuclease sites present in all 3 *rrn* group isolates (except BCT2) are marked with an ^a, and those present in all isolates examined (except BCT2) and SY are marked with a ^b.

Although *ApaI*, *HindIII*, *KpnI* and *SacII* cannot be used to distinguish between leptospirilla, these restriction enzymes can be used as a diagnostic tool in distinguishing between *Leptospirillum*, *Acidithiobacillus caldus*, *At. ferrooxidans* and *At. thiooxidans*. To confirm the usefulness of this approach, 16S rDNA of leptospirilla strains for which the 16S rDNA had not been sequenced, but for which the number of copies of *rrn* had been determined, was amplified by PCR. Restriction enzyme digests for *NcoI*, *SmaI*, *BfrI*, *EcoRV*, *SspI* and *StuI* were carried out and in each case the *Leptospirillum* isolate could be correctly placed into the group with 2 or 3 *rrn* gene copies based on the restriction enzyme digests (Figure 2.12).

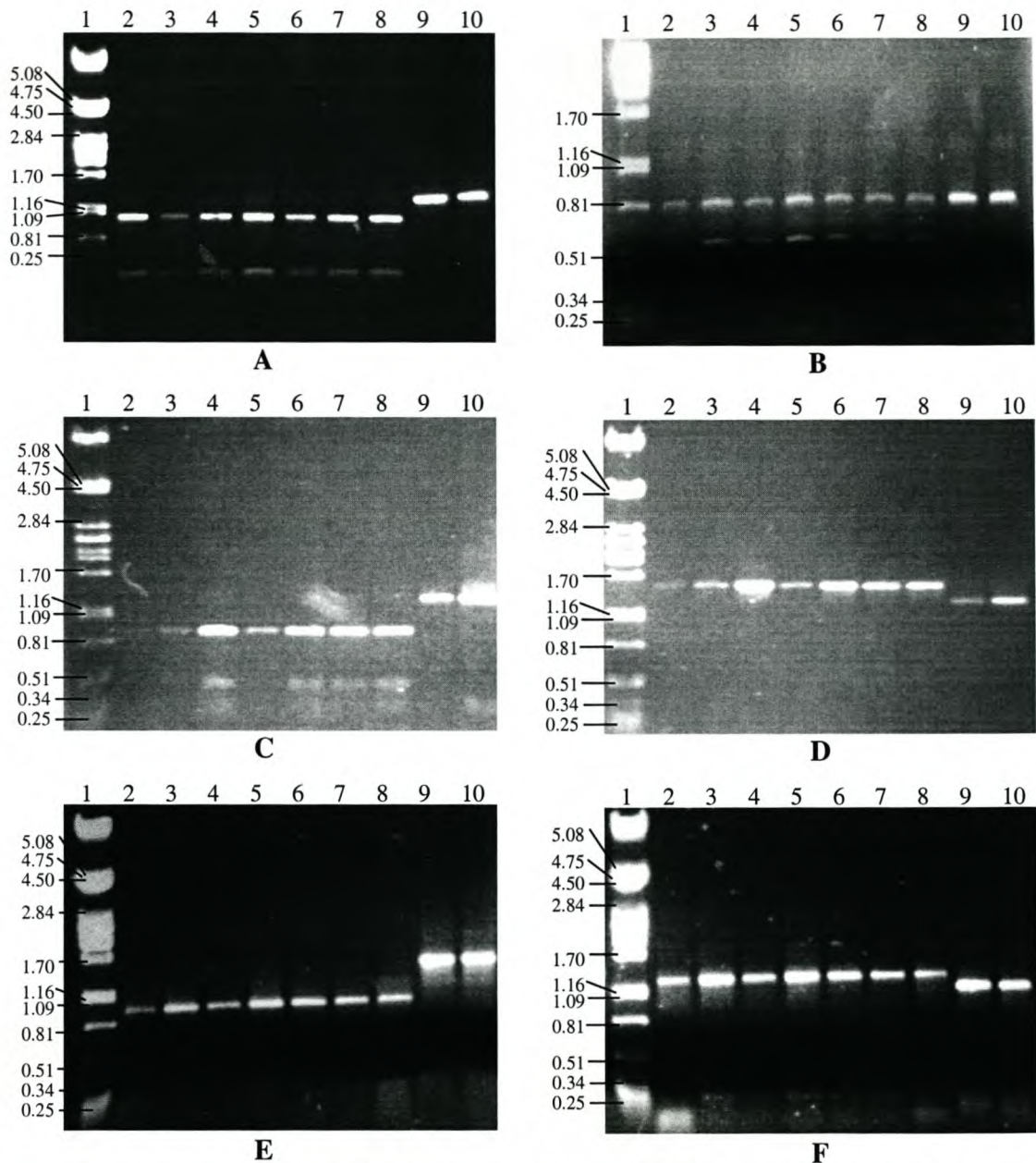


Figure 2.12: Restriction endonuclease patterns of 16S rDNA PCR products obtained from *Leptospirillum* isolates for which the 16S rRNA gene sequence was not available. Expected sizes of the fragments generated for the 3 *rrn* and 2 *rrn* groups are indicated in brackets (kb). Underlined fragment sizes could not be seen in the photographs due to either obstruction by the loading dye, or the gel being run too far. (A) *Age*I, (3 *rrn*, 1.3 and 0.2; 2 *rrn*, 1.01 and 0.49). (B) *Eco*RV, (3 *rrn*, 0.78, 0.325, 0.250, and 0.145; 2 *rrn*, 0.78, 0.575, and 0.14). (C) *Nco*I, (3 *rrn*, 1.26, and 0.24; 2 *rrn*, 0.87, 0.39, and 0.24). (D) *Bfr*I, (3 *rrn*, 1.25 and 0.25; 2 *rrn*, 1.5). (E) *Sma*I, (3 *rrn*, 1.4 and 0.1; 2 *rrn*, 0.95, 0.45, and 0.1). (F) *Stu*I, (3 *rrn*, 1.1, 0.2, 0.15, and 0.05; 2 *rrn*, 1.23, 0.2, and 0.07). Lanes: 1, λ -*Pst*I (relevant sizes are given in kilobases); 2, Bionic 3.1; 3, Mont 4; 4, ATCC49880; 5, 617; 6, Adapt; 7, BN Mod; 8, Warwick (repeat as sequence is available); 9, N₂5; 10, Crys13. Isolates in lanes 2-8 fall into the 2 *rrn* copy group, whereas isolates in lanes 9 and 10 fall into the 3 *rrn* copy group.

2.3.4 Amplification product profiles of the 16S-23S intergenic region (IR)

The intergenic regions between the 16S and 23S rRNA genes were amplified in all 19 *Leptospirillum* isolates. Single and multiple banding patterns ranging in size from 3.0 kb-0.47 kb were obtained (Figure 2.13). PCR product profiles consisted of both intense, highly reproducible fragments (primary products), as well as weaker fragments (secondary products). Attempts to reduce the intensity of the secondary products were made by altering PCR parameters. An increase in MgCl₂ concentration (from 3 mM to 5 mM) and annealing temperature (from 45°C-50°C) saw a reduction, if not removal, of secondary products. On submission of this work to Applied and Environmental Microbiology (Coram and Rawlings, 2002), reviewers pointed out that secondary products are not used for classification purposes, and so secondary products that could not be eliminated, were ignored.

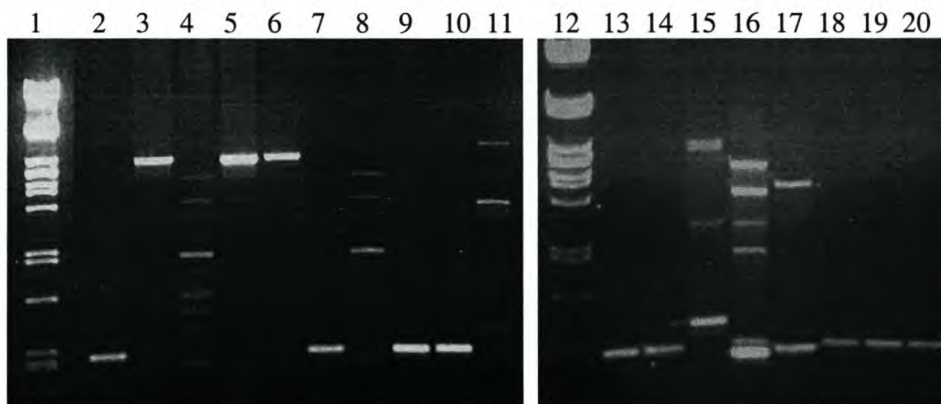


Figure 2.13: 16S-23S IR product profiles for *Leptospirillum* isolates used in this study. Lanes: 1, λ -*Pst*I; 2, Fairview; 3, Parys; 4, P₃a; 5, CF12; 6, Chil-Lf2; 7, Warwick; 8, ATCC49879; 9, ATCC49880; 10, ATCC49881; 11, SY; 12, λ -*Pst*I; 13, Bionic 3.1; 14, Mont 4; 15, Crys13; 16, N₂5; 17, BCT2; 18, Adapt; 19, 617; 20, BN Mod. 16S-23S product profiles for *L.ferrooxidans* type strain DSM2705 were obtained and were the same as those of isolates P₃a and N₂5 (results not shown).

A single 0.5kb IR spacer was amplified from leptospirilla of the group with two *rrn* gene copies whereas IR spacers of a variety of sizes were amplified from leptospirilla of the group with 3 *rrn* gene copies. Isolates P₃a, N₂5, DSM2705, ATCC49879, and Crys13 produced three different primary IR products, presumably a different sized product from each of the 3 *rrn* gene copies (Table 2.4). These results are in

agreement with existing evidence that multiple IR of varying sizes may be present within a single species (Gurtler, 1999).

Table 2.4: Fragments sizes of the 16S-23S IR amplification products

Isolate (<i>3rrn</i> group)	Fragment size (kb)	Isolate (<i>2rrn</i> group)	Fragment size (kb)
P _{3a}	2.3, 1.75, 1.0	Fairview	0.5
N ₂₅	2.3, 1.75, 1.0	Adapt ^a	0.5
DSM2705	2.3, 1.75, 1.0	BN Mod ^a	0.5
ATCC49879	2.3, 1.75, 1.0	Warwick	0.5
BCT2	1.9, 0.47	ATCC49880	0.5
Crys13	3.0, 2.84, 1.6	ATCC49881	0.5
SY	3.0, 1.6	617 ^a	0.5
Parys	2.84	Bionic 3.1	0.5
CF12	2.84	Mont 4	0.5
Chil-Lf2	2.84		

^aIsolate did not form part of the original study but was added during the course of the investigation.

2.3.5 mol% G+C

Mol% G+C was obtained for fifteen of the original *Leptospirillum* isolates. Although these values do not take into account the linear sequence of bases within the DNA molecules, these results did indicate differences in relationship amongst the isolates. G+C contents grouped into two definite temperature ranges, 48.8-51.9°C and 55.0-58.0°C, for the 3 *rrn* and 2 *rrn* isolates respectively (Table 2.5). These ranges were consistent with those of a previous study by Harrison and Norris (1985), where six isolates comprising *L. ferrooxidans* type strain DSM2705 and other *Leptospirillum*-like bacteria were found to form two G+C content groups of ca. 51% and 55-56% respectively. Theoretically, bacterial genomes with differences greater than 20-30 mol% have virtually no sequences in common (Rosselló-Mora and Amann, 2001). It has been shown that organisms that differ by more than 10 mol% are not likely to belong to the same genus, where a 5 mol% difference is the common range found within a given species. The mean differences between the two mol% G+C ranges within this sample group was 5.63 mol%, and is well within the limit to justify a

species separation. Although differences in mol% are taxonomically useful, the criterion can only be used negatively (similarities in base composition alone may not indicate similarity in linear base sequences), and should therefore be used in conjunction with a more thorough analytical genomic parameter such as total DNA-DNA hybridization.

Table 2.5: *Leptospirillum* G+C contents (mol%)

Isolate	Mol% G+C ($\pm 1\%$) ^a
3 <i>rrn</i> subgroup	
P ₃ a	51.9
N25	51.9
DSM2705	51.7
ATCC49879	51.7
BCT2	51.0
Crys13	50.7
SY	48.8
Parys	51.5
CF12	51.2
Chil-Lf2	50.1
2 <i>rrn</i> subgroup	
Fairview	58.0
Warwick	54.9
ATCC49880	57.8
ATCC49881	56.6
Bionic 3.1	56.1

^a isolates for which mol% G+C were not determined: Mont 4, Adapt^b, BN Mod^b, and 617^b.

^b Isolate did not form part of the original study but was added during the course of the investigation.

2.3.6 DNA-DNA hybridization

Although sequence analysis of the 16S rRNA is a valuable tool in investigating phylogenetic relationships, it has been shown in several cases that almost identical 16S rRNA sequences have yielded DNA-DNA hybridization values of less than 70%, indicating separate species (Stackebrandt and Goebel, 1994). For this reason DNA-DNA hybridization was used in conjunction with 16S rRNA sequence analysis. DNA-DNA hybridization percentages were obtained for 16 isolates using genomic DNA from 13 leptospirilla as hybridization probes. Hybridization signals were quantified by chemiluminescence detection, and percentages estimated by comparison of hetero- to homo-duplex formation. Figure 2.14 illustrates the differences in hybridization patterns obtained when a probe from each *rrn* group was used against the collective set of isolates. Only Southern hybridization blots from probes

DSM2705 (3 *rrn* group) and ATCC49881 (2 *rrn* group) are shown here, a more complete complement of blots is presented in Appendix A. The higher the DNA sequence similarity of probe to target DNA, the greater the binding affinity will be, resulting in high hybridization percentages.

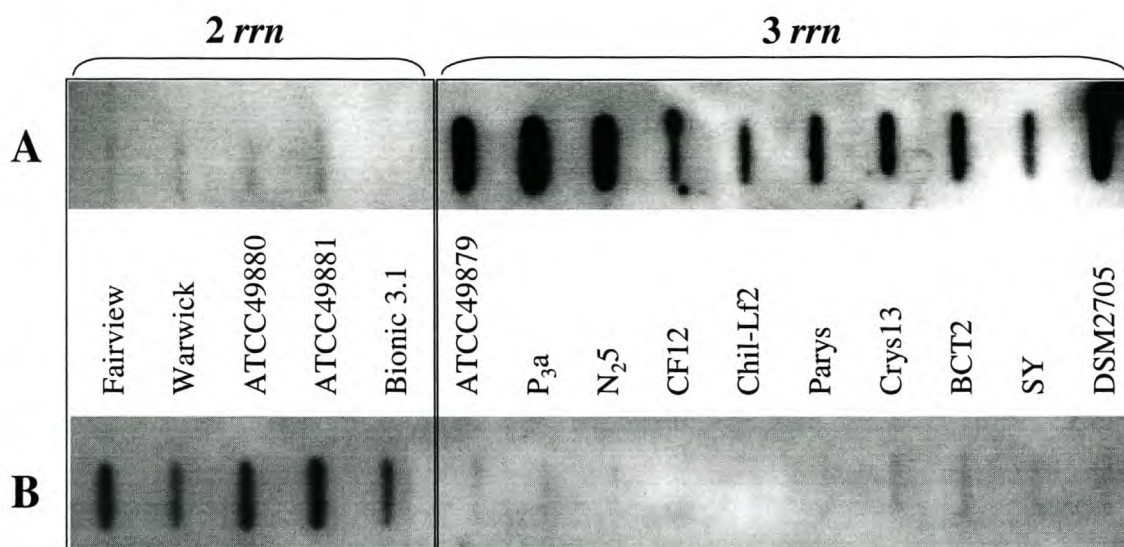


Figure 2.14: Autoradiographs of slot blot hybridizations between fifteen *Leptospirillum* isolates and representative DIG-labeled total genomic DNA probes from the respective *rrn* subgroups. Probes: (A) 3 *rrn* subgroup: *L. ferrooxidans* type strain DSM2705; (B) 2 *rrn* subgroup: ATCC49881. Target DNA is labelled accordingly. 2 *rrn* and 3 *rrn* groups are indicated by means of brackets. Mont 4 DNA is not included in these slot blots, however both probe and target DNA hybridization results are available in Appendix A.

Hybridization results are given in Table 2.6. The 3 *rrn* leptospirilla (group I) could be divided into two DNA-DNA hybridization subgroups with 94-100% and 93-100% similarity within a subgroup and 60-79% similarity between the two subgroups. We have named the subgroups I.1 and I.2. The 2 *rrn* leptospirilla (Group II) formed a single DNA-DNA hybridization subgroup with 81-100% similarity. However, there was only < 5-11% similarity between subgroups I.1 and I.2 of the group I leptospirilla and the group II leptospirilla.

Table 2.6: DNA-DNA hybridization values between *Leptospirillum* *rrn* groups^a

Probe isolate	Hybridization (%)															
	Group I (3 <i>rrn</i>)										Group II (2 <i>rrn</i>)					
	Subgroup I.1					Subgroup 1.2										
ATCC49879	P _{3a}	DSM2705	N ₂₅	CF12	Chil-Lf2	Parys	Crys13	BCT2	SY	Fairview	ATCC49880	ATCC49881	Warwick	Bionic 3.1		
ATCC49879		100	95	96	94	63	64	67	75	74	72	<5	<5	<5	<5	<5
P _{3a}		96	100	100	97	65	63	65	65	65	65	<5	<5	<5	<5	<5
DSM2705		100	100	100	100	68	64	64	72	78	64	8	5	11	8	<5
N ₂₅		98	100	100	100	75	76	75	76	76	79	<5	<5	<5	<5	<5
CF12		62	68	75	76	100	98	100	96	98	93	<5	<5	<5	<5	<5
Crys13		69	70	66	69	97	98	98	100	94	90	<5	<5	6	6	8
BCT2		69	68	60	70	94	97	94	97	100	95	7	7	10	6	10
Fairview		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	100	97	99	81	100
ATCC49880		5	7	<5	<5	<5	<5	7	9	9	6	100	100	100	89	100
ATCC49881		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	95	99	100	82	100
Warwick		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	84	84	87	100	97
Bionic 3.1		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	96	97	97	95	100
Mont 4		<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	98	97	97	97	100

^aLabelled genomic DNA from each probe isolate was hybridized against genomic DNA from all target isolates. Each hybridization was carried out at least three times. Mont 4 DNA was used as a probe against all target DNA samples shown but served as target for only a few of the probes shown. These results were the reciprocal of what is shown in this table, that is, DNA hybridization was 98 to 100% with isolates Bionic 3.1 and ATCC49881 and <5% with ATCC49879, DSM2705 and CF12 (not shown). With one exception, standard deviations of DNA-DNA hybridization values ranged from 0 to 8%.

2.3.7 *Leptospirilla* capable of growth at 45°C

One of the few physiological differences reported between leptospirilla is that some isolates are capable of growth at temperatures of > 40°C (Golovacheva *et al.*, 1993; Schrenk *et al.*, 1998). We have previously investigated the bacteria present in pilot plants operating at 45°C and found that large numbers of leptospirilla were present (Rawlings *et al.*, 1999). Furthermore there is a report of a *Leptospirillum* that is capable of iron-oxidation at 55°C and which is considered to constitute a separate species, *Leptospirillum thermoferrooxidans*. We wished to determine to which group the leptospirilla adapted in pilot plants to grow at 45°C belonged and whether any of the non-adapted isolates of leptospirilla were also capable of growth at 45°C. Each of the sixteen original isolates was tested for the ability to oxidize ferrous iron at 30°C

and 45°C. In addition, the three leptospirilla isolated from bioreactors operating at 45-55°C were included (Adapt, BN Mod and 617). Several members of the leptospirilla group with two copies of *rrn* genes including those not previously exposed to bioreactors operating at 40°C or above were able to oxidize iron at 45°C (Table 2.7). However, the rate of iron oxidation was slower than at 30 or 40°C and no leptospirilla from the group with three *rrn* gene copies were able to oxidize iron at 45°C.

Table 2.7: Ability of leptospirilla to grow at 45°C

Isolate	Growth at 45°C (±)
3 <i>rrn</i> subgroup	
P ₃ a	-
N25	-
DSM2705	-
ATCC49879	-
BCT2	-
Crys13	-
SY	-
Parys	-
CF12	-
Chil-Lf2	-
2 <i>rrn</i> subgroup	
Fairview	+
Warwick	-
ATCC49880	+
ATCC49881	+
Bionic 3.1	-
Mont 4	-
Adapt ^a	+
BN Mod ^a	+
617 ^a	+

^a Isolate did not form part of the original study but was added during the course of the investigation.

2.3.8 Physiological and physical analysis of the two groups of leptospirilla

We examined the type strain of *L. ferrooxidans* (DSM2705) and a representative of the type II leptospirilla (ATCC49881) for physiological and physical differences besides temperature tolerance. Both species/groups had properties similar to those reported for *L. ferrooxidans* (Hippe, 2000; Johnson, 2001). Both leptospirilla were catalase negative and peroxidase positive. They were of similar size (0.3 - 0.5 µm in width, 0.9 – 3.0 µm in length) with ATCC49881 being at the narrower end of the

width range. Both species were vibrio-shaped in young cultures (up to 4 days), helical (2 to 5 turns) in older cultures and motile by means of a single polar flagellum.

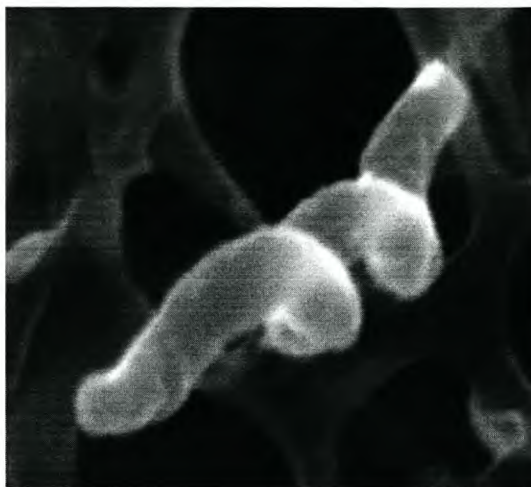


Figure 2.15: Electron micrograph of type II leptospirilla (isolate Fairview).

Both species grow autotrophically at the expense of pyrite mineral with doubling times in the range of 12-15 h. Whether an appreciable difference in growth rate between the two groups exists has not been established. Owing to the small size and number of leptospirilla cells, it is difficult to accurately determine growth rate. However, the rates of ferrous iron oxidation between isolate ATCC49881 and *L. ferrooxidans* DSM2705 were compared. Prior to this comparison, ferrous iron oxidation for each isolate was optimized with regard to temperature and pH; testing ranges of 20-45°C and 0.8-2.0 respectively. For ATCC49881 optimum ferrous iron oxidation occurred between 37-40°C, but could be extended at both ends of this range to 30°C and 45°C respectively. The temperature range for ferrous iron oxidation of DSM2705 was more limited, occurring within the range of 30-40°C, while the temperature optimum (37-40°C) appeared to be similar. Both isolates were capable of oxidizing ferrous iron over a narrow pH range (1.4-2.0), with optimum pH values for each isolate as follows: pH 1.4 to 1.8 for ATCC49881 and pH 1.6 to 2.0 for *L. ferrooxidans*. From this data, optimum temperature and pH values of 37°C and pH 1.8 were selected for use in the comparison of ferrous iron oxidation between ATCC49881 and *L. ferrooxidans* DSM2705 (Figure 2.16).

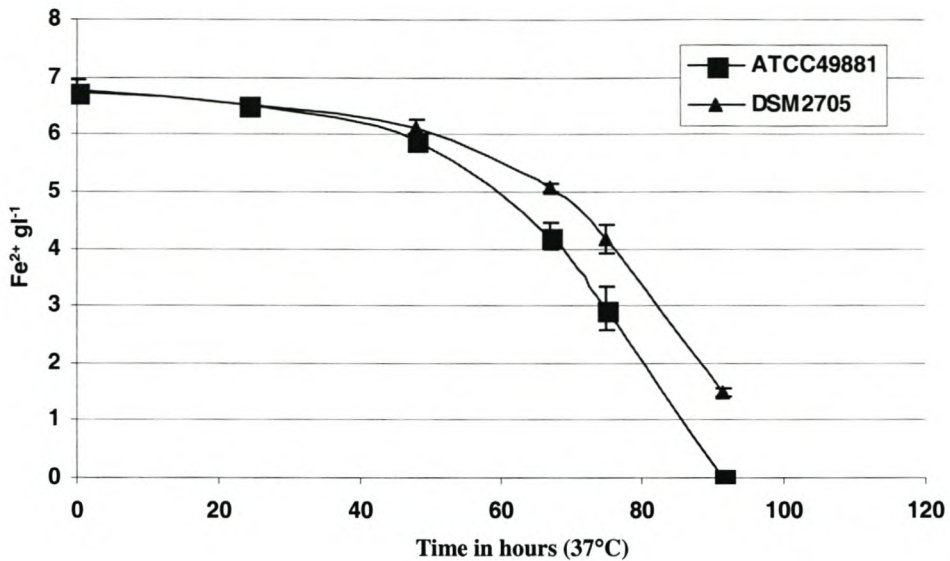


Figure 2.16: Ferrous iron oxidation (in hours). Graphs are drawn from a mean of four different experiments.

From these results it appears as though ATCC49881 was able to oxidize ferrous iron faster than DSM2705. Whether this is a strain phenomenon or applicable to each species as a whole was addressed by performing batch culture iron oxidation experiments on other strains selected randomly from each species group. Figure 2.17 shows the comparison of ferrous iron oxidation between ATCC49879 (*L. ferrooxidans*) and Fairview (type II leptospirilla).

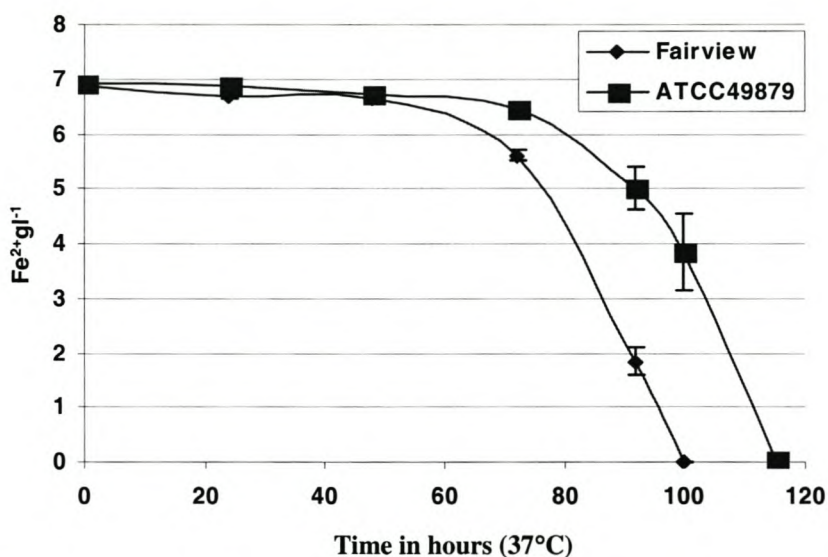


Figure 2.17: Ferrous iron oxidation (in hours). Graphs are drawn from a mean of three different experiments.

Again the type II leptospirilla (Fairview) seemed to oxidize ferrous iron at a higher rate than the type I leptospirilla (ATCC49879- *L. ferrooxidans*). Therefore, the same trend as with ATCC49881 and DSM2705 appeared evident. However, when the data from these two experiments which were carried out under as near identical conditions as possible were combined, there was no clear indication that one *Leptospirillum* species oxidized iron more rapidly than the other (Figure 2.18).

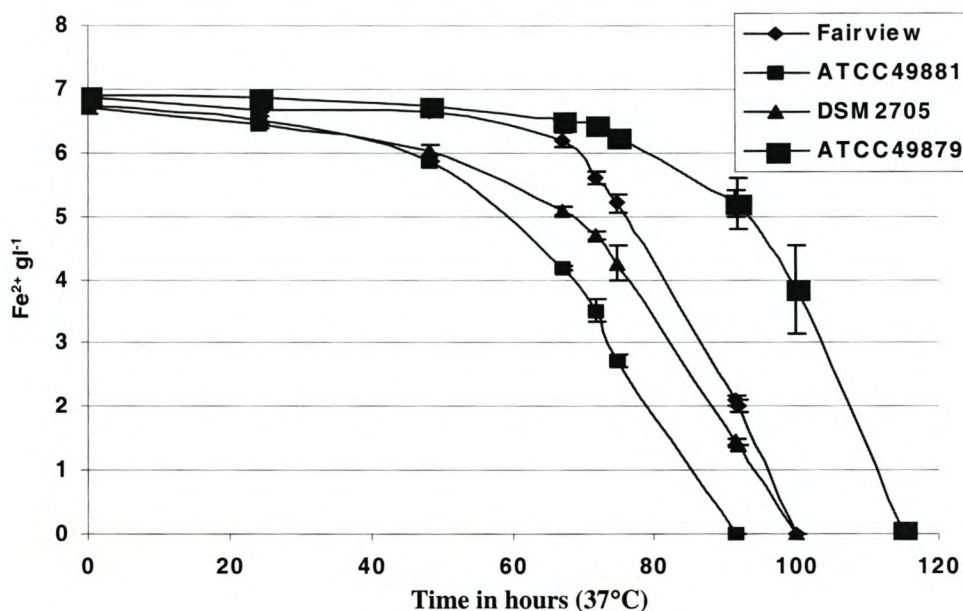


Figure 2.18: Comparison of ferrous iron oxidation ability of type I (DSM2705 and ATCC49879- *L. ferrooxidans*) vs type II (ATCC49881 and Fairview) leptospirilla.

Therefore, it seems that the rate of ferrous iron oxidation is unique to each strain, and is not a consistent characteristic of either *Leptospirillum* species.

2.4 Discussion

Studies on mesophilic leptospirilla by several workers (Goebel and Stackebrandt, 1995; Goebel and Stackebrandt, 1994; Hallman *et al.*, 1993; Harrison and Norris, 1985; Lane *et al.*, 1992) have indicated that more than one species of *Leptospirillum* exists. Nevertheless, all mesophilic leptospirilla have been generally referred to as *L. ferrooxidans* or *Leptospirillum*-like bacteria as there has been insufficient physiological grounds or molecular information to decide whether these leptospirilla represented more than one species. Genomic criteria commonly used to identify two bacteria as belonging to the same species are mol% G+C values that differ by 5% or less and genome DNA-DNA hybridization of about 70% or greater (Stackebrandt and Goebel, 1994). Comparison of 16S rRNA sequence data has been reported to be a somewhat less reliable criterion for separation of organisms into species. As a result of the compilation of data carried out by Stackebrandt and Goebel (1994), it was suggested that organisms with 16S rRNA sequence identities of less than 97% are unlikely to have DNA-DNA hybridization values of above 60%. Similar comparisons have been carried out by Rosselló-Mora & Amann (2001) and they suggested a slightly more relaxed interpretation, that genomes should have less than 50-70% DNA-DNA hybridization before being considered as belonging to different species.

Unlike genomic comparative techniques, where grounds for species separation is governed by specific homology/similarity percentages, phenotypic analysis is designed to reflect the degree of similarity represented within the organisms under analysis through the comparison of a large set of independently varying characters. One or two phenotypic characteristics are inadequate to define a species, and many individual characteristics have been shown to be insufficient as parameters for determining genetic relatedness. Nevertheless, taken as a group, phenotypic characteristics do provide descriptive information, which aid in the recognition of taxa. Evidence of this application is available in Bergey's Manual of Systematic Bacteriology (1984-1989), where bacteria have been divided into 33 different sections, comprising different taxa and genera, described according to a variety of characteristics. Although genetic information often provides sufficient information to justify a species-level separation on its own, the Committee on Reconciliation of Approaches to Bacterial Systematics recommended that a bacterial species

classification should also provide diagnostic phenotypic characteristics (Wayne *et al.*, 1987).

Both genomic, and to a lesser extent, phenotypic characteristics were analysed for the leptospirilla represented in this study. Phenotypic analysis proved difficult due to the metabolic limitations of the bacterium (ferrous iron or ferrous iron-containing sulfide minerals, such as pyrite serves as the sole energy source). Of the characteristics that could be tested (morphology and growth at various temperature and pH values), minor differences in pH ranges were obtained. Differences were however discovered when testing the ability for growth at 45°C. Only members of the type II leptospirilla were able to oxidize iron at 45°C. These isolates included leptospirilla both previously exposed (Fairview, Adapt, BN Mod, and 617) and unexposed (ATCC49880 and ATCC49881) to bioreactors operating at 40°C. Although not all members of the type II group were able to grow at 45°C, none of the type I leptospirilli (*L. ferrooxidans*) grew at 45°C. The differences obtained in growth temperature cannot be used as a diagnostic tool in distinguishing between the two species since the ability to grow at 45°C was not universal within the type II leptospirilla. Nonetheless, the capability of some type II leptospirilla to grow at elevated temperatures was a clear difference, although not taxonomically useful on its own.

The late arrivals to this study (Adapt, BN Mod, and 617) were isolated from pilot plant tanks operating at 45-55°C. This raised the possibility that they could have been isolates of the previously reported but now lost *Leptospirillum thermoferrooxidans* (Golovacheva *et al.*, 1993). However, when grown in pure culture, none of these isolates were capable of growth above 45°C. In addition to this, results from the analysis of 16S rRNA sequence, *rrn* copy number, 16S-23S rRNA intergenic spacer region, and DNA-DNA hybridization, indicated that these were type II leptospirilla. These isolates, although able to grow at 45°C, only achieved elevated temperature tolerance by raising the temperature in small increments over the period of a few weeks. Therefore the ability of the type II isolates to grow at 45°C reflected an ability to tolerate rather than thrive at this temperature. Why some strains could be isolated from the mixed culture, particulate environment of a stirred tank, which was

maintained at 55°C, but struggled to grow at 45°C when in a pure culture, non-particulate environment is uncertain.

We suggest that the mesophilic leptospirilla investigated in this study may be subdivided into two groups and that the differences between these groups are sufficient for them to be regarded as separate species. Although differences in the phenotypic traits tested between the species were minor, the genomic diversity was vast. The mean difference between the mol% G+C ranges of 49-52 vs 55-58% (average~ 6%) is within the recommended 5% or greater mol% G+C difference needed to justify a separate species. The 16S rRNA sequence identity of 91-93% between the two groups was also sufficiently low, which suggests that separation into two species is warranted (< 97%). In addition, the groups differ in that one group has two copies of *rrn* genes while the other group has three copies. Based on the amount of difference between the 16S rRNA sequences of the two groups, one would expect DNA-DNA hybridization values of less than 60% (see Figure 2.4) (Stackebrandt and Goebel, 1994). DNA-DNA hybridization values between group I and group II leptospirilla were much lower than this, falling in a range of < 5 to 11%. Since genomes that display less than 50-70% DNA-DNA hybridization homology are considered as belonging to a different species (Rosselló-Mora and Amann, 2001), the low degree of homology further supports the separation into two species. Conversely, differences in hybridization between subgroups I.1 and 1.2 (60 to 79%) fall within the suggested guidelines for organisms to be considered as a single species (50-70%).

The differences in size of the intergenic region between the 16S-23S rRNA genes, namely 0.5 kb for all type II- and 0.47- to 3.0-kb for all type I- leptospirilla, also adds support for the separation of type II leptospirilla into a separate species. However, whether the type I leptospirilla represent only a single species is not as clear. Within the size range of the group I 16S-23S rRNA IR amplification products, definite subgroups were identified. Isolates P_{3a}, N₂₅, DSM2705, and ATCC49879 grouped together with the same number and size fragments as well as isolates Parys, CF12, and Chil-Lf2. The remaining isolates BCT2, Crys13, and SY were more varied, however Crys13 had a fragment in common with Parys, CF12, and Chil-Lf2. DNA-DNA hybridization results only supported the division of isolates into subgroups I.1

(ATCC49879, P_{3a}, DSM2705, and N₂₅) and I.2 (CF12, Chil-Lf2, Parys, Crys13, BCT2, and SY) within the type I leptospirilla. 16S-23S rRNA intergenic region analyses might therefore be a technique, which could be used to identify, isolate grouping at levels finer than subgroup/subspecies. Whether the differences between these subgroups are sufficient to support a further species level division is doubtful.

Based on the above evidence we propose that the leptospirilla used in this study should be divided into two species, one of which consists of two distinct subgroups or genomovars (Rosselló-Mora and Amann, 2001). The name, *L. ferrooxidans* should be used for group I because the *L. ferrooxidans* type strain (DSM 2705) belongs to this group and we propose that a new species name is required for group II. In the absence of a distinguishing physiological property between all members of both species, we suggest that the name *Leptospirillum ferriphilum* (*ferri* iron; *philum* loving) could be used for the group II leptospirilla. This name reflects a common property of all leptospirilla, which is that they use only ferrous iron as their electron donor. The name *L. ferriphilum* has been validated by the International Committee on Systematic Bacteriology (Validation List No. 86, 2002).

One of the aims of this study was to determine which *Leptospirillum* type dominated industrial biooxidation tanks. This would help to identify which species should be the focus of long-term molecular biology research. Five of the nineteen strains tested in this study were obtained from industrial pilot plants (Fairview, Bionic 3.1, Adapt, BN Mod, and 617). The isolates from the commercial biooxidation tanks at the Fairview mine, and the nickel pilot plant (Billiton) were from the group II leptospirilla (*L. ferriphilum*). Likewise the 45°C adapted 617 and 55°C adapted BN Mod and Adapt isolates belonged to the group II leptospirilla and are therefore also *L. ferriphilum*. These five isolates are representative of the bacterial dominance in South African industrial biooxidation plants operating at 40°C, and do not include overseas plants or plants operating at a different temperature. In a continuous culture study on a culture being prepared for a commercial cobaltiferous pyrite ore bioleaching operation, a *Leptospirillum*-like bacterium (strain L8) was isolated with an optimum pH of 1.3 to 1.6 and temperature of 37.5°C but which could grow at 45°C (Battaglia *et al.*, 1994). This bacterium had a G+C ratio of about 55.6 mol%, which suggests that it was also a

group II *Leptospirillum* (*L. ferriphilum*) rather than *L. ferrooxidans*. These findings confirm the importance of *L. ferriphilum* in industrial operations, but do not necessarily negate the importance of *L. ferrooxidans*. It would be interesting to determine whether strains of the 3 *rrn* gene copy *L. ferrooxidans* group are found in industrial heap leaching or aeration tank type processes that operate at temperatures of lower than 40°C. Since none of the *L. ferrooxidans* strains examined in this study were capable of growth at 45°C, it may be that these bacteria were non-competitive in the tanks that we investigated, all of which operated at temperatures of 40°C or higher. However, *L. ferrooxidans* may well be important in industrial processes that operate at temperatures lower than this.

Description of *Leptospirillum ferriphilum* sp.nov.

Leptospirillum ferriphilum (*ferri* iron; *philum* loving). This description is based on this study and that reported by Sand (Sand *et al.*, 1992). Cells are small curved rods or spirilla, measuring 0.3-0.6 µm wide and 0.9 to 3.5 µm long. Young cells are vibrio-shaped but in cultures older than 4 days cells are mostly spiral-shaped with 2 to 5 turns. Cells are Gram-negative, spore-forming and motile by means of a single polar flagellum. Growth is aerobic and chemolithotrophic with ferrous iron or pyrite but not sulfur serving as the energy source. Optimum pH is 1.4 to 1.8, and temperature 30-37°C with some isolates having the ability to grow at 45°C. Cells are catalase negative and peroxidase positive. G+C content of the DNA is 55-58%, 2 copies of *rrn* genes, and based on 16S rRNA sequence analysis cells form phylogenetic cluster which is separate from *Leptospirillum ferrooxidans*. Size of the 16S-23S rRNA intergenic region is conserved among isolates at 500 bp. The type strain is strain ATCC 49881 that is the same as strain P3A provided by Sand and originally isolated in Peru (Sand *et al.*, 1992).

Chapter 3

A survey of plasmids from the genus *Leptospirillum* and the identification of a plasmid that contains DNA, which is present in all *L. ferrooxidans* isolates

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3.0. Summary

A plasmid search was carried out for all *Leptospirillum* isolates used in this study by means of Pulsed Field Gel Electrophoresis (PFGE). Owing to the instability of *Leptospirillum* total DNA when set in agarose plugs, only three isolates, which contained plasmids, were identified with certainty (49879, CF12, and Parys). The inconsistency in plasmid detection prevents ruling out the presence of plasmids in the remaining isolates. Regardless of the ability to detect plasmids, isolation proved difficult. Plasmid p49879 was the only plasmid to be isolated successfully. This plasmid was DIG-labeled and used as a probe against the remaining fifteen isolates. All members of *L. ferrooxidans* species gave a positive signal on hybridization whereas *L. ferriphilum* species members showed little or no homology. The apparent species bias was investigated in an attempt to determine whether plasmid homology further contributed towards the phylogenetic relationship already shown to exist amongst isolates. The region of homology present in all *L. ferrooxidans* strains was localized to an area showing high amino acid identity to a transposase/putative transposase of *Methanosarcina acetivorans* and plasmid CP1 from *Deinococcus radiodurans* R1 respectively. The origin of the region of homology could therefore be either plasmid or chromosomal. Whether these regions of homology indicate that complete, functional transposons are present in all *L. ferrooxidans* isolates still remains to be determined.

This chapter describes the preliminary mapping, sequencing, and characterization of plasmid DNA isolated from *L. ferrooxidans* ATCC 49879, and a study of the homologous region shared amongst *L. ferrooxidans* isolates. Since mobilization genes and Tn21-like regions were found on the plasmid DNA a brief review of plasmid transfer and some characteristics of the widespread transposon Tn21 are given.

3.1. Introduction

Bacterial plasmids are genetic elements involved in the spread of information within the environment. A plasmid by definition is an extra chromosomal genetic element that confers a local selective advantage (i.e. in a specialized ecological niche or a specific set of environmental conditions), as opposed to the essential activities of the bacterial cell encoded on the chromosome (Coplin, 1989). The selective or

phenotypic trait conferred by the plasmid is not always a mere competitive advantage, but is often crucial for bacterial survival in a particular niche. Examples of such traits are antibiotic and heavy metal resistance, pathogenicity factors, degradation of aromatic compounds, and ultraviolet (UV) resistance. These functions provided by the plasmid are referred to as accessory since they are not essential for plasmid existence, but may select for plasmid retention and possible transfer to other hosts. Some plasmids are cryptic and have acquired mechanisms to ensure that they are stably inherited, or rely on a high copy number to prevent them from being lost. Genes such as these form part of what is collectively referred to as the “plasmid backbone”, which includes genes and sites required for typical plasmid associated functions such as replication, conjugation and stability (reviewed in Rawlings and Tietze, 2001).

The minimum requirement for the existence of a plasmid is the ability to replicate; the genes and sequences, which play a role in replication and its control, are referred to as the basic replicon. Replicons consist of an *oriV* (vegetative origin), and typically one or more proteins essential for plasmid replication. The *oriV* constitutes the site at which replication commences and is required *in cis* for replication to occur (Rawlings *et al.*, 1993). The genes that code for the essential replication proteins may be provided *in trans*. A number of different replication protein configurations exist. Despite the knowledge accumulated on plasmid replication only a limited number of replicons have been studied in detail. Plasmids may be categorized by the different strategies that they employ to initiate and control replication. Two general strategies to facilitate the loading and assembly of the replisome multicomplex at the origin of replication exist.

3.1.1 Plasmid replication systems

3.1.1.1 Theta replication: This involves strand opening at the origin, followed by initiation of leading strand synthesis (Figure 3.1). Two subgroups occur within the theta replicons; one requires host encoded DNA Polymerase I for initiation of DNA synthesis (PolA-dependent) and the other relies on plasmid-encoded proteins for initiation (PolA-independent). Within the DNA PolA-dependent subgroup there are plasmid families whose replication relies on host-encoded proteins alone (i.e.: ColE1 family). Replisome structure and the mechanism of DNA elongation and termination

are likely to be common in PolA-dependent and PolA-independent plasmids, differences arise in the mechanism of initiation (Marians, 1992).

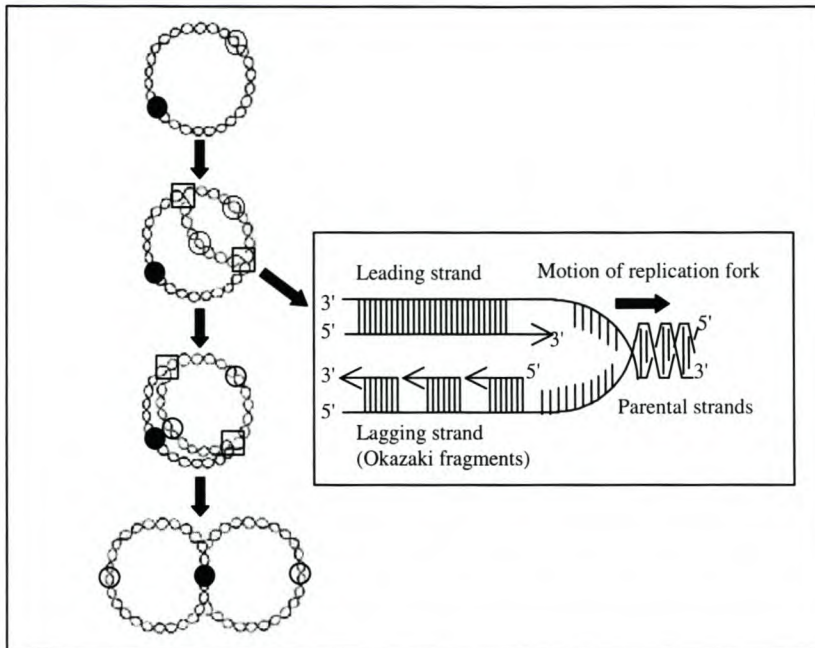


Figure 3.1: A diagrammatic representation of the theta mode of replication. An enlargement of DNA synthesis at the replication fork is shown in the inset. Open circle, origin of replication; closed circle, terminus; open square, replication forks

(<http://www.urmc.rochester.edu/smd/mbi/med/lecZ.html>).

3.1.1.1.1 PolA-independent plasmids

The overall structures of the origins of replication of PolA-independent plasmid replicons have common features (Figure 3.2). These origins typically consist of: (i) recognition sites for the site-specific DNA binding protein (Rep initiator); in most cases a set of short repeated DNA sequences of approximately 20 bp known as iterons serve as binding sites. In IncFII replicons, imperfect palindromes have been shown to occur (Giraldo and Diaz, 1992). (ii) One or more sites for the *E. coli* DnaA protein (*dnaA* boxes); (iii) an A+T rich region adjacent to the iterons; and commonly (iv) GATC sequences. The GATC sequences are target sites for the host Dam methylase which, when methylated, are thought to positively affect initiation (Crooke, 1995). The exact mode of interaction is unknown; direct contact of the methylase to initiation factors is possible, or perhaps methylated DNA is more easily unwound promoting a better DnaA protein interaction. Methylation is not essential for replication; its role is primarily post-replication. The presence of methylation on the parental strand ensures

that changes induced by the proofreading function of DNA-PolII, occur on the daughter strand.

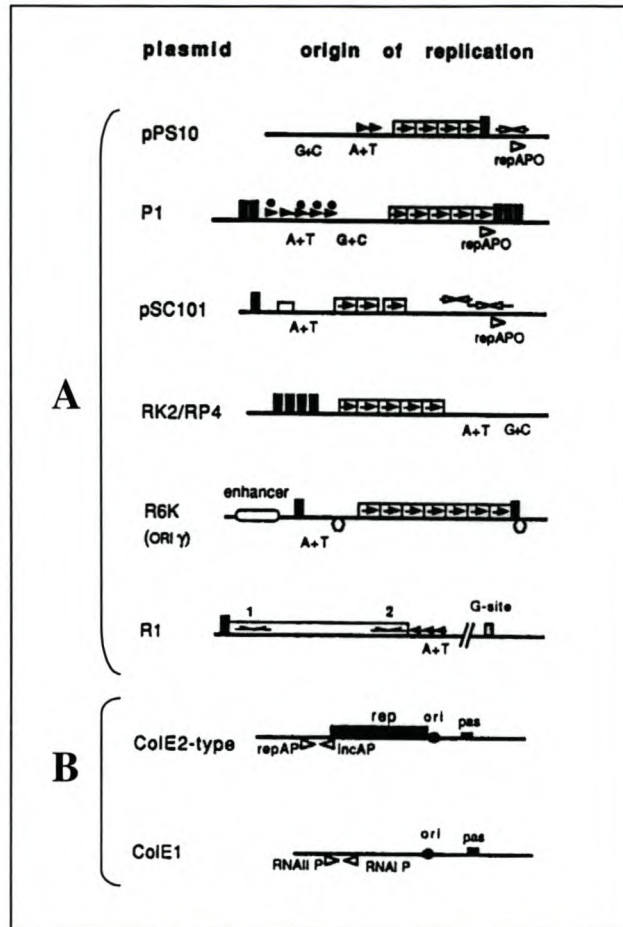


Figure 3.2: Origins of replication of some representative theta-replicating plasmids from gram-negative bacteria. (A) PolA-independent replicons. (B) PolA-dependent replicons. The symbols used are as follows: Arrowheads within boxes, iterons found in origins of replication (*ori*) and incompatibility (*inc*) regions; A+T or G+C, regions rich in these bases; arrowheads over A+T-rich regions, repeats (n-mers). Promoters are indicated as open arrowheads. Solid rectangles indicate *dnaA* boxes, and solid circles, *dam* methylation sites (del Solar *et al.*, 1998).

Initiation of replication generally follows the model proposed for replication at *oriC* of *E. coli* (Kornberg and Baker, 1992). The initiator Rep protein binds to the iterons of the origin and distorts DNA conformation causing a localized "melting" of the double helix at the adjacent A+T rich region (Figure 3.3). This occurs with varying degrees of assistance from DnaA. Opening of the DNA helix facilitates entry and incorporation of the DNA helicase (DnaB) to one of the strands to form the pre-

priming complex. Rep proteins can also interact directly with the helicase (Ratnaker *et al*, 1996) and can play a role in the delivery and activation of the helicase in the replication fork (Konieczny and Helinski, 1997). Binding of the DNA helicase extends the initial replication bubble enabling entry of the primase. Association of the primase (DnaG) at the replication fork is the result of a direct interaction with the DNA helicase, which is necessary for optimal primer synthesis (Lu *et al*, 1996). Entry of DNA Polymerase III holoenzyme completes primosome construction, priming and synthesis of leading and lagging strand extension follows (Kornberg and Baker, 1992).

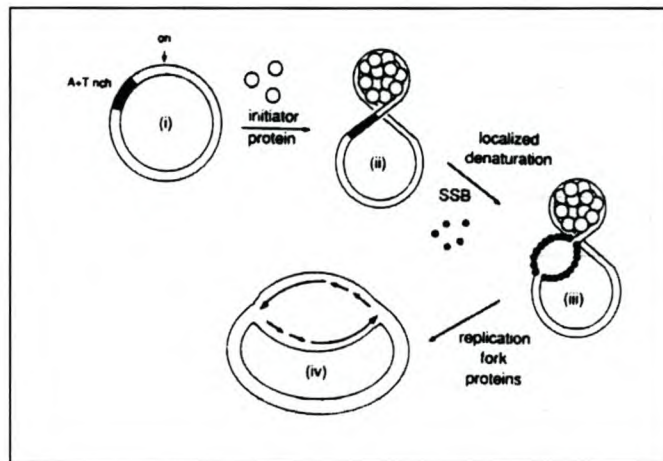


Figure 3.3: Initiation of theta-type replication in PolA-independent plasmids. (i) Plasmid replicon with iterons at *ori*, (ii) initiator proteins bind to iterons forming a nucleoprotein complex at the origin, (iii) localized denaturation at A+T rich region, host encoded single stranded binding proteins bind ssDNA, (iv) open helix facilitates binding of primosome and progression of replication fork as in Figure 3.1 (Marians, 1992).

The IncQ plasmid family, whose prototype is RSF1010, replicate via a strand displacement mechanism (a subgroup of theta replication). Members of this family require three different plasmid-encoded proteins, an initiator protein (RepC), a helicase (RepA), and a primase (RepB), for initiation of DNA replication. These proteins promote initiation at a complex origin, and replication then proceeds in either direction by strand displacement. The first few steps in replication initiation are similar to those of the theta replicating PolA-independent plasmid replicons, however different proteins drive them. For the sake of completion an explanation of these

steps will be repeated, including a detailed description of the roles of the respective IncQ plasmid-encoded replication proteins.

The minimal *ori* region of RSF1010 includes 3.5 20 bp iterons plus a 174 bp region containing a 28 bp GC-rich stretch and a 31 bp AT-rich segment (Figure 3.4) (reviewed in Rawlings and Kusano, 2001; del Solar *et al*, 1998). The origin extends further incorporating two small 40 bp palindromic sequences containing the plasmid-specific single stranded DNA initiation sites, *ssiA* and *ssiB* (Sakai and Komano, 1996). The *ssiA* and *ssiB* sites initiate the priming of single stranded DNA synthesis on opposite strands in leftward and rightward directions, respectively (Miao *et al*, 1993). Palindromes favor the formation of hairpins. The upper part of the putative stem is essential for replication, whilst base complementarity and sequence specificity in the lower part of the stem are important for primer synthesis (Sakai and Komano, 1996; del Solar *et al*, 1998). The start point for plasmid synthesis was mapped to the 3' flanking region of each inverted repeat.

RSF1010 replication is independent of *E.coli* DnaA, DnaB, DnaC, and DnaG proteins as well as RNA polymerase, but is dependent on host DNA Polymerase III and DNA gyrase. Replication initiation begins with binding of the 31 kDa RepC protein, which is active as a dimer, to the iterons in the *oriV*. Binding of RepC causes the localized melting of the DNA at the A+T-rich region in *oriV* (Kin and Meyer, 1991). A secondary interaction of RepC to a site within the A+T-rich region, approximately 60 bp away from the iterons has been suggested. RepC induced DNA melting facilitates the entry of the RepA protein. RepA has a molecular mass of 30 kDa in its monomeric form, and exists as hexamers in an active form. It has two activities: a ssDNA dependent ATPase (stimulated by non-specific ssDNA) and a 5'-3' helicase (del Solar *et al*, 1998). RepA helicase activity unwinds the dsDNA in an ATP-dependent manner, promoting the exposure and activation of the *ssi* sites in a ssDNA configuration. The single strand *ssiA* and *ssiB* sites are now ready for specific recognition and priming of synthesis of the complementary single strand by RepB' primase.

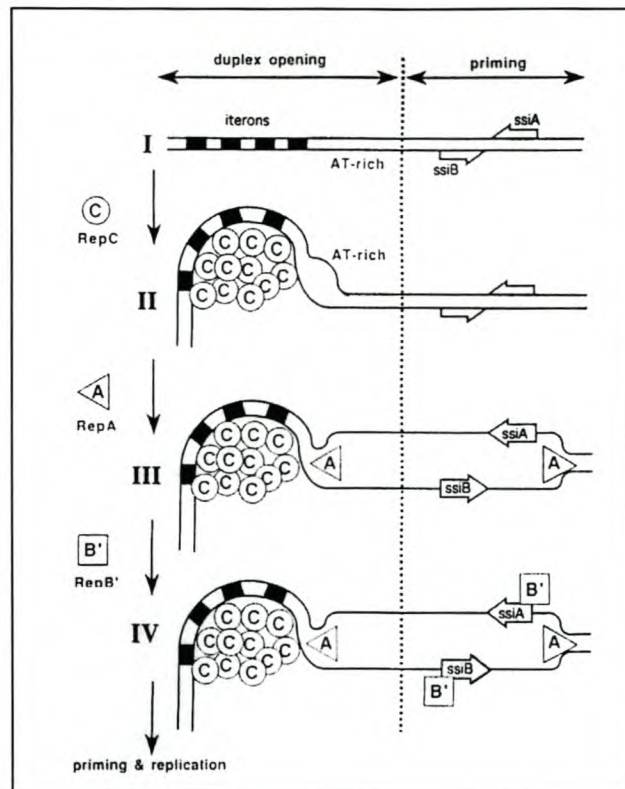


Figure 3.4: Replication of plasmid RSF1010 by the strand displacement mechanism. (I) Replicon of RSF1010, sites of RepB (inverted repeat [convergent open arrows]) and RepC (iterons [boxes]) interaction are indicated. AT-rich indicate regions rich in these bases. Dotted line separates regions of duplex opening and DNA-priming. (II) RepC binds to the iterons at *oriV*, causing a localized melting of the DNA at the AT-rich region. (III) RepA binds at the open DNA complex, unwinding the DNA and exposing single strand *ssiA* and *ssiB* sites. (IV) RepB' binds to *ssiA* and *ssiB*, priming and replication follows (Sakai and Komano, 1996).

The RepB primase occurs in two forms, a 78 kDa MobA-RepB fusion protein (Figure 3.5) and a 36 kDa RepB'. Both are encoded by the same reading frame, the translation start codon of RepB' is downstream from that of RepB (Rawlings and Kusano; Sakai and Komano, 1996). RepB' has a primase activity on *ssiA* and *ssiB*. No other template sequences are utilised by this enzyme (Haring and Scherzinger, 1989). Exposure of the stem-loop structure in the *ssi* sites is probably required for RepB' to initiate replication (Miao *et al*, 1993). It is strongly suggested that RepB' primase is responsible for the initiation of DNA synthesis on both strands by the formation of DNA primers. Initiation at either *ssi* can occur independently and replication proceeds continuously. RepA helicase facilitates displacement of non-replicated parental strand forming a D-loop. Opposed continuous replication from

each *ssi* results in the formation of single stranded displaced circles carrying either *ssiA* or *ssiB*, these sequences are used to initiate synthesis of the complementary strand, converting ssDNA templates to double stranded supercoiled circles (del Solar *et al*, 1998).

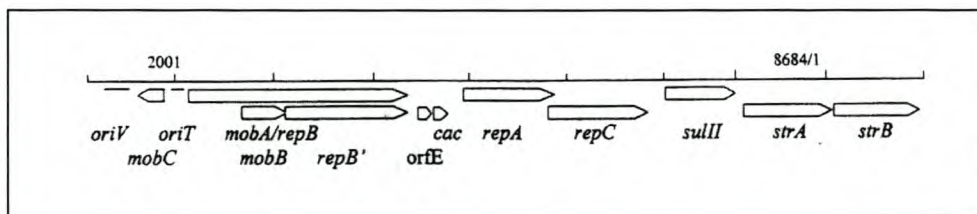


Figure 3.5: Genetic map of plasmid RSF1010 of the IncQ family. Genes and structural features: *oriV*, origin of vegetative replication; *oriT*, origin of transfer; *mobA*, *mobB*, and *mobC*, mobilization genes; *repA*, *repB*, *repB'* and *repC*, replication genes; *cac*, control of *repA* and *repC* regulator; *orfE*, open reading frame of unknown function; *sulII*, sulfonamide-resistant dihydropteroate synthase; *strA* and *strB*, streptomycin aminoglycoside phosphotransferase (Rawlings and Tietze, 2001).

3.1.1.1.2 PolA-dependent plasmids

The PolA-dependent plasmid replicons carry origins of replication that display a lesser degree of similarity to those of the PolA -independent replicons (Figure 3.2B), and consequently include diverse modes of replication initiation. In contrast to the PolA-independent replicons, where replisome assembly occurs before DNA replication, replisome assembly occurs after an initial synthesis of the leading strand carried out by host PolA (DNA Polymerase I). The reason for this is that the "starting points" of initiation are replication signals that are functional only in the single-stranded conformation and are exposed and activated by a strand displacement reaction (Figure 3.6) (Marians, 1992). Following primer synthesis, PolA attaches to the displaced single strand at a 3'OH terminus to initiate DNA synthesis and replisome assembly. While the latter two steps appear to be highly conserved, the mechanism of primer formation differs greatly.

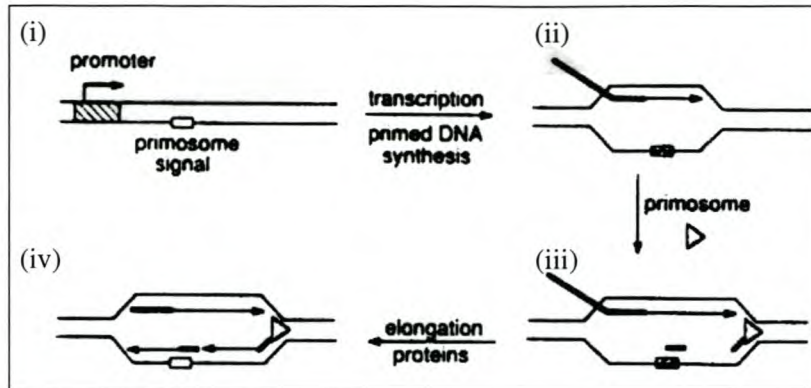


Figure 3.6: Initiation of theta-type replication in PolA-dependent plasmids. (i) Recognition of promoter at origin by either host RNAP (ColE1) or plasmid-encoded initiator proteins (ColE2), (ii) RNA transcript is synthesized, (iii) replisome assembly, (iv) DNA replication proceeds normally from replication fork as in Figure 3.1 (Marians, 1992).

In the ColE1 family the host-encoded RNAP and RNaseH perform primer synthesis. RNAP recognizes the external promoter (RNAII P_>) (Figure 3.2B) and synthesizes an RNA transcript that extends beyond the initiation site of DNA replication. An RNA/DNA hybrid is formed on one template strand, either transiently or in a stable D-loop. RNaseH cleaves the RNA moiety at one of three consecutive A residues, providing a 3'OH terminus for initiation of PolA-directed DNA synthesis (Espinosa *et al*, 2000).

The ColE2 family relies on the priming function of the plasmid encoded initiator protein. The Rep protein recognises and binds to the origin where it synthesizes a short RNA molecule, with a unique structure of 5'-ppApGpA (Takechi, 1995). The hosts DNA Pol A recognize this primer.

Primer formation in the pAM β 1 family is not yet fully understood, although thought to involve RNAP transcript synthesis followed by plasmid encoded Rep processing (Reeder and Lang, 1994). There are two models which tentatively describe the role of Rep. Once the RNA transcript has been synthesized the Rep protein acts either as an "annealase" or "stop-protein", in both cases halting RNA chain elongation and inducing cleavage of the nascent transcript approximately 10 nucleotides from the 3' end. This processing releases the 5' end of the transcript, generating a short cleavage product for use as a primer.

Once the first step of initiation is achieved no more specific events are required and the different replicons can take advantage of the host replication enzymes to fulfil remaining steps of replication and eventually termination.

3.1.1.2 Rolling circle replication (RCR)

A large number of small multicopy plasmids in different bacteria have been found to replicate by a rolling-circle (RC) mechanism. Although differences in the mode of replication exist between the different families, a basic pattern does exist. Replication is initiated by the plasmid-encoded Rep protein, which introduces a site-specific nick in the parental [+] strand, at a DNA region termed double strand origin, *dso*. The nick introduces a 3'-OH end, which serves as a primer for leading strand synthesis. In some plasmid families the Rep protein becomes covalently attached to the 5' phosphate end of the DNA through a phosphotyrosine linkage. Elongation from the 3'-OH, probably done only by host proteins, is accompanied by the displacement of the parental [+] strand. This continues until the replisome reaches the reconstituted *dso*.

Lagging strand synthesis, which occurs at single strand origin (*sso*), and generally depends on the host RNAP, converts ssDNA intermediates into dsDNA forms. Finally replication products are supercoiled by the host DNA gyrase (Figure 3.7).

Essential factors required for RC-replication in plasmids are collectively referred to as, the leading strand initiation and control region (LIC). This comprises the double strand origin (*dso*), the rep gene, and the plasmid elements involved in control (del Solar *et al*, 1993). Based on homologies observed in the essential LIC module, five plasmid families have been defined, their prototypes being (i) pT181, (ii) pMV158, (iii) pC1941, (iv) pSN2, and (v) pIJ101/pJVI. (major RC families are underlined). A complete list of plasmids in each family is reported in Khan (1997).

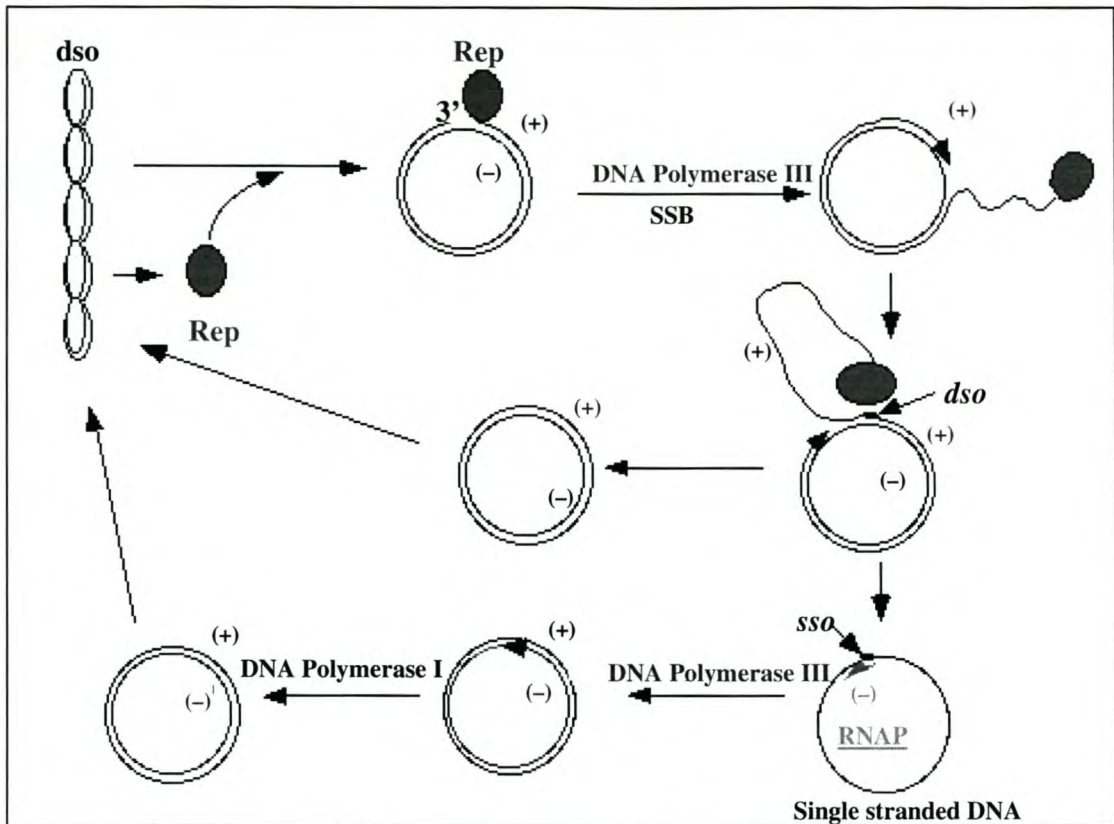


Figure 3.7: Diagrammatic representation of rolling circle replication (RCR). *dso*, double strand origin; *ssso*, single strand origin (Modified from Espinosa *et al.*, 1995).

3.1.1.2.1 Double strand origins (*dso*)

Replication of the parental [+] strand is initiated from the leading-strand origin (*dso*) and proceeds in a unidirectional manner. Two loci have been defined within the *dso*, namely, the *bind* and *nic* regions (del Solar *et al.*, 1998). The former is the binding site of the Rep initiator protein, whereas the latter contains the site where Rep cleaves the plasmid DNA (nick site). These two regions can either be adjacent to each other (pT181 family) or can be separated by a spacer region of 13 to 91 nucleotides (pMV158 family) (Khan, 1997). Most origins, with the exception of the pUB110 origin (pC194 family), contain sequences that have the potential to form secondary hairpin structures (Figure 3.8). Sequence flexibility in the *nic* and *bind* regions promotes a DNA topology, which facilitates Rep binding (Gruss and Ehrlich, 1989; Alonso *et al.*, 1988). The nick region contains inverted repeats able to generate one or two hairpin structures, the Rep *nic* site is generally located on unpaired regions (loop) of the hairpin.

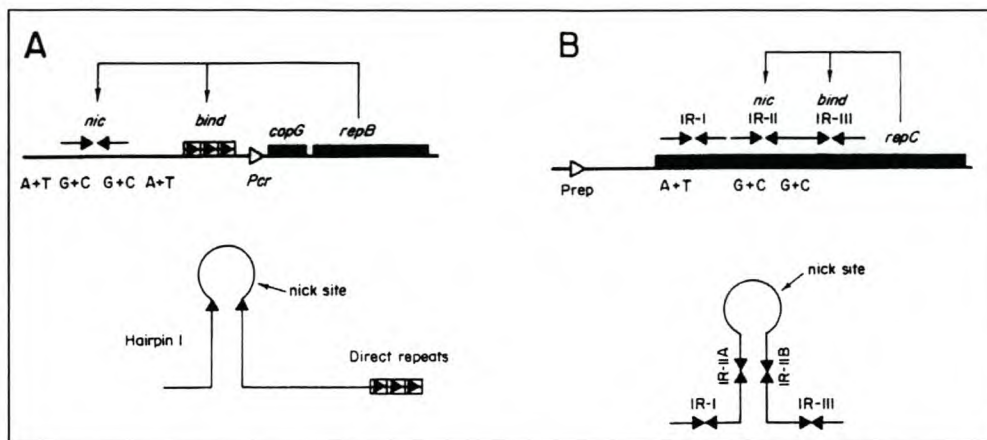


Figure 3.8: Secondary structure created at the *dso* origins of replication of plasmids pMV158 (A) and pT181 (B) for the initiation of rolling circle replication. The *bind* and *nic* regions are indicated, as are the approximate sites where nicking takes place (nick). Thin arrows indicate sites where the initiator Rep proteins act; iterons, arrowheads in boxes; IR, inverted repeats; open triangles, promoters; A+T/G+C, areas rich in these bases; *capG*, transcriptional repressor of *repB*. (del Solar *et al*, 1998).

DNA sequences of the *bind* regions consist of either an inverted repeat contiguous to the nick site (IRIII in the pT181 family), or a set of two or three direct repeats (iterons), which are separated from the nick site by intervening sequences (pMV158 family). Figure 3.8 shows a structural comparison between the *bind* regions of these two plasmid families (del Solar *et al*, 1998). It seems that only the pMV158 family contain directly sequences within their *dso* (del Solar, 1993).

Essential regions involved in the interaction with the plasmid initiator protein vary and have been determined for the different plasmid families (Wang, *et al* 1993; Gruss and Ehrlich, 1989; del Solar *et al*, 1998). A typical feature however is that the *nic* regions are highly conserved among replicons of the same family, whereas differences are found at the *bind* loci.

Not only is the *dso* a site for replication initiation, but for termination as well. For plasmids of the pT181 family it has been shown that after a round of replication the replication fork proceeds approximately 10 nucleotides beyond the regenerated Rep *nic* site (due to Rep-DNA association at the RH IRII and IRIII), where replication is arrested and Rep cleaves at *nic* (Khan 1997). Only the *nic* site (not the *bind* site) has been shown to be necessary for termination, via a specific, albeit weak, interaction. Finally the Rep protein is inactivated by covalent attachment of an oligonucleotide

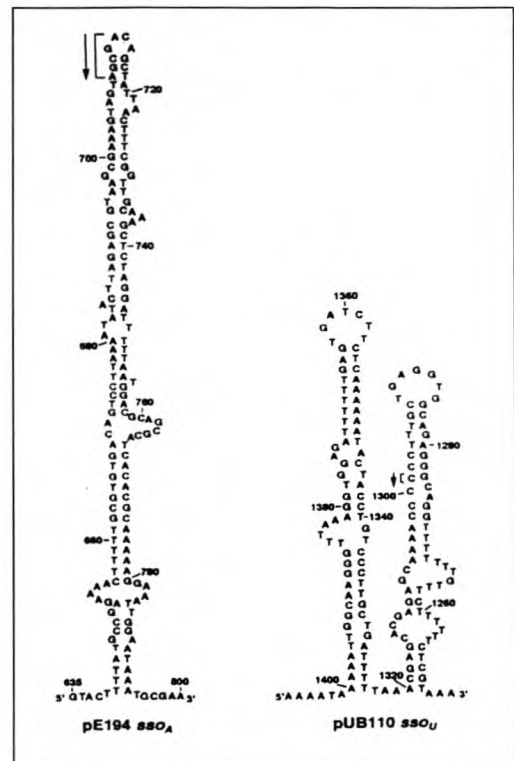
(10mer) to the active tyrosine residue, which is responsible for nicking-closing of the DNA. Prevention of Rep initiation recycling is critical in the control of over-replication (del Solar *et al*, 1998). The model for pT181 termination does not apply to the pMV158 family since the Rep protein does not remain covalently bound to the DNA, no alternative models for leading-strand termination have been reported.

3.1.1.2.2 Single strand origins (*sso*)

Lagging strand synthesis initiates and terminates at the *sso*. The SSOs are non-coding DNA regions able to generate large stem-loop structures, which function in an orientation dependent manner. This suggests that the ssDNA to dsDNA conversion requires unpaired sequences within the secondary structure that constitute the *sso* (Espinosa *et al*, 2000). Unlike DSOs, SSOs are generally not homologous among plasmids belonging to the same family. Several SSOs have been identified based on their secondary structures, such as *sso_A* (pT181 and pC194), *sso_U* (pUB110), *sso_T* (pTA1060 and pBAA1) and *sso_W* (pWV01) (Boe, 1989; Khan, 1997; del Solar *et al*, 1998 and Meyer *et al*, 1998). Plasmid pMV158 bears both a *sso_A* and a *sso_U*. SSOs are generally host specific (*sso_A*-type origins), however some *sso_U* origins have been shown to display a wider host range (Boe, 1989), which could contribute to plasmid promiscuity and horizontal transfer amongst related bacteria.

Analysis of the DNA sequence and structure of the *sso_A* of various plasmids showed the presence of two conserved unpaired regions (Figure 3.9) (Novick, 1989). A 6-nucleotide sequence 5' TAGCGA/T 3' (*CS₆*) located in the central loop of the secondary structure and a recombination site, *RS_B*, involved in plasmid recombination. The RNA polymerase has been shown to bind to ssDNA only if an intact *RS_B* sequence is present, which is expected to be in the dsDNA formed by the secondary structures, and synthesis of an ~20 nucleotide RNA primer which is subsequently terminated at *CS₆*. *CS₆* marks the site for RNA primer synthesis termination and the point at which the host DNA Polymerase I initiates DNA synthesis (Khan, 1998). Other sequence structures reported in SSO's include two inverted repeats (IR1 and IR2) of pWV01 *sso_W*, and sequence that weakly resembles -35 and -10 regions in plasmid pLS1 and pT181. Following replication initiation, lagging strand synthesis commences until replication is terminated.

Figure 3.9: Comparison between folded structures of *ss_{oA}* (pE194) and *ss_{oU}* (pUB110). The brackets and arrows indicate the major start sites of lagging-strand replication from these SSOs. The bracket in pE194 *ss_{oA}* also corresponds to the conserved CS₆ sequence. The first stem-loop structure located at the bottom of the pE194 *ss_{oA}* corresponds to RS_B, which is the binding site for RNA polymerase (Khan, 1997).



3.1.1.2.3 Initiator proteins

The role of the plasmid encoded Rep protein in RCR is two-fold. Firstly a nicking activity is essential for initiation of replication, whilst cleavage and joining (in a type I topoisomerase-like fashion) is essential for replication termination. The Rep proteins for each plasmid family, regardless of their differences, display both of these qualities. Rep proteins encoded by RC-replicating plasmids have several conserved motifs that are shared with the Tra/Mob proteins involved in plasmid transfer through conjugation (discussed later) (Pansegrau and Lanka, 1991; Waters and Guiney, 1993). The presence of two other domains corresponding to enzymatic activity and metal binding were also shown. Interestingly plasmids of the pT181 family do not have this last motif.

The **RepC of pT181** functions as a dimer although dimerisation may occur after RepC has bound to its target DNA (Zhao and Khan, 1997). The conserved phosphodiester bond 5'-ApT-3' is cleaved by residue Tyr191 of RepC, which remains covalently bound to the 5'-phosphate end generated by the cleavage reaction (Thomas *et al*, 1995). Once a RepC dimer has been used it becomes inactive for a few rounds of replication because of the attachment of an oligonucleotide to one of its subunits, generating a heterodimer, RepC/C* (Rasooly, 1994). Biochemical analysis has shown

RepC to be identical to RepC*, except for the absence of the active tyrosine residue in the modified protein. Since the pT181 RepC assembles as a dimer on the DNA, the active Tyr191 residues of both subunits probably participate in the transesterification reactions during the termination step.

The **RepA of pC194** was shown to have two different catalytic residues, one (Tyr 214) involved in nicking of the DNA during initiation and the second (Glu 210), activates a water molecule causing the hydrolysis of a phosphodiester bond resulting in termination. Re-initiation in this case is prevented by the utilization of a glutamate residue in place of a tyrosine during the termination step (Khan, 1997).

The **RepB of pMV158** does not form a stable covalent tyrosyl-phosphodiester bond with its target DNA (Moscoso *et al*, 1997). A transient bond between RepB and a DNA sequence containing the cleavage site of RepB (5'-GpA-3') could however mediate the initiation of replication. A weak covalent interaction between RepB and the nick site that could mediate nicking and closing was predicted from analysis of the chirality of the phosphate involved in cleavage (del Solar *et al*, 1998). Figure 3.10 illustrates the functional organization of the pT181-, pMV158-, and pC194-family replicons.

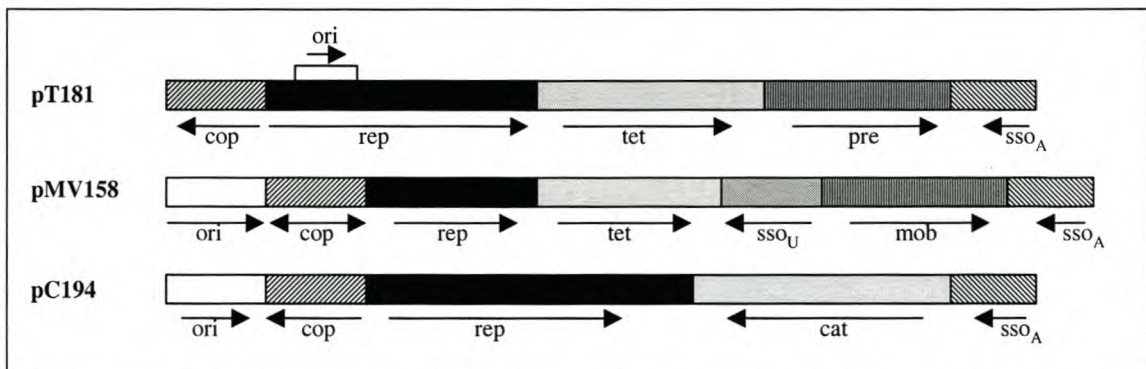


Figure 3.10: Functional organization of RC plasmids belonging to three different families. Maps are not drawn to scale. Direction of gene transcription and direction of replication from the origins (DSO's) are shown. Plasmid pMV158 encodes two copy control genes (an antisense RNA and a repressor), which are transcribed in opposite directions. Plasmid pMV158 contains both *ssoA* and *ssoU* sequences, whereas the other plasmids contain only one SSO. Ori, double-strand origin; *sso*, single-strand origin; *rep*, initiator gene; *cop*, copy control gene(s); *pre*, recombinase gene; *mob*, mobilization function; *tet*, tetracycline resistance gene; *cat*, chloramphenicol resistance gene (Khan, 1997).

3.1.1.3 Control of plasmid replication

The basic strategy employed by plasmids to maintain themselves in host cells is the coupling of plasmid DNA replication to the growth of the host cell. This is achieved through the synthesis of inhibitors (negative regulators) to curtail replication, should plasmid copy number rise above a certain level. Mechanisms controlling replication have been studied in various systems, and several types of inhibitors have been detected. (i) Antisense RNA (ColE1 and pT181); (ii) both antisense RNA and a repressor protein (pMV158 and R1), and (iii) DNA sites for binding of initiator proteins (F, P1, RK2, and R6K).

3.1.1.3.1 Control by antisense RNA

Control by antisense RNA can be either direct (ColE1), or indirect (pT181). Direct regulation occurs when the inhibitor prohibits the synthesis of an RNA primer, while indirect regulation controls the level of a rate-limiting Rep protein. In both cases control is performed by antisense RNAs (80-150 nt), complementary to a region in the 5' end of the target transcript (preprimer RNA for replication or *rep* mRNA), also called "countertranscribed" RNAs (ctRNAs) (Novick, 1987; Novick, 1989). The inhibitory interaction involves complementary base pairing between inhibitor and target transcripts at the unpaired hairpin loop region of their secondary structures. The complex formed by this interaction is called a "kissing" complex, which modifies the secondary structure of the target transcript, thereby interfering with its function (Tomizawa, 1982; Wong, 1985; Masukata, 1986). Figure 3.11 shows a diagrammatic comparison of direct versus indirect regulation of inhibitor-target plasmids, and the mutational effects of ctRNA on plasmid replicons.

In plasmid ColE1 (as previously mentioned) initiation of replication depends on the formation of an RNA primer (RNAII), which is made by host encoded RNAP and processed by RNaseH. Processing of the DNA-RNA hybrid is required for the transition from RNA to DNA synthesis, by generating a 3'-OH end recognized by PolII. The availability of the 3'-OH end is rate limiting for initiation, and therefore the target of ctRNA binding (del Solar *et al*, 1998). Hybrid formation between the antisense RNAI and the preprimer RNAII occurs only when the secondary structure of the nascent RNAII transcript comprises three stem-loop domains (I, II, and III), which are complementary to the hairpin structures formed by RNAI (Figure 3.11A).

This association alters the secondary structure of the preprimer, the 3'-OH end of which is no longer available to hybridize to the DNA, preventing DNA-RNA hybrid formation. This removes the target for RNaseH processing and inhibits the initiation of replication (Tomizawa, 1981). Formation of stem-loops I, II, and III depends on the associations formed between areas α , β , and γ which occur along the length of the RNAII transcript (Cesareni *et al*, 1991). When RNA polymerase has transcribed approximately 200 nucleotides, I, II, and III are formed, this conformation is only transient as the polymerization of several more nucleotides disrupts stem III and creates a new, longer stem (IV), which is stabilized by hydrogen bonding between α and β sequences. As transcription proceeds, two mutually exclusive structures become possible, depending on whether β remains paired with α , or instead contributes to the formation of a new stem-loop structure (V), by pairing with the downstream complementary sequence γ . A $\beta\gamma$ complex causes hairpins I, II, and III to reform, RNAI can therefore associate with RNAII- $\beta\gamma$ and as a consequence reduce the plasmid copy number. An auxiliary role is provided by the Rop protein (Som and Tomizawa, 1983). Rop acts as a stabilizer, increasing the formation efficiency of the "kissing" complex and thus decreasing the efficiency of replication.

In the model for the control of replication of pT181 it is proposed that synthesis of the Rep initiator (RepC) is inhibited by two ctRNAs (RNAI and RNAII), which are complementary to the untranslated 5'-end of the *rep* mRNA (Kumar, 1985; Novick, 1987; and Novick, 1989). Both RNA species can form the ctRNA-mRNA hybrid, but act independently of each other. Hybrid formation results in a conformational change at a region disassociated from the region of complementarity (Figure 3.9B). As the ct-Rep mRNA complex is transcribed, hairpin structures resembling transcription termination sites are formed, resulting in the premature termination of transcription. Therefore transcriptional attenuation is the mechanism for controlling the copy number of pT181 (del Solar *et al*, 1998). Overexpression of the RepC protein of pT181 has also been shown to inhibit plasmid replication (Khan, 1998), due to the formation of inactive RepC-origin complexes. Thus while initiator proteins are normally rate limiting for replication, regulation of replication by the initiators may also occur under conditions in which the Rep proteins are overexpressed.

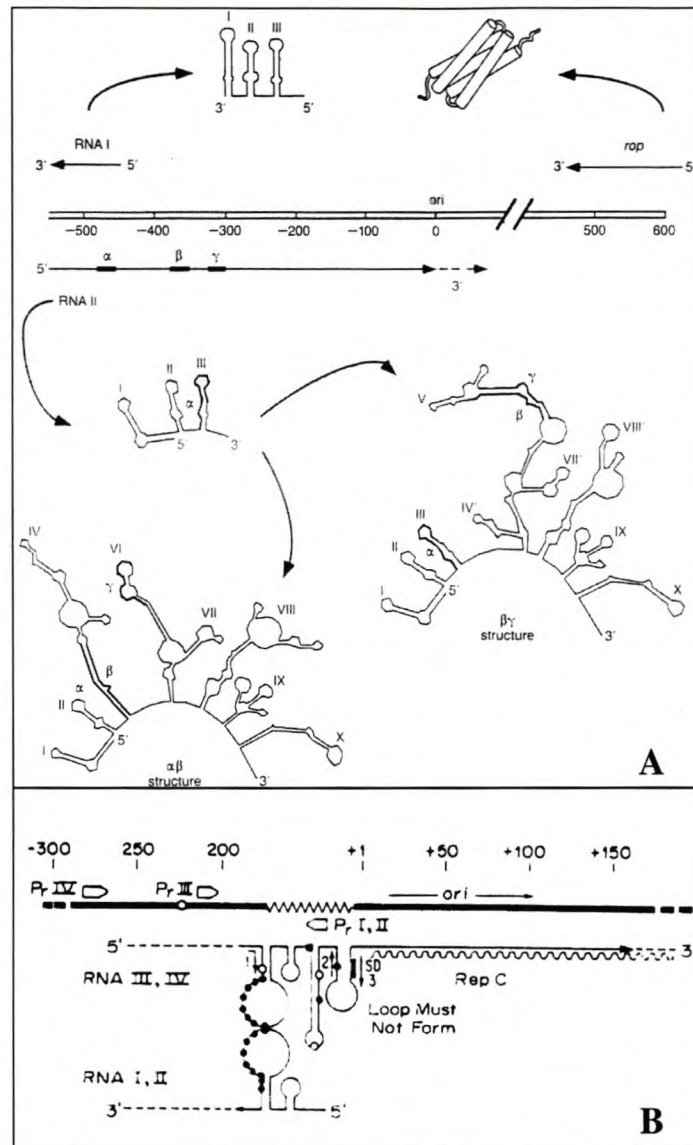
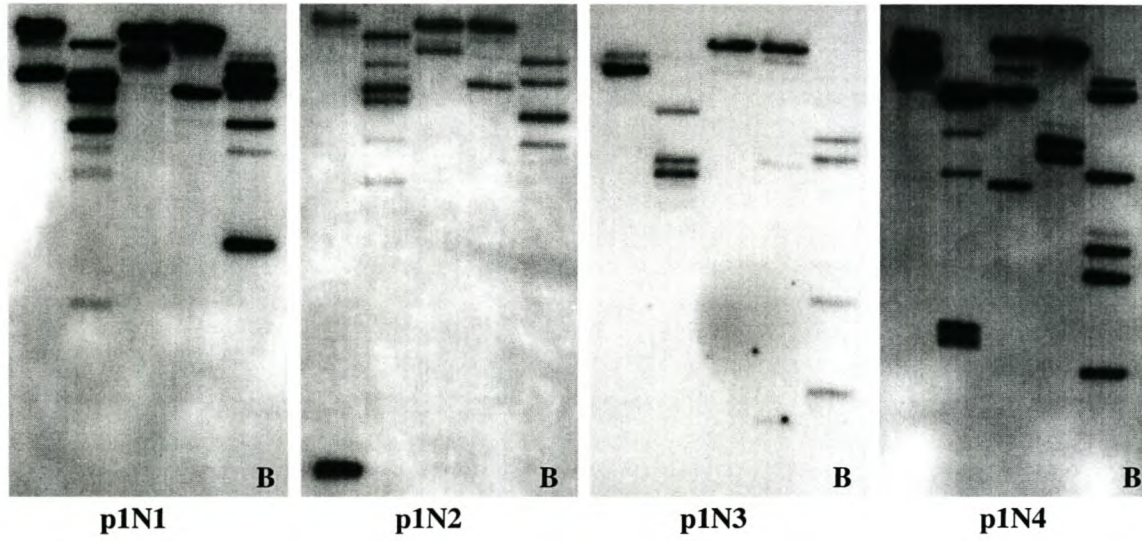


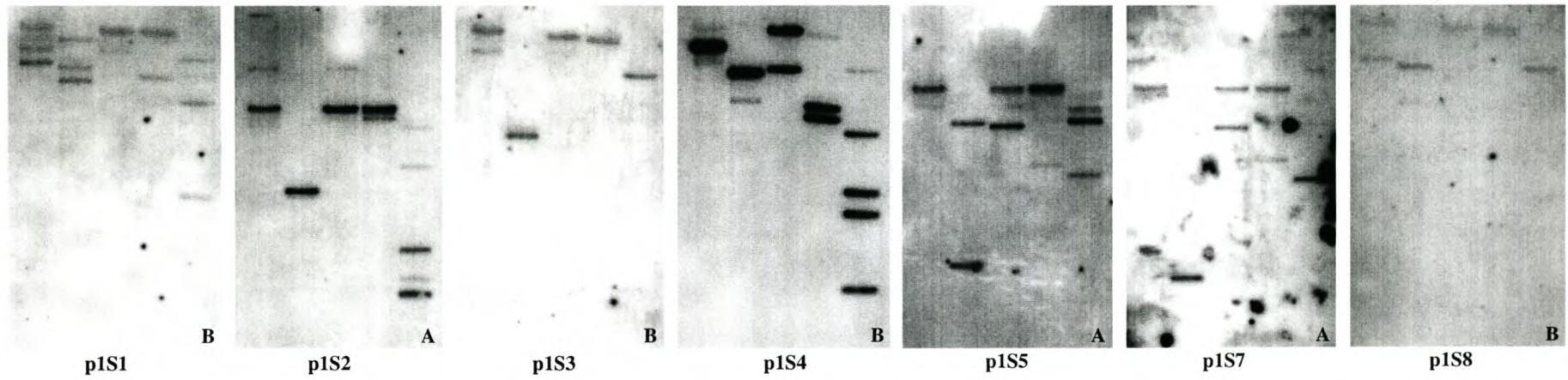
Figure 3.11: Direct versus indirect regulation of inhibitor-target plasmids. (A) ColeE1: Two convergent promoters PrI and PrII. Synthesis from PrII (RNAII) results in the formation of the preprimer necessary for replication initiation. Synthesis from PrI (RNAI) yields a short ~108 nucleotide transcript. Pairing of the preprimer sequences, α and β , is required for the formation of the downstream hairpin necessary for preprimer processing; binding of RNAI to RNAII prevents this (Tomizawa *et al*, 1981). Formation of the $\beta\gamma$ complex reinstates hairpins I, II, and III necessary for RNAI binding, and as a consequence results in a reduction of plasmid copy number (Cesareni *et al*, 1991). (B) pT181: Three different promoters PrI/II, PrIII, and PrIV. RepC mRNA is transcribed from promoters PrIII and PrIV. Two small RNA species (RNAI and II) are transcribed from a single promoter PrI within the leader from the opposite strand. Pairing of sequence 1 and 2 (labeled arrows) prevents the formation of the 2-3 stem, which is thought to interfere with translation of the initiator protein *repC* (wavy line), and it is the 1-2 pairing that is blocked by RNAI/II binding to RNAPIII/IV (Kumar and Novick, 1985). Pr, Promoter; SD, Shine-Dalgarno site; *ori*, replication origin (Novick, 1987).

B.1 Plasmid p49879.1 hybridization results

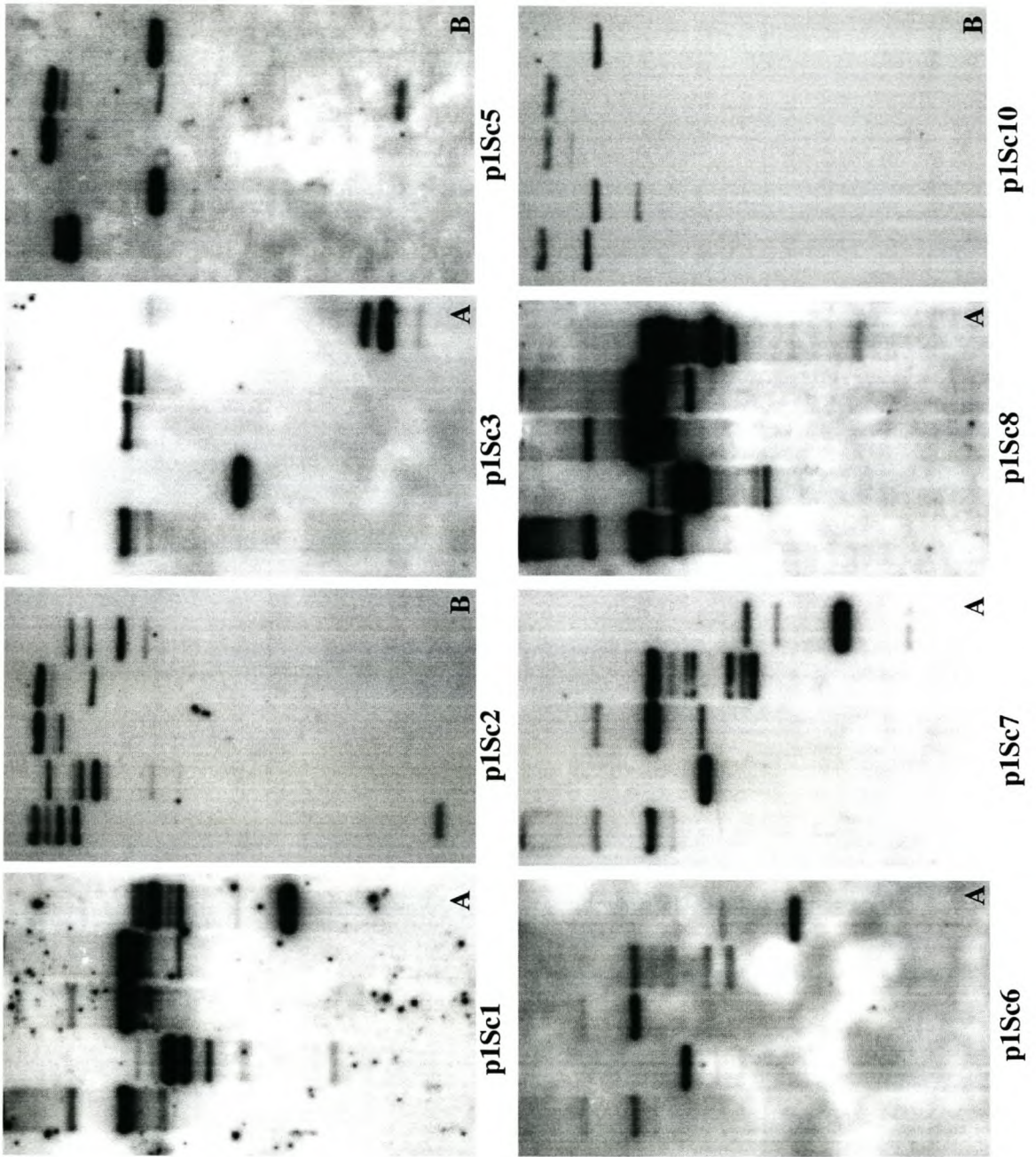
B.1.1 *NotI* probes



B1.2 *SalI* probes

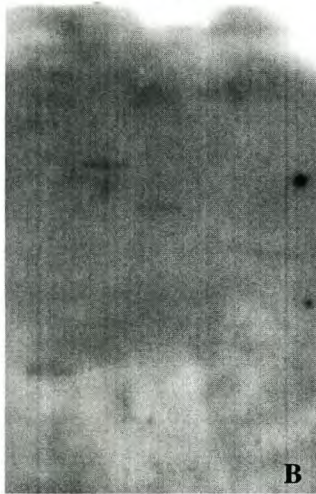


B.1.3 *Sac*II probes



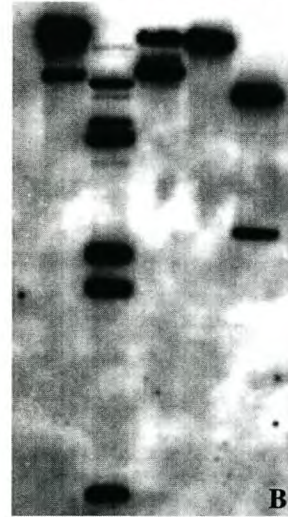
B.2 Plasmid p49879.2 hybridization results

B.2.1 *NotI* probes

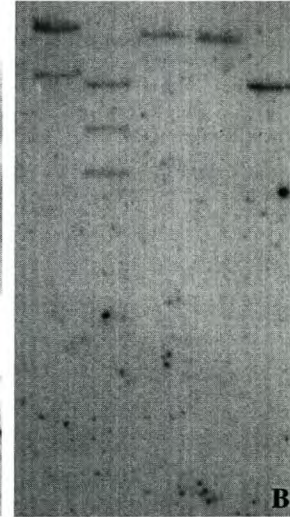


p2N1

B.2.2 *SacII* probes



p2Sc3

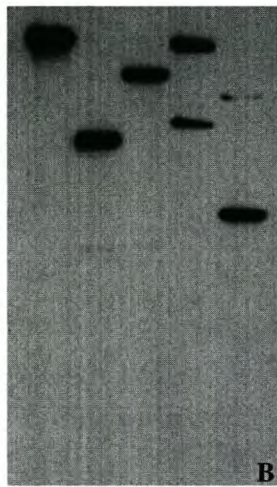


p2Sc4

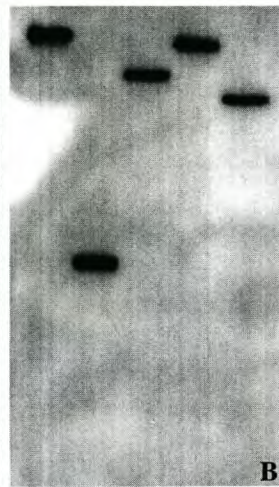
B.2.3 *SalI* probes



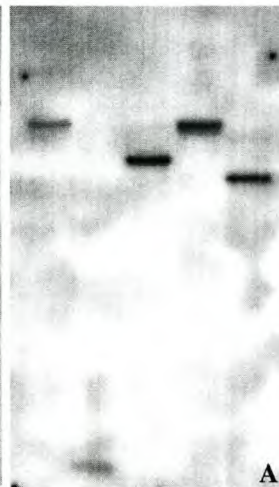
p2S1



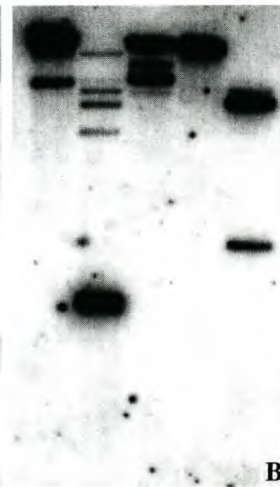
p2S2



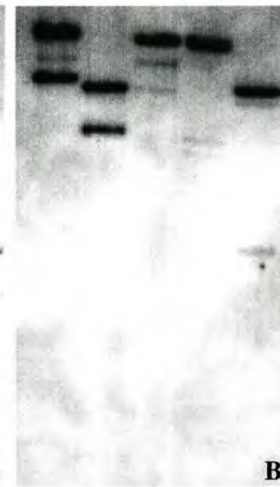
p2S4



p2S5



p2S6



p2S7

3.1.1.3.2 Control by both antisense RNA and a transcriptional repressor

Control in these systems is two-fold, inhibiting expression at both transcriptional (repressor protein) and post-transcriptional (ctRNA) levels. Post-transcriptional mediated inhibition also involves a “kissing” complex between target and inhibitor transcripts, blocking translation initiation of the respective *rep* mRNAs.

Replicative control of plasmid R1 is modulated by the products of the copy number control genes *copA* and *copB* (Wagner and Simons, 1994). CopA is an unstable antisense RNA complementary to the leader region (CopT) of the *rep* mRNA, and is the primary inhibitor (Figure 3.12). The CopA-CopT hybrid inhibits RepA synthesis by hindering the translation of a leader peptide-encoding gene *tap*, which is translationally coupled to RepA (Blomberg *et al.*, 1992). The CopB protein plays a secondary role by repressing transcription of *repA* from a promoter, P₂, located downstream of *copB*. Under steady-state conditions CopB is in abundance and totally represses transcription from P₂. The *repA* mRNA synthesized at this time takes the form of a polycistronic *copB-tap-repA* mRNA. Inhibition at P₂ is derepressed when the copy number drops or is low at the early stages of plasmid establishment, and *repA* is transcribed as *tap-repA* mRNA.

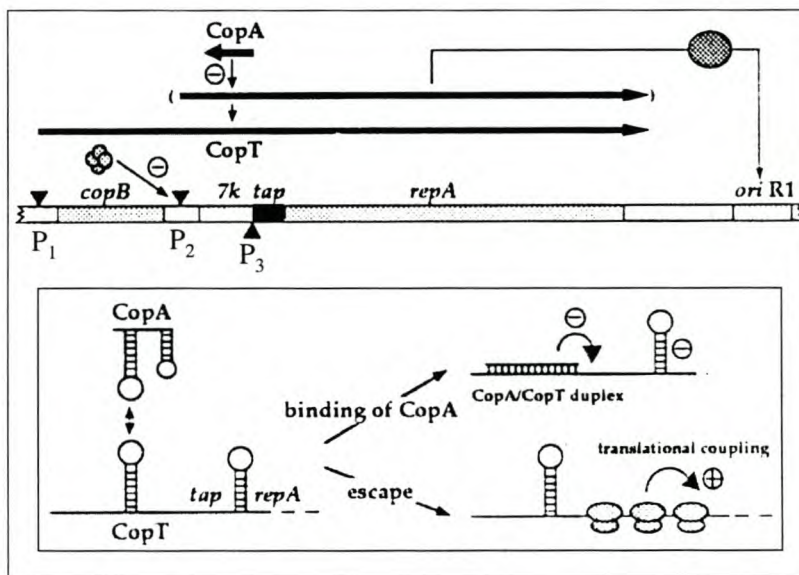


Figure 3.12: Control of replication of plasmid R1 by CopA RNA and CopB. Genes and sites of the basic replicon are shown. Promoters (filled triangles): P₁, *copB* promoter, P₂, *repA* promoter, P₃, *copA* promoter. Negative regulation is indicated by minus signs. The consequences of antisense RNA binding or of RepA mRNA escape are shown schematically. Details are discussed in the text (Wagner and Simons, 1994).

In the case of plasmid pMV158 the inhibition is carried out primarily by CopG, a transcriptional repressor, which binds to and represses transcription from a single promoter for both the *copG* and *repB* genes (Figure 3.13). The ctRNA (RNAII), which is complementary to a region of the *cop-rep* mRNA between the *copG* and *repB* genes, is the secondary element involved in copy number control (del Solar *et al*, 1998).

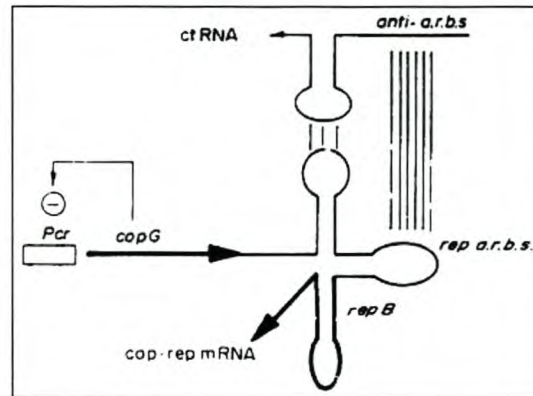


Figure 3.13: Control of plasmid replication by both antisense RNA and a transcriptional repressor in pMV158. Transcripts are shown as continuous lines, with arrowheads indicating direction of synthesis. mRNAs are shown with thicker lines than antisense RNAs; ctRNA, thin line with small arrowhead. Other symbols: rectangles, promoter; a.r.b.s., atypical ribosome-binding site; parallel vertical lines, mRNA-ctRNA interaction; minus, inhibitory RNA-RNA or protein-DNA interactions. *Pcr*, promoter for *copG* and *repB* genes (del Solar *et al*, 1998).

3.1.1.3.3 Control by iterons

In theta-replicating iteron containing plasmids, both the Rep protein and iterons are involved in regulation of replication. The Rep proteins of most iteron-containing plasmids are autoregulated (del Solar *et al*, 1998; Nordström, 1990). Two mechanisms for the involvement of iterons in inhibition have been proposed. (i) The titration model, which assumes that the Rep protein is rate limiting for initiation and that the iterons titrate the Rep protein away from the origin thus limiting the frequency of initiation (Pal and Chattoraj, 1988; Tsutsui *et al*, 1983). Whether iterons are present at the *oriV* alone (pSC101, R6K, and RK2), or both within and outside of the *oriV* (P1), this model is only plausible if two different forms of the Rep protein are involved in autoregulation and initiation respectively. This would ensure that inhibitor titration could not be overcome by derepression of *rep* (Chattoraj *et al*,

1988). In these replicons it has been shown that replication is initiated through the binding of a monomeric form of Rep to the iterons at the *oriV*, whereas autoregulation occurs through binding of a dimeric form of Rep to an inverted repeat sequence partially similar to the iterons. (ii) The second model is based on the initiator protein having at least two DNA binding sites, one on each monomer, and that the protein has the ability to bind simultaneously at both sites (Mukherjee *et al*, 1988). When Rep proteins bind to and saturate the iterons of the origin, initiation occurs if the plasmid copy number is low. As the number of copies increases, Rep molecules bound to the iterons of one origin begin to interact with similar complexes generated on other origins (Khatri *et al*, 1989; McEachern *et al*, 1989). Plasmid molecules pair through Rep-Rep interactions and cause a steric hindrance to the function of both origins resulting in inactivation of replication (Figure 3.14). This is called the “handcuffing”, or steric hindrance model.

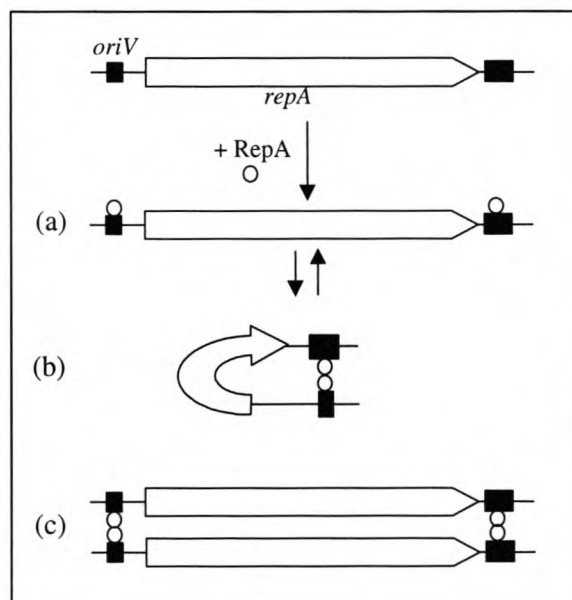


Figure 3.14: Model for copy number control in plasmids using iterons. The figure shows the *ori* region of plasmid P1. (a) RepA protein binds to iterons both within and outside of *ori*, protein-protein interactions occur between RepA proteins of the same origin (b), and origins of different plasmids (c), both of which are reversible (Nordstrom, 1990).

3.1.2 Replicon-associated incompatibility

Not only is the replicon involved in plasmid replication and control, but it also determines plasmid compatibility status. Plasmid incompatibility is the inability of two co-resident plasmids to be stably inherited in the absence of external selection (Novick, 1987; reviewed in Rawlings and Tietze, 2001), and cannot be assigned to the function of a single gene, but is the result/consequence of the normal activities of certain plasmid maintenance and replication functions. Incompatibility may be either symmetric or vectorial. Symmetric incompatibility (where co-resident plasmids are lost with equal probability), results when co-resident single replicons share essential replication and maintenance functions and are unable to correct fluctuations arising from the random selection of plasmid copies for replication and partitioning events. Vectorial incompatibility (where one plasmid is lost exclusively or with higher probability) is usually the result of interference of replication by cloned plasmid fragments containing replication control or maintenance systems, or by certain copy control mutations of directly regulated plasmids (Novick, 1987). Incompatibility can result either directly or indirectly from plasmid determined negative regulation systems designed for copy number control and maintenance. These systems may be grouped into inhibitor-target- and iteron binding regulation. Negative mechanisms for the control of plasmid copy number via antisense RNA and iteron binding have both been discussed in the previous section, therefore only the incompatibility conferred by plasmids utilizing these systems will be addressed.

3.1.2.1 Inhibitor-target regulation

The synthesis of inhibitory RNA's is constitutive and responsible for controlling copy number fluctuations in bacterial cells. Should any two plasmids using inhibitor-target regulation be isogenic for the ctRNA coding region, they will be incompatible. Plasmid selection for replication inhibition is random, however in a study of a system containing cloned ctRNA determinants strong, vectorial incompatibility was expressed as such clones displace any sensitive plasmid (Molin and Nordstrom, 1980). The secondary regulatory factors for ColE1 and R1, the Rop and CopB proteins respectively, do not cause incompatibility alone, but enhance the incompatibility activity of the cloned inhibitor (Tomizawa, 1984).

3.1.2.2 Iteron Regulation

The two modes for iteron mediated regulation are (i) titration of the initiator protein which reduces replication initiation and/or (ii) replicon “handcuffing”, which prohibits replication (Chattoraj, 2000). Deletion of these repeats leads to an increased but still controlled copy number (Pal *et al*, 1988; Nordstrom, 1990), while the introduction of extra copies reduces the replication frequency, i.e., the repeats cause incompatibility (Gardner *et al*, 2001; Lin *et al*, 1987; Persson and Nordstrom, 1986; Tsutsui, 1983). Plasmids that share iteron sequences are incompatible as they compete for the binding of a limiting, essential replication protein and plasmid replication becomes random.

3.1.3 Secretion systems

The transfer of cellular components, such as proteins, toxins, hydrolytic enzymes including genetic material from one bacterial cell to another may occur. Directed secretion pathways exist which involve some means of translocation of the exported substrate across the cell membrane. Substrate transfer may occur directly (cell to cell), or indirectly, where substrates are transported to the extracellular milieu in order to make contact with the target cells. Mechanisms responsible for such movements have been described, and are referred to as Type I-V secretion systems. Systems I, II, III, and V (reviewed by Linton and Higgins, 1998; Sandqvist, 2001; Plano *et al*, 2001; and Jacob-Dubuisson, 2001 respectively), will not be addressed here.

3.1.3.1 Type IV secretion systems

Type IV secretion systems are classified by their capacity to transfer protein substrates intercellularly (Christie, 2001). Transfer occurs via macromolecular transfer systems that share a common ancestry with the conjugation machinery (mating pair formation, mpf, complex) of Gram-negative bacteria. Thus, conjugation systems appear to form a sub-group of type IV systems that have the additional ability to translocate DNA-protein complexes.

Type IV secretion systems are present in several pathogens of plants and mammals for example: *Agrobacterium tumefaciens* (transfer of oncogenic T-DNA and several effector proteins to the nuclei of plant cells); *Helicobacter pylori* (the delivery of the 145kDa CagA protein to mammalian cells); and *Bordetella pertussis* (exports the

pertussis toxin, PT) (Christie, 1997; Christie, 2001). Each of these systems requires the formation of a macromolecular protein complex, which spans the cell membrane, consisting in part of a coupling protein which ATP-dependently “docks” the substrate for transfer at the receptor/pore of the plasma membrane. Substrate translocation differs between systems. The *A. tumefaciens* T-DNA transfer system and the *H. pylori* CagA export system are thought to export substrates in one step across the membrane directly to the eukaryotic cytosol. Included in this group of transfer proteins that are non-covalently associated with ssDNA are the Sog primase by plasmid Col1b-P9, and RecA by the F and RP4 plasmid transfer systems (Rees and Wilkins, 1990). The *B. pertussis* PtI system is thought to export PT in two steps across the cell envelope to the extracellular milieu. Secreted holotoxin then binds to the mammalian cell membrane (Christie, 2001).

Regardless of whether DNA-protein complexes or proteins alone are transferred, sequence analyses of the genetically defined transfer regions have revealed that many of the deduced proteins are highly similar to the Tra proteins of broad host range (BHR) plasmids. In addition, the genes encoding homologous proteins are usually located at similar positions in the respective transfer operons (Table 3.1).

T-DNA transfer in *A. tumefaciens* has been studied extensively (Christie, 1997; Sundberg, 1999; Christie, 2001), and is used as a reference point for the type IV systems. It also provides a convincing link between protein transfer and plasmid conjugation systems. Comparative studies between *A. tumefaciens* T-DNA transfer and RP4 conjugation systems were performed (only parallels are drawn here). For a detailed account of conjugation see section 3.1.3.2. Substrates for the nicking enzymes of both systems, T-DNA border sequences and the RP4 origin of transfer (*oriT*) exhibited a high degree of sequence similarity. The nicking enzymes VirD2 of pTi and TraI of RP4, possessed conserved active-site motifs located within the N-terminal regions of these proteins. Purified forms of both proteins were found to complement nick site cleavage in the opposing system. Both VirD2 and TraI were found to remain covalently bound to the 5'-phosphoryl end of the nicked DNA at conserved tyrosine residues Tyr-29 and Tyr-22 respectively, and both proteins also displayed type I topoisomerase rejoining activity (Christie, 1997; Pansegrau, 1993a; Pansegrau, 1993b, Pansegrau, 1994).

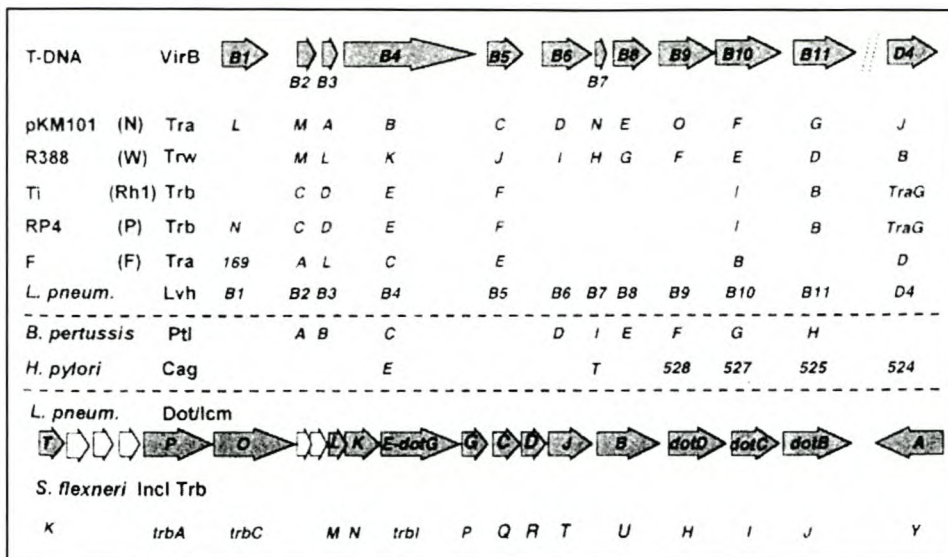


Table 3.1: Gene organisations of type IV systems known or postulated to translocate macromolecular substrates intercellularly. The *A. tumefaciens virB* gene products shown across the top assemble as the T-DNA transfer system. The underlying examples of type IV systems are composed of homologues of some or all of the VirB proteins. The top group (above dashed lines), transfer DNA between bacteria, the *B. pertussis* and *H. pylori* systems deliver known substrates (PT and CagA respectively) to mammalian cells. The *L. pneumophila dot/icm* gene products are homologues of the *Shigella flexneri* ColIb-P9 (IncI) transfer proteins. This system can conjugally transfer DNA, but its proposed role in virulence is to export effector proteins (part of a diagram from Christie, 2001).

3.1.3.2 Conjugation

Plasmids of *E. coli* have been classified into approximately twenty incompatibility (Inc) groups according to the specificity of their replication machinery (Couturier, *et al*, 1988). With the exception of the IncF and IncI complexes, little or no DNA/protein similarity among the transfer systems of the other Inc complexes exists. Of the twenty only six have been analysed in detail with regard to conjugation. A general trend in the mode of conjugation amongst Gram-negative bacteria does however exist, with minor differences.

Conjugation is the process whereby genes are transferred from one bacterial cell to another through plasmid-encoded functions. This occurs in both Gram-positive and -negative bacteria. The conjugative transfer process requires a *cis*-acting site, the origin of transfer (*oriT*), and two other *trans*-acting complexes: the mating pair formation system (Mpf), responsible for donor-recipient contact, and the DNA transfer and replication system (Dtr). Conjugative plasmids may be either self-transmissible or mobilizable. The former carry a self-sufficient transfer system, whereas the latter are not self-transmissible but can be transferred via conjugation in the presence of a transmissible “helper” plasmid. Self-transmissible plasmids therefore have the unique ability to promote an intimate cellular association between donor and recipient cells facilitating the formation of a “mating bridge” through which plasmid DNA may be transferred (Figure 3.15).

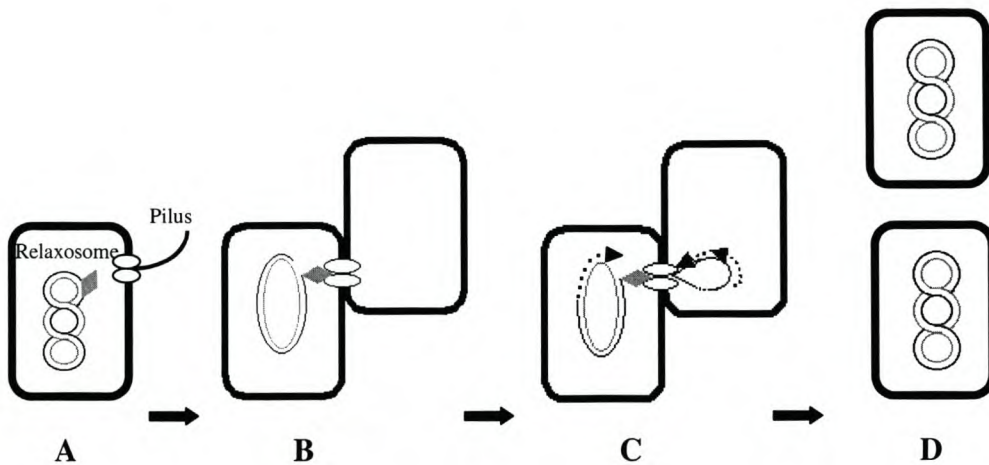


Figure 3.15: Diagrammatic representation of the process of conjugation. (A) Plasmid-carrying donor cells express sex pili; (B) Pilus mediated pair formation, relaxosome (◆) catalyzed *oriT* cleavage; (C) ssDNA transfer, complementary strand synthesis in donors and recipients; (D) T-DNA recircularises and supercoils.

3.1.3.2.1 Origin of transfer (*oriT*)

The origin of transfer plays a central role in both initiation and termination of DNA transfer, and is the only site required *in cis* for DNA processing and transfer. 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 (Zechner *et al*, 2000).

Although the sequence of each *oriT* is unique to its cognate plasmid type, similarity in the structure at *oriT* often allows plasmids from different families to cross-complement each other (Furuya and Komano, 2000). The *oriT* not only includes binding sites necessary for the nicking (*nic*) and initiation of DNA transfer, but single strand initiation sites (*ssi*) that are required for the triggering of complementary DNA synthesis in both donor and recipient cells as well (Rohrer and Rawlings, 1993). The *oriT* region is characterised by the presence of one or more sets of inverted repeats. Divergent promoters are also a common feature within the *oriT* of most plasmids studied, and encode proteins involved in *oriT* binding and relaxosome formation (Drolet *et al*, 1990). Divergent transcription is capable of creating a region of negative supercoiling between the two advancing RNA polymerases, which would stimulate unwinding of the DNA. In the case of pTF1 (isolated from *Acidithiobacillus ferrooxidans*, Holmes *et al*, 1984), this would promote the formation of a single-stranded DNA structure in the minimal *oriT* region for binding of the plasmid encoded DNA transfer protein MobL (Drolet and Lau, 1992). In contrast, the two proteins (TraI and TraJ) that react with the *oriT* of RP4, interact only with supercoiled DNA, although the site of nicking is single stranded (Pansegrau *et al*, 1990). Initiation of DNA processing at *oriT* has been studied extensively *in vivo* and *in vitro* using plasmids of the incompatibility group P and will be used (specifically RP4) to give a more detailed account of the reactions which take place at the *oriT*.

The generation of a single DNA strand is the first step in DNA transfer and is initiated by the formation of a nucleoprotein complex at *oriT* of plasmid encoded proteins (TraJ, -I, -H, and -K). TraJ binds asymmetrically to the 19 bp imperfect inverted repeat sequence in *oriT* by recognising a 10 bp palindromic sequence (*srj*) in the right half of the repeat (Figure 3.16) (Pansegrau and Lanka, 1996; Pansegrau *et al*, 1994; Pansegrau *et al*, 1993b; Ziegelin *et al*, 1989). TraI (DNA relaxase) then binds to the

TraJ•*srj* (TraJ bound at its binding site) complex via a protein-protein interaction with TraJ, as well as recognising and binding to *sri*, a 6 bp sequence in the *nic* region in between the right end of the 19 bp repeat and *nic*.

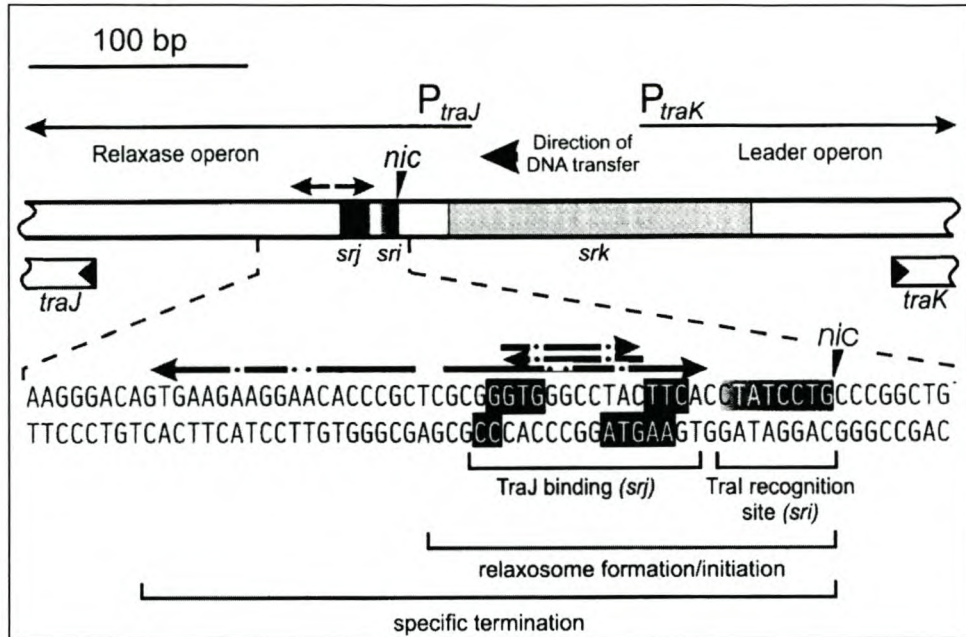


Figure 3.16: Organisation of the transfer origin of RP4. Transcription of relaxase and leader operons initiating at divergent promoter sites within *oriT* is indicated by horizontal arrows. Bold horizontal arrows mark an inverted repeat sequence adjacent to the cleavage site (*nic*). Binding sites for transfer gene products TraI, TraJ and TraK (*sri*, *srj*, and *srk* respectively) are drawn as shaded bars. Open bars represent the 5' terminal regions of the transfer genes *traJ* and *traK*. Arrowheads show the 5' ends. Part of the nucleotide sequence of *oriT* is depicted below: inverted repeat sequences are indicated by horizontal arrows, dots mark deviations from the symmetry. Black regions within *srj* indicate nucleotides that are protected against attack by hydroxyl radicals in the presence of TraJ. Nucleotides that are recognised by TraI are drawn with a shaded background. A wedge marks the position of the cleavage site (Zieglin *et al*, 1989).

Two additional proteins encoded by the relaxase and leader operon, TraH and TraK, also participate in relaxosome formation and influence cleavage at *oriT*. TraH does not bind DNA itself but stabilises the complex at *oriT* by binding to the TraJ and TraI proteins. TraK interacts specifically with *oriT* by wrapping *srk*, a 180 bp region downstream of *nic*, around a core of TraK (Ziegelin *et al.*, 1992). TraK•*srk* induces additional supercoiling which is thought to influence the local *oriT* topology, exposing the single stranded *nic* region, thereby facilitating the interaction of TraI with its target site (Pansegrau *et al.*, 1993b). The TraI-mediated cleavage reaction at *nic* of *oriT* consists of a reversible transesterification initiated by nucleophilic attack of the phosphodiester moiety at *nic* by the hydroxyl group of TraI tyrosine 22. Cleavage results in covalent attachment of TraI to the 5'-terminal 2'-deoxycytidine residue at *nic* (Pansegrau *et al.*, 1993b).

Active site characterisation of the relaxases of both RP4 (TraI) and RSF1010 (MobA) indicated that they rely on only one active tyrosine for DNA strand transfer (Pansegrau *et al.*, 1993b). The monomeric form of relaxase is therefore insufficient for second cleavage and hence for termination. A model has been proposed suggesting that the relaxosome functions as a dimer, each catalytic site being responsible for either initiation or termination of DNA transfer (Pansegrau and Lanka, 1996). This model is similar to that of replication termination in RCR plasmids discussed earlier. Termination at *oriT* has been studied extensively and a common sequence/structure requirement is evident in each case. For plasmid RSF1010 (R1162) the *oriT* consists of a specific nick site and a 10 bp inverted repeat with one mismatch situated 8 bp from the nick site. The 10 bp IR as well as the sequence around the nick site was shown to be required for termination (Furuya and Komano, 2000). Similar results were obtained for plasmid R64 whose *oriT* consists of two inverted repeats (17 bp and 8 bp respectively) and a nick site (Furuya and Komano, 1991). The nick site and both inverted repeats are required for efficient termination, however the 8 bp IR probably serves as an enhancer of protein binding to 17 bp IR. It has been postulated that during termination of DNA transfer, formation of a hairpin loop structure at the inverted repeat is required for resealing of the transferred DNA at the reconstituted *nic* site by the respective plasmid relaxase, which is still covalently bound to the 5' end of the transferred DNA (Furuya and Komano, 2000). This model is comparable to replication termination in RCR plasmids, discussed earlier.

3.1.3.2.2 Mating pair formation systems:

The current model for bacterial conjugation involves the formation of a stable interaction between donor and recipient cells. This entails an intimate association through the formation of physical intercellular contacts, which take the form of extracellular filaments called sex pili. Studies on the F pilus revealed that it is composed of a major subunit, pilin, of 70 amino acids (Anthony *et al*, 1999). Pilin is processed from a 121-amino-acid precursor, propilin, encoded by the *traA* gene of plasmid F. Propilin is inserted in the inner membrane through the action of TraQ, an F-pilin-specific chaperone in a process which requires ATP and an active proton motive force (Majdalani *et al*, 1996). Mature pilin is stored in the inner membrane where it is assembled into a functional pilus filament by the assembly proteins to form the Mpf-complex (Anthony *et al*, 1999). Cell contact occurs between the pilus tip and surface of the recipient cell. Pilus retraction, caused by depolymerisation of pilin subunits, ensures direct cell-to-cell contact, followed by the formation of a “mating bridge” through which DNA may be transferred. Pilus-recipient contact also enables the donor to detect whether the recipient harbours an identical or related plasmid, as plasmid-bearing status is displayed through surface or entry exclusion (Zechner *et al*, 2000). Exclusion proteins expressed by the plasmids are presented on the cell surface as an abundant outer membrane lipoprotein, which halts the interaction of the pilus tip with the recipient cell. Proteins TraT (OM) and TraS (IM) of the IncFI plasmid F are involved in blocking mating pair stabilisation (surface exclusion), and the signal for DNA transfer (entry exclusion), respectively between donor cells (Achtman *et al*, 1977; Manning *et al*, 1980; Perumal and Minkley, 1984; Anthony *et al*, 1999). Entry exclusion is followed by disengagement of the two bacterial cells.

Most models of the physical association of bacteria during conjugation are based on F plasmid mediated conjugation (Silverman, 1997). The thick, flexible F pili have been described as the “universal” mating type correlated with mating on either liquid or solid substrates. Recent studies on RP4 mediated conjugation, which is approximately four orders of magnitude greater on solid substrate than on liquid, disputes the role of a “universal” pilus mediated physical association (Samuels *et al*, 2000). The presence of junctions in matings mediated by Tra2 mutant donors (*trbC*, pilin mutant), suggested that pili are not essential for close contact to occur in RP4 mediated matings. However, no DNA transfer was shown to take place through these

“mutant” junctions, suggesting a role in correctly targeting a protein needed for junction stabilisation and DNA transfer for the pili of RP4 (Haase *et al*, 1995).

In all plasmid families the mating pair formation genes (*tra*) are proposed to be responsible for pilus production, cell to cell contact, and the formation a trans-membrane channel through which DNA is transferred. The exact identity of these trans-membrane channels is not known, however, electron microscopy has revealed electron dense regions of cell envelope termed “conjugational junctions”, protein complexes that span the cell envelopes of donor and recipient (Samuels *et al*, 2000). Many questions about the assembly process of the Mpf-complex remain unanswered.

Conjugation mediated by the IncP α plasmid RP4 has become a model system for several reasons. (i) The promiscuity of RP4 transfer between diverse taxa is analogous to the movement of plasmid-borne antibiotic genes in clinical situations. (ii) Horizontal gene transfers due to plasmid movement are important for evolutionary and ecological study. And (iii), RP4 bears similarities to other Type IV secretion systems (Samuels *et al*, 2000). A detailed study of the IncP α RP4 conjugative transfer apparatus has therefore contributed to a better understanding of the cellular interactions that take place.

Two distinct regions of RP4, Tra1 and Tra2, encode essential transfer functions (Pansegrau and Lanka, 1996). The Tra1 region mainly encodes DNA processing functions (TraH, TraI, TraJ, TraK, and TraM) for the generation of the single-stranded DNA molecule, which is transferred to the recipient cell. The Tra2 core region (11 genes) and *traF* of Tra1 belong to the mating pair formation system (Lessl *et al*, 1994). TraG, also encoded by Tra1, is thought to connect the gene products of Tra1 (relaxosome), and Tra2 (Mpf complex). Since a close cell-to-cell interaction is needed for DNA transfer from donor to recipient cell, mpf components were proposed to form a membrane pore or channel. Sequence alignments of Tra2 gene products indicated that proteins TrbC, -G, -H, and -L fulfill some requirements for outer membrane localisation, such as signal sequences and membrane spanning regions, suggesting that most, if not all, of these proteins are membrane associated (Lessl *et al*, 1992). Disruption of the Mpf-containing cells and subsequent separation of cell

membranes in density floatation gradients led to the discovery of an additional membrane fraction, Mpf-containing membranes (MCM), whose density was close to that of the outer membrane (Grahn *et al*, 2000). This fraction contained all Mpf components, except TrbB, the only soluble protein of the Tra2 region. Interestingly cytoplasmic (LPase) and outer membrane (OmpA) marker proteins were also found in this fraction, suggesting a rearrangement of the cell envelope architecture. This evidence strongly suggests that the Mpf proteins form a complex that connects the cell- and outer membrane fractions by binding them together. TrbB belongs to a group of proteins called the PulE family (Motallebi-Veshareth *et al*, 1992). They are typically involved in the export and building of type IV pili, and serve as the energy-delivering protein for the export of components through the outer membrane. The TrbB protein also contains a signature sequence common in lipoproteins targeted to the outer membrane (Lessl *et al*, 1992a).

From the protein localisation experiments performed by Grahn *et al* (2000), results indicate that the RP4 relaxosome is located in the cytoplasm and is associated with the cytoplasmic membrane (Figure 3.17). This association is independent of the membrane spanning Mpf-complex, since TraI and TraL were partially localised to the cytoplasmic membrane in the absence of the Tra2 region proteins. Even in the presence of Mpf components, TraL was found in the outer membrane fraction. Relaxosome proteins TraH and TraK were found to be associated with the cytoplasmic fraction of the floatation gradients. TraG associated with the cytoplasmic membrane independent of the presence of the Tra2 region. Interestingly, during floatation experiments TraG was found in an intermediate band, supporting its potential role as an interface between the RP4 Mpf system and the relaxosome.

Whether this model can be extended to other Gram- negative Inc plasmid systems is still to be determined.

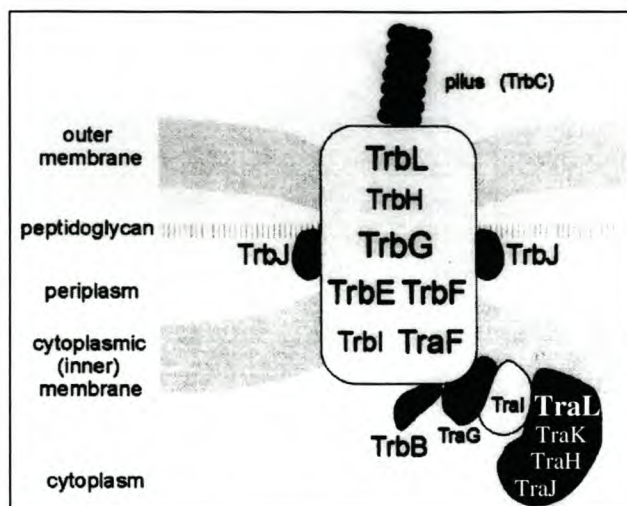


Figure 3.17: A model of the membrane spanning and membrane associated DNA transfer apparatus of RP4 across the *E.coli* membrane (Grahn *et al.*, 2000).

3.1.3.2.3 DNA transfer and replication systems

The systems involved in DNA transfer and replication require the formation of a multi-protein complex at the origin of transfer called the relaxosome. Each Inc family has different relaxosome components, but generally share a common method for initiation and replication. DNA relaxases are the key enzymes in the initiation of transfer DNA replication and catalyse site- and strand-specific cleavage at the nick site (*nic*) of a transfer origin (*oriT*). Relaxases do not initiate transfer *in vivo* in the absence of other proteins, so together with accessory proteins that may be either plasmid- or host- encoded, they form the stable nucleoprotein complex at *oriT* (Byrd and Matson, 1997; Zechner *et al.*, 2000). The accessory proteins assist in; i) nick site recognition, binding and initiating relaxosome formation, and ii) stabilization of the complex at *oriT*. DNA transfer is supposed to occur via a ssDNA intermediate generated by a rolling circle-type DNA replication mechanism, followed by re-circularisation in the recipient cell. It is thought that re-circularization is mediated by the molecule of relaxase that is linked covalently to the 5' end of the transferring DNA. The 3' end of this strand is believed to undergo continuous extension by the replicative DNA polymerase of the donor cell, thereby generating an internal *nic* site. Relaxase bound to the leading end of the intermediate recognises the site, and through a cleavage-rejoining transesterification, releases a monomeric circle of DNA (Lanka

and Wilkins, 1995). DNA transfer and replication (DTR) systems of the Inc groups most studied will be addressed below.

IncP-type: DNA relaxases of the IncP-type appear to be the most widely distributed. They display a common domain structure consisting of an N-terminal catalytic domain and a C-terminal domain of variable size that apparently mediates interactions with other components of the transfer machinery (Zechner *et al*, 2000). The N-terminal catalytic domain contains three conserved motifs, which include at least one invariant residue. Motif I has a conserved tyrosine residue (Tyr-22) whose exchange with a leucine resulted in complete loss of DNA cleavage activity (Pansegrau *et al*, 1994). This result is in agreement with the expected role of Tyr-22 in the formation of the covalent linkage to the DNA 5' terminus, the nucleophilic agent of the relaxase centre (Pansegrau *et al*, 1993b). Tyr-22 is in fact a universally conserved residue in all listed relaxases, and is supposed to be pivotal for catalytic activity. Mutations in motif II (serine 74) did not abolish cleavage activity, but when exchanged with an alanine exhibited a strong topoisomerase type I-like activity on the negatively supercoiled origin of transfer of RP4. Thus motif II is thought to be involved in tight binding of the nick region (Pansegrau *et al*, 1994). Motif III (histidine 116) is also involved in catalytic activity, as mutational analysis drastically reduced the DNA cleavage activity of TraI. His-116 is thought to be involved either in activation of the Tyr-22 hydroxyl group by proton abstraction (Pansegrau *et al*, 1994), or it could mediate the proton transfer reaction to the 3' terminus that creates the leaving 3' hydroxyl group in the cleavage reaction (Pansegrau and Lanka, 1996). Therefore it has been proposed that motifs I and II are in close contact and together form part of the catalytic center of TraI, whilst motif II sustains the topology needed for efficient cleavage at *nic*. Figure 3.18 gives a diagrammatic representation of how motifs I, II, and III work together in the catalytic center of TraI. Assembly of the relaxosome complex of RP4 has already been discussed in a previous section.

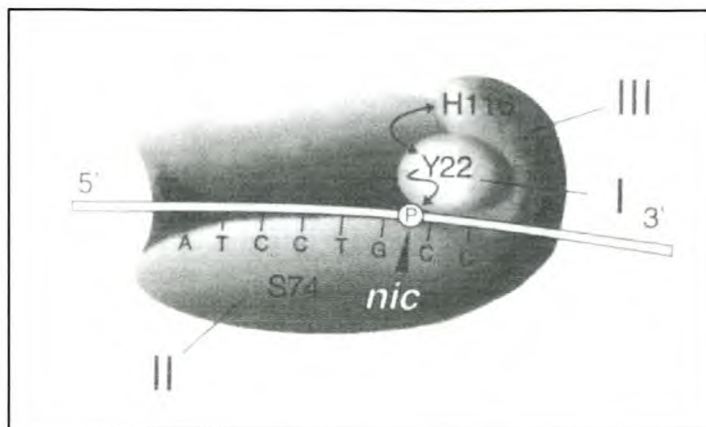


Figure 3.18: A model of the TraI catalytic center. Proposed locations of motifs I, II, and III are indicated, interactions between amino acid residues or between amino acid residues and the DNA are represented by arrows. The nick site (*nic*) is marked by a wedge (Pansegrau *et al*, 1994).

IncF and IncW type: The relaxases encoded by IncF and IncW plasmids have a common domain structure consisting of an N-terminal relaxase and a C-terminal 5'→3' DNA helicase domain (Byrd and Matson, 1997). Physical linkage of these domains was shown to be important, as dissection of the two domains from each other lowered the transformation frequencies by several orders of magnitude (Llosa *et al*, 1996). Binding via the relaxase domain could possibly enhance the helicase concentration at the transfer origin, or the helicase activity could also be responsible for providing the motive force for DNA transport across the bacterial membranes. The catalytic domain also carries a tandem arrangement of tyrosine residues. Recent evidence indicates that both tyrosines are actively involved in the cleavage reaction (Zechner *et al*, 2000). Virtually nothing is known about the role of protein-protein interactions in relaxosome assembly and will therefore not be addressed any further.

IncQ type: IncQ and other plasmids of this subgroup have a basic mobilization system which consists of MobA, MobB, and MobC proteins, which congregate to form a nucleoprotein complex at an *oriT*-containing region. MobA is a multifunctional protein: the N-terminal domain encodes the DNA relaxase (MobA), while the C-terminal domain encodes a primase (RepB). The RepB primase occurs in two forms, a 78 kDa MobA-RepB fusion protein and a 36 kDa RepB' protein, which is translated from an initiation codon downstream of and in the same reading frame as the fusion protein (Figure 3.5) (reviewed in Rawlings and Tietze, 2001). Although

the two domains appear to function independently of each other, the influence of RepB presence in DNA transfer is different for the various plasmid families. In some cases, such as IncQ plasmid pTfFC2 (Rohrer and Rawlings, 1993), the absence of RepB does not affect transfer or the frequency thereof. In contrast, in a study of R1162, the MobA-linked form of the primase increased the efficiency of the transfer system (Henderson and Meyer, 1999). MobA recognises the IR sequences at the *oriT*, binds, and induces a site-specific cleavage at the *nic* site via its active site tyrosine residue (Tyr-24). MobA remains covalently attached to the single-stranded DNA substrate and guides the displaced strand into the recipient cell. Although MobA does not require any accessory protein for cleavage of single- or double- stranded DNA substrates *in vitro*, the reaction is enhanced in the presence of plasmid encoded accessory proteins MobB and MobC.

MobB is synthesised from within the *mobA* gene, in a different reading frame, while MobC is transcribed divergently from a gene that lies on the opposite side of *oriT* (Rawlings and Tietze, 2001) (Figure 3.5). MobC assists in unwinding of the DNA at the region of the *nic* site, allowing easier access to the *oriT* for the larger MobA protein. MobB enhances the reaction between MobA and the *oriT* by stabilizing the relaxosome and shifting the DNA cleavage-joining equilibrium to the cleaved state (Perwez and Meyer, 1996). Initiation of DNA synthesis in the recipient cell is dependent on the specific plasmid-encoded primase RepB (RepB') (Henderson and Meyer, 1996).

Members of the IncQ group have been isolated which deviate from the three-protein mobilization region. Plasmid pTF1 appears to have only two mobilization proteins, MobL and MobS. Amino acid sequence alignments identify MobL as having sequence related to the N-terminal relaxase region of MobA and MobS being related to MobC (Drolet *et al*, 1990; Rawlings and Kusano, 1994). No protein equivalent to the MobB, or to a DNA primase has been identified on plasmid pTF1 (Rawlings and Tietze, 2001).

3.1.4 Comparison of systems

Parallels may be drawn between the plasmid-associated rolling circle replication (RCR), T-DNA transfer in *A. tumefaciens*, and bacterial conjugation. All three

involve plasmid replication of the rolling circle type. The first takes place within a cell while the latter two take place during DNA transfer between cells. In each case not only are the processes of initiation, DNA transfer and termination similar, but also the specific reaction sites required for each process display high degrees of sequence similarity (Waters and Guiney, 1993; Guzman and Espinosa, 1997). Interestingly *A. tumefaciens* plasmid pTi, in addition to the virulence genes (*vir*) that mediate the process of T-DNA transfer, encodes a bacterial conjugation system called Tra. This system mediates transfer of the entire plasmid between agrobacteria (Lanka, 1995). Although it has been suggested that these systems evolve from a common ancestor (conjugation and Type IV transfer systems), is there a possibility that they might be converging to form one multi-system with a lower energy demand on the cell?

3.1.5 Transposons

In addition to genes required for maintenance and transfer, plasmids frequently carry embedded mobile genetic elements called transposons. Transposons have the ability to move from one carrier replicon to another, providing the bacteria with genome flexibility, which is often necessary to cope with the stresses of a continuously changing environment. Three classes of transposable elements have been defined on a structural basis. Class I includes all known insertion sequences (IS) and the composite transposons which consist of two insertion sequences flanking additional DNA. Class II include transposons that do not contain any recognisable IS. Class III includes mutator bacteriophages that amplify their genome using a transposition mechanism (Merlin *et al*, 2000). The first recognized largest and most widely distributed example of a transposon is the multiply composite transposon Tn21, which belongs to a subgroup of the Tn3 family of transposable elements (Liebert *et al*, 1999). Interest in this transposon has been stimulated due to the involvement of the Tn21-family in the distribution of antibiotic resistance in clinical environments.

The Tn3 family of transposable elements (class II) is characterized by flanking inverted repeats of about 38 bp, a transposase (*tnpA*) and a resolvase (*tnpR*) gene, and a *res* site of approximately 130 bp at which the resolvase acts (Clennel *et al*, 1995; Liebert *et al*, 1999). The organization of genes in the Tn3 family falls into two classes (Figure 3.19). Transposons with a divergent gene arrangement (B) are referred to as the Tn3 transposon subfamily, whilst transposons with *tnpR* and *tnpA*

transcribed in the same direction (A) are described as the *Tn501* subfamily (Sherratt, 1989). *Tn21* falls into the latter subgroup, which also have a variable region (XX), which frequently contain genes encoding for metal or antibiotic resistance (reviewed in Rawlings and Kusano, 1994).

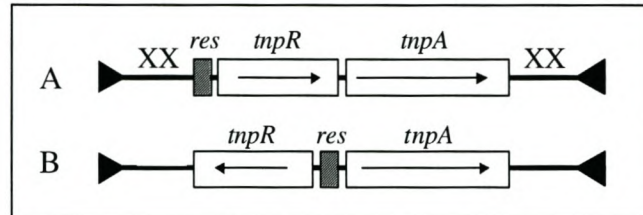


Figure 3.19: Gene organization of class II transposons. (A) *Tn501* subfamily: transcription of *tnpR* and *tnpA* in the same direction; *res* is 5' of *tnpR* and *tnpA* (*Tn501*, *Tn1721*, *Tn21*, *Tn551*, *Tn917*). (B) *Tn3* subfamily: divergent transcription of *tnpR* and *tnpA* from a central *res* site (*Tn3*, *Tn1000*, *Tn2501*, and *Tn4430*). Solid triangles, IR sequences; XX, variable DNA segment; → orfs for transposition functions; hatched boxes, resolution sites (*res*) (Sherratt, 1989).

3.1.5.1 Transposon Tn21

Transposon Tn21 carries genes involved in its own transposition (*tnp*), a mercury resistance operon (*mer*), and a potentially independently mobile DNA element called an integron, which will be described later (Figure 3.20).

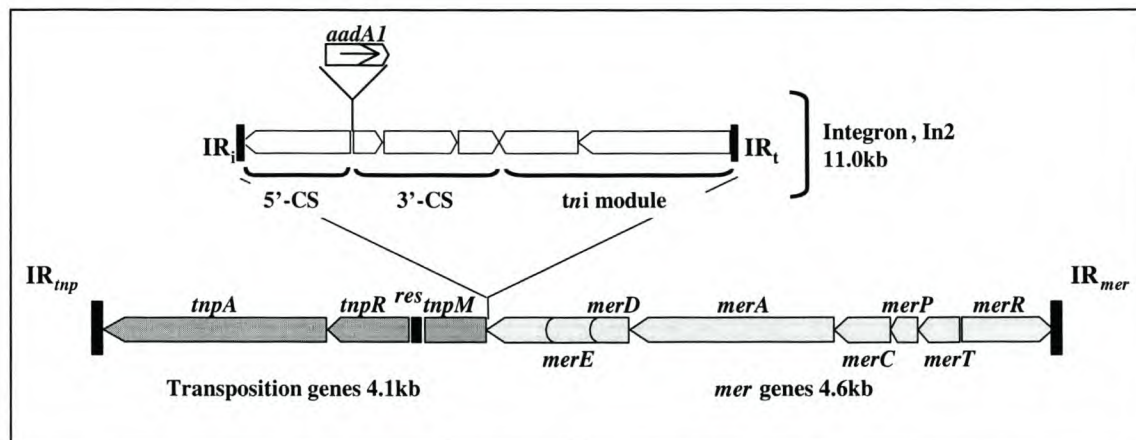


Figure 3.20: Structural organization of transposon Tn21. The three main sections of Tn21 are shown, the transposition region (*tnp*), the integron, and the *mer* operon. The *tnp* region consists of genes for the transposase (*tnpA*), the resolvase (*tnpR*), the putative transposition regulator (*tnpM*), and the resolution site (*res*). The integron consists of the 5' and 3' conserved insertion sequences, and remnants of the *tni* transposition gene module; it carries only one integrated gene cassette, *aadA1*. The mercury resistance operon consists of regulatory and structural genes (unlabelled) (Part of diagram from Liebert *et al*, 1999).

3.1.5.1.2 The transposition module (*tnp*) and mode of interaction

Transposition of transposon Tn21 is carried out by the transposase, TnpA. Transposases of this family are at least 70% homologous to each other and share, as do many other transposases, a DD-E motif (Liebert *et al*, 1999). The two conserved aspartates (D) are usually separated from the conserved glutamate (E) by approximately 35 amino acids; the DD-E motif is part of the enzyme catalytic site and is involved in binding bivalent metallic ions (Polard and Chandler, 1995). Replicative transposition of Tn21 involves specific recognition of and binding to the terminal inverted-repeat (IR) elements of Tn21 by TnpA. Repeats are fairly conserved amongst members of the same transposon family however; the interchangeability of transposases in transposition is limited (Sherratt, 1989). The DD-E motif traps two

magnesium ions destabilizing the phosphodiester bond at the 3' ends of the transposable element (IR). The weakened bond is now susceptible to nucleophilic attack by a water molecule, liberating the transposons 3'-OH end. A second nucleophilic cleavage occurs on the target DNA resulting in transposon-target linkage and ultimately cointegrate formation. The sites of insertion, defined by a 5 bp duplication of target DNA, are usually rich in AT residues with little other conservation in sequence (Liebert *et al*, 1999).

The resolvase (*tnpR*) catalyzes site-specific recombination between supercoiled DNA and two directly repeated copies of the transposon DNA at the *res* site, resulting in both the donor and target replicons containing one copy of the transposon (Stark *et al*, 1989). The resolvases from the Tn3-like transposons fall into a series of complementation groups characterized by their specificities for the DNA sequence at *res*. Within each group, the resolvases have a greater than 80% amino acid identity and can act at *res* sites from all members of the same group (Soultanas *et al*, 1995). The *res* site is approximately 115 bp long and contains three highly conserved subsites (I, II, and III), which are involved in binding of the resolvase. Each of the three sites in the Tn3 subfamily constitutes a pair of inverted 9 bp repeats, with the consensus TGTCYRTTA, which spans a central spacer (Sherratt, 1989). The sizes of the spacer regions for each site is different; site I, 10 bp; site II, 16 bp; and site III, 7 bp. Each subsite is believed to bind a resolvase dimer, this occurs at both recombination loci. Protein-DNA complexes at each *res* site interact to form a synaptic complex, where strand exchange takes place at the center of subsite I, the "crossover site" (Stark *et al*, 1989). Sites II and III stabilize the complex via protein-protein interactions. The function of the putative *tnpM* gene (351 bp) is unknown. Although it is not necessary for transposition, it has been proposed that *tnpM* produces a protein, which could enhance Tn21 transposition and suppress cointegrate resolution (Hyde and Tu, 1985).

3.1.5.1.3 In2 Integron

Integrans are genetic units that carry determinants for a site-specific recombination system allowing them to capture genes, which can determine antibiotic resistances, and are located on mobile DNA fragments known as "gene cassettes" (Recchia and Hall, 1997). Integrans also carry promoter/s that are functional in a wide range of

bacteria, ensuring not only expression of the trapped gene cassette, but ready mobility. Since integrons often occur on plasmids, the extent of mobility is determined by plasmid host range. Broad host range plasmids therefore contribute to the interspecies spread of integron-associated resistance (Stokes and Hall, 1989). Integrons in Gram-negative bacteria have been classified into three different classes based on similarities between the integron-encoded integrases, which are involved in transposition (Merlin *et al*, 2000).

The integron (In2) found in Tn21 is a class I integron carrying only one integrated gene cassette, *aadA1*. The *aadA1* gene determines resistance to streptomycin and spectinomycin (Liebert *et al*, 1999). In2 is 11 kb and is flanked by 25 bp imperfect inverted repeats (IR_i and IR_j), which are in turn bound by a 5 bp direct duplication of the sequence between the transposition and mercury genes. Since a 5 bp duplication is the target site for transposition, Tn21 must be the result of the entry of integron In2 into an ancestral *tnp-mer* transposon. In2 encodes a RecA-independent, site-specific integration system that is responsible for the acquisition of gene cassettes that encode antibiotic resistance genes. In2 has an incomplete set of transposition (*tni*) genes and cannot transpose itself, movement is ensured by the use of transposition proteins provided in *trans* (Brown *et al*, 1996). The backbone of In2 consists of three distinct regions: 5'-conserved segment (5'-CS) the 3'-conserved segment (3'-CS), and remnants of the *tni* transposition gene module (Figure 3.19). The 5'-CS and 3'-CS lie on either side of the cassette insertion point.

The 5'-CS includes three repeats at its lefthand side that are predicted to bind the *tni* transposase (Radstrom *et al*, 1994). It also carries a secondary promoter, P2, found only in In2 and close relatives. The 3'-CS contains three ORFs: *qacEΔ1*, *sul1*, and *orf5* (Stokes and Hill, 1989). The *qacEΔ1* confers marginal resistance to quaternary ammonium compounds. The *sul1* gene encodes a 279aa sulfonamide-resistant dihydropteroate synthase, which is able to replace the bacterial enzyme targeted by this group of drugs. The function of *orf5* is not known (Liebert *et al*, 1999). In conclusion, the 3'-CS contains genes associated with resistance to antiseptics and disinfectants. The *tni* transposition module carries only one complete gene, *tniA*, and

a truncated version of the *tniB* of Tn5053; deeming it redundant for transposition (Radstrom *et al*, 1994).

3.1.5.1.4 The *mer* operon

The *mer* region is flanked by two inverted repeats, Tn21 IR_{mer} on the left and In2 IR_t on the right (Figure 3.19). The mercury resistance operon of Tn21 contains regulatory genes (*merR* and *merD*) and structural genes (*merT*, *merP*, *merC* and *merA*), which encode the NADH-dependent flavin oxidoreductase, mercuric reductase (MerA), two inner membrane proteins (MerC and MerC), and a periplasmic protein (MerP). This region is sufficient to confer resistance to Hg (II) (Liebert *et al*, 1999). In general the resistance mechanism is based on the reduction of mercuric (Hg²⁺) ions to volatile elemental mercury (Hg⁰) (Silver and Phung, 1996). The model for mercuric reduction is as follows: Hg (II) enters the periplasm where it is bound by a pair of cysteine residue in MerP. Transfer from MerP to the cysteine residues in MerT and MerC occurs; MerT/MerC facilitate movement of the ion across the cytoplasmic membrane via a series of ligand exchange reactions. Hg (II) is delivered to the active site of MerA, which catalyses the reduction of Hg (II) to Hg⁰. This less reactive monoatomic gas diffuses from the cell and surrounding medium (Silver and Phung, 1996).

This chapter describes the screening of all *Leptospirillum* isolates analyzed in this study for plasmids, with the long-term goal of the further development of *Leptospirillum* for industrial purposes. These findings represent the first isolation of plasmids from *Leptospirillum ferrooxidans*.

3.2 Materials and Methods

3.2.1 Bacterial strains

Leptospirillum strains used in this plasmid study are shown in Table 3.3. Plasmid detection within isolates is indicated. *E. coli* strains used in mobilization experiments are shown in Table 3.2.

Table 3.2: *E. coli* strains used in mobilization experiments

Strain	Genotype	Reference
<i>E. coli</i> S17-1	<i>rec</i> ⁻ deriv of 294 (<i>hsdR</i> , <i>pro</i>) with RP4-2Tc::Mu Km::Tn7	Simon <i>et al.</i> , 1983
<i>E. coli</i> CSH56	F ⁻ <i>ara</i> Δ(<i>lacpro</i>) <i>supD nalA thi</i>	Cold Spring Harbour (CSH)

3.2.2 Pulsed Field Gel Electrophoresis (PFGE)

PFGE was conducted using a Beckman Geneline Transverse Alternating Field system. Total genomic DNA was prepared by resuspending cell cultures in SET buffer to OD₆₀₀ =1-2.5 (1.6 is optimal). Cells were incubated with 20 mg/ml proteinase K (Merck) for 30-60 minutes at 37°C. Samples were added to a low melting point agarose (Seaplaque) at 1.1% final concentration and applied to a 2x2x25 mm “plug” mold. Samples were extracted and incubated in TE containing 1% (w/v) SDS until opaque appearance of plugs started to clear. Agarose entrapped cells were lysed within the plugs by incubation in ES solution (Na-lauroyl sarcosine 10 g/l, 168 g/l EDTA, pH 8) containing 1 mg/ml proteinase K at 37°C for 30 minutes and then overnight at 50°C. Overnight washes in ESP solution at 50°C were repeated to ensure clean plugs. To remove proteinase K, plugs were washed in ES solution overnight at 50°C. Rinsing in TE buffer (2x 30 min) at room temperature inactivated any residual proteinase K. Plugs were then incubated in Pefabloc proteinase inhibitor (Boeringer Mannheim) at 4°C overnight and washed in TE. Plugs were stored in ES or TE₅₀ at 4°C until used. Plugs were equilibrated in 1x PFGE running buffer prior to use.

Agarose gels (1% w/v) were made with 1xPFGE running buffer. Gels were electrophoresed at 140 mA for 15.5 h with a 2-second pulsed time.

3.2.3 Preparation of plasmid DNA (gentle lysis technique)

Cells were harvested from 10 litres of 9K medium at 9000 rpm for 35 min, and washed in 10 ml 1× mineral salts medium. Cells were collected at 9000 rpm for 10 min, and resuspended in 10 ml SET buffer (50mM Tris, 2mM EDTA, 25% sucrose, pH 8.0). Incubation for 1 hour at -20°C to weaken the cell walls followed. 1 ml 10% SDS was added and incubated at 4°C for 10 min followed by 37°C for 10 min. The lysate was then centrifuged at 18 000 rpm for 1 hour to remove debris and chromosomal material. Supernatant was collected. 1 g/ml CsCl was added; the refractive index was left unaltered at approximately 1.4. Owing to the high sucrose content lowering of the refractive index to 1.396 required large amounts of TE, which resulted in a diffused plasmid band after ultra-centrifugation. 200 µl EtBr (10 mg/ml) was added and centrifuged at 10 000 rpm for 10 min to remove residual protein. Supernatant was loaded into Vti65 tubes and centrifuged overnight at 55 000 rpm (20°C) in a Beckmann Ultracentrifuge (RCF average 275444, RCF maximum 297805). Plasmid bands were extracted from the CsCl gradient. EtBr and CsCl were removed using salt-saturated isopropanol and ethanol washes respectively. Plasmid DNA was quantified against a known Lambda DNA standard.

3.2.4 DNA sequencing and analysis

All plasmid subclones were sequenced on an ABI 3100 Genetic Analyzer, using Sequencing Analysis 3.7 software. Computer analysis of nucleic acid sequence as well as deduced protein sequence was carried out using the DNAMAN for windows program, Version 4.13 (1994-99). BLAST searches (Altschul *et al.*, 1990) were conducted on the World-Wide-Web (<http://www.ncbi.nlm.nih.gov>).

3.2.5 Mobilization experiments

Sub-clone p1S1 in pUC19 was transformed into *E.coli* S17-1, and transformants were selected for growth on LA plates with ampicillin (100 µg/ml) and streptomycin (50 µg/ml). Single colonies of donor (S17-1) and recipient (CSH56) bacteria were grown separately overnight at 37°C. Antibiotics were added to all donor cultures to maintain

selection of the plasmids. Cells were washed three times in 0.8% NaCl solution and mixed in a 1:1 ratio. 100 µl of the mixed sample was spotted onto an agar plate without antibiotics and incubated at 37°C overnight. Agar plugs were excised, and the cells were resuspended in 10 ml 0.8% saline by shaking at room temperature for 30 min. Cells were collected from all 10 ml of saline and washed three times to remove extracellular β-lactamase and prevent the growth of satellite colonies at low dilutions. Serial dilutions were plated onto LA ampicillin (100 µg/ml)-streptomycin (50 µg/ml) plates to count donors and onto LA ampicillin (100 µg/ml)-nalidixic acid (50 µg/ml) plates to count transconjugants.

3.3 Results and Discussion

3.3.1 Screening of *Leptospirillum* isolates for plasmid presence

Undigested total DNA from cells of all *Leptospirillum* isolates was separated using Pulsed Field Gel Electrophoresis (PFGE). DNA stability within the agarose plugs was not consistent, often resulting in degraded DNA on electrophoresis, which obscured the detection of plasmid bands (Figure 3.21A). However, degradation patterns varied between isolates and experiments, this enabled the identification of plasmid DNA with certainty from *L. ferrooxidans* isolates ATCC49879, CF12, and Parys (Figure 3.21B). DNA from plasmid 49879 was DIG-labelled and used as a probe against the pulsed field gels of undigested total *Leptospirillum* DNA, plasmid bands could be identified for isolates 49879, Parys and CF12 (Figure 3.22). This implies that regions with homologous sequences must exist between the three plasmids. Since uncut samples were run, this result cannot be used to size plasmid bands. Inconsistency in plasmid detection prevented ruling out the possibility that plasmids may also be present in some of the remaining isolates (Table 3.3).

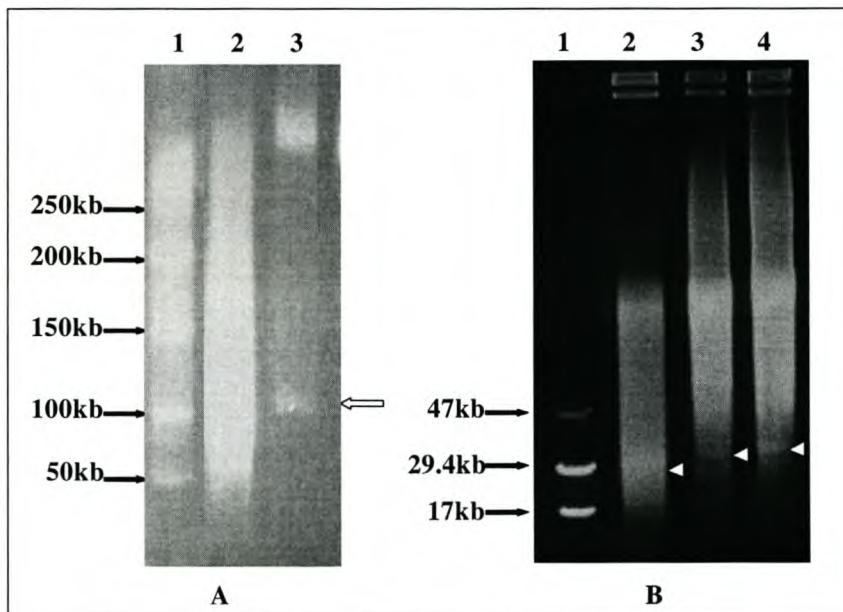


Figure 3.21: Pulsed-Field gel of uncut cells of a selection of *Leptospirillum* isolates. (A) Lane 1, λ concatamers (respective band sizes are indicated by solid arrows); Lane 2, Parys; Lane 3, ATCC49879. A plasmid band is clearly present in Lane 3 (isolate ATCC49879), and is indicated by an open arrow. An example of the degraded DNA smears often obtained is shown in Lanes 2. (B) Lane 1, molecular weight marker λ cut with *Kpn*I. Lane 2, ATCC49879; Lane 3, CF12; Lane 4, Parys. The top λ band is a combination of the 29.4kb and 17kb bands due to association via the cohesive ends.

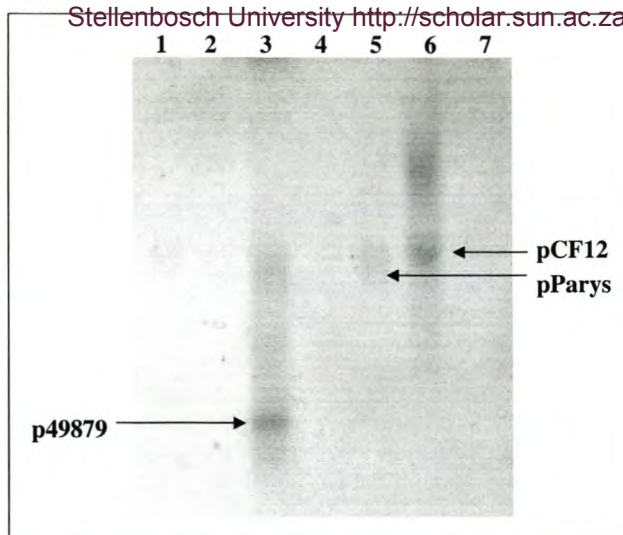


Figure 3.22: Southern blot of undigested total DNA from cells of selected *Leptospirillum* isolates probed with DIG-labeled plasmid 49879 DNA. Lane 1, Fairview; lane 2, DSM2705; lane 3, ATCC49879; lane 4, P_{3a}; lane 5, Parys; lane 6, CF12; lane 7, BCT2.

Table 3.3: Summary of plasmid presence detected in this study using PFGE.

Strains	Species (<i>L. ferrooxidans</i> or <i>L. ferriphilum</i>)	Origin	Plasmid presence detected in this study using PFGE
P _{3a}	<i>L. ferrooxidans</i>	Coal mine, North Wales, UK	No
ATCC49879	<i>L. ferrooxidans</i>	Romania	Yes
SY	<i>L. ferrooxidans</i>	Sygun Cu mine, North Wales, UK	No
N ₂ 5	<i>L. ferrooxidans</i>	New Zealand	No
Crys13	<i>L. ferrooxidans</i>	Ag mine, Montana	No
BCT2	<i>L. ferrooxidans</i>	Birch Coppice, UK	No
Parys	<i>L. ferrooxidans</i>	Parys Mountain, Anglesey Cu mine, Wales	Yes
CF12	<i>L. ferrooxidans</i>	Idaho Co mine, USA	Yes
Chil-Lf2	<i>L. ferrooxidans</i>	Cu mine, Chile	No
DSM2705	<i>L. ferrooxidans</i>	Markosyan strain, (1972), Cu mine, Armenia	No
Fairview	<i>L. ferriphilum</i>	South Africa	No
Warwick	<i>L. ferriphilum</i>	Warwick, UK	No
ATCC49880	<i>L. ferriphilum</i>	Romania	No
ATCC49881	<i>L. ferriphilum</i>	Peru	No
Bionic 3.1	<i>L. ferriphilum</i>	Nickel pilot plant, South Africa	No
Mont. 4	<i>L. ferriphilum</i>	Pyrite column, Montana, USA	No

3.3.2 Plasmid isolation

Although plasmid presence was identified in three of the *L. ferrooxidans* strains (Figure 3.21B), isolation was complicated. A variety of standard isolation procedures and modifications thereof were applied to these strains with limited success. In order to reduce the likelihood of size being the limiting factor in plasmid isolation, each method was tested with a series of control plasmids ranging in size from 3 kB (pSK) to 60 kB (RP4). Although the size of plasmid p49879 was not known, the method ultimately chosen for plasmid isolation yielded workable (μ g) amounts of each control plasmid. The procedure involved a gentle lysis technique, to minimize DNA nicking, and was applied to isolates ATCC49879, CF12, and Parys. Success was varied and plasmid DNA, only from strain ATCC49879, could be consistently isolated in very small (ng) amounts. A possible reason for the difficulty in plasmid isolation from strains CF12, Parys, and ATCC49879, could be the result of a low plasmid copy number. As only plasmid 49879 was isolated, this plasmid was studied further, and used to investigate whether similar plasmids or part thereof, exist in the other *Leptospirillum* isolates represented in this study.

3.3.3 Restriction endonuclease digestion of plasmid 49879

In order to size the plasmid, restriction endonuclease digestions were carried out with rare cutting enzymes; *NotI*, *PacI*, *PmeI* and *XbaI* were identified as suitable. Plasmid DNA was digested and separated via PFGE.

Digestion with *NotI* generated nine bands (approximate sizes: 0.7-, 1.3-, 7/8.0-, 10.0-, 11/12.0-, 17.0-, 20.0-, 23.0-, and 30.0 -kb), a collective size of ~120 kb. Band intensities were not uniform (Figure 3.23), suggesting either partially digested bands or the presence of two plasmids, each with a different copy number.

Although six of the nine *NotI* fragments were cloned (bands 1-4, 8 and 9 in Figure 3.23) and used at various stages of this study (discussed later), subclones 1-4 were instrumental in understanding the reason for the existence of different plasmid band intensities.

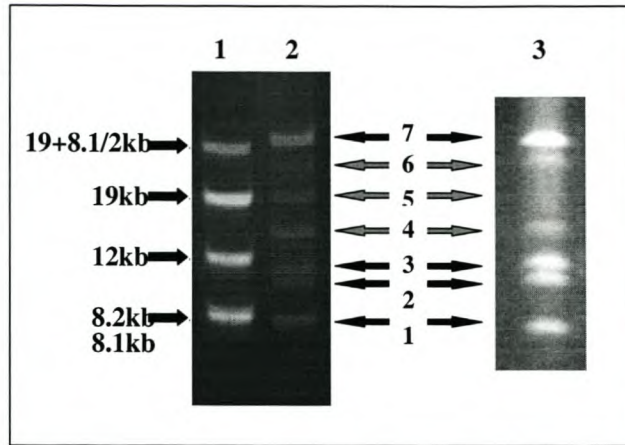


Figure 3.23: Restriction endonuclease analysis of p49879 with *NotI*. Lane 1 contains molecular weight marker λ cut with *SmaI*. The top λ band is a combination of the 19.4 kb and 8.2 kb bands due to association via the cohesive ends. Lanes 2 and 3 *NotI* digested p49879. Lane 3 clearly shows the different band intensities. Bands of equal intensity are marked by shaded arrows, high intensity ($\blackleftarrow\blackrightarrow$) and low intensity ($\leftarrow\rightarrow$). The 0.7 kb and 1.3 kb bands (bands 8 and 9) are not visible on this gel.

Subclones 1-4 were digested with *NotI* and separated on a pulsed field gel along with *NotI* digested p49879 (Figure 3.24A). A Southern blot was prepared and probed with each individually labelled subclone. Results are shown for subclones 1, 2, 3, and 4 in Figure 3.24-B, -C, D, and -E respectively. Subclone 2, as expected hybridized only to itself and to the corresponding band within the p49879 *NotI* digestion. This same hybridization loyalty was achieved for each of the three remaining subclones; however additional hybridization signals were also obtained. Subclone 1 (~7/8.0 kb) and 3 (~11/12.0 kb) both hybridized to subclone 4 (~17/18 kb) and vice versa.

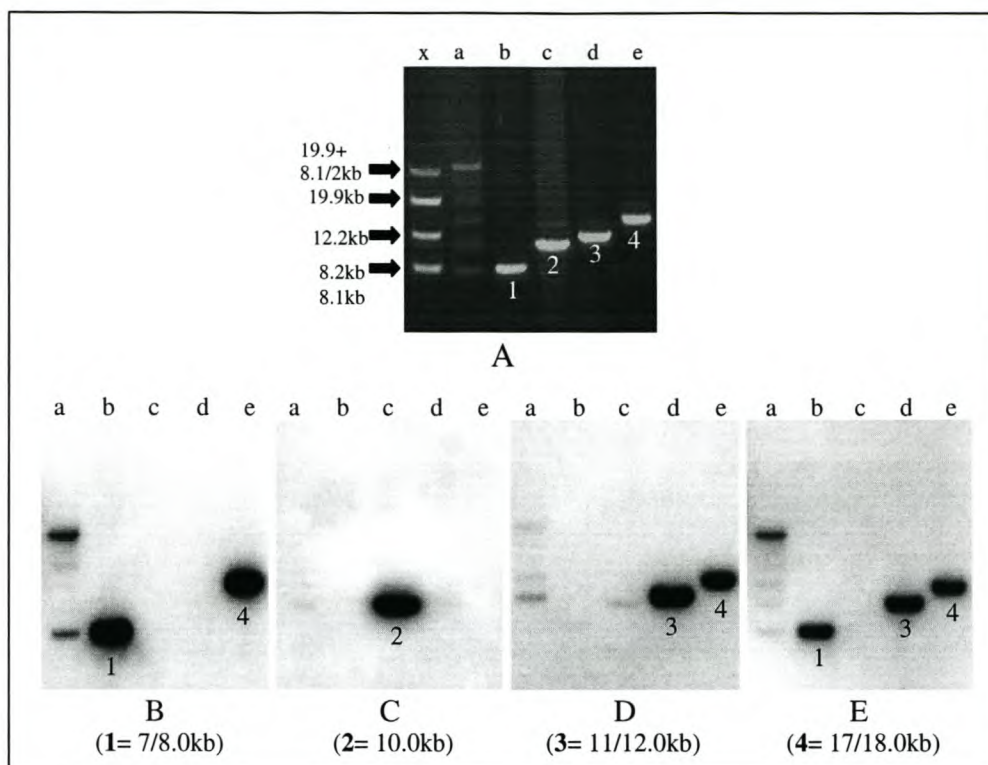


Figure 3.24: (A) Pulsed Field Gel of whole p49879 DNA and four p49879 subclones corresponding to bands 1-4 in Figure 3.23, all digested with *NotI*. Lane x contains molecular weight marker λ cut with *SmaI*; lane a, whole p49879 DNA; lane b, subclone 1; lane c, subclone 2; lane d, subclone 3; lane e, subclone 4. Gels (B)-(E), Southern blot of (A) probed with, (B) Subclone 1 (~7/8.0 kb), (C) Subclone 2 (~10.0kb), (D) Subclone 3 (~11/12 kb), and (E) Subclone 4 (~17/18 kb). Fragments are identified on the various gels with their corresponding subclone numbers.

From this result we suspected that subclone 4 (17/18.0 kb) was a partially digested plasmid band comprising subclones 1 (7/8.0 kb) and 3 (11/12.0 kb). This was later confirmed through sequencing data. Although subclone 4 was the only plasmid band of lower intensity to be successfully cloned, the same theory of "partial banding" was applied to all lower intensity bands. Faint hybridization signals were obtained in lane 1 of Figures 3.24-B, -C, and -D to the ~20.0 kb and ~23.0 kb plasmid bands. This could be the result of a partial association between subclone 4 and subclones 8 (~0.7 kb) and 9 (~1.3 kb), which were not represented on this gel.

NotI restriction endonuclease sites are prone to methylation by an independent methyltransferase system present in *E.coli* K strains, with a target sequence of CG

(*mcrA*). This system is different to the *dam* and *dcm* methylases which modify the adenine residues in the sequence GATC to N⁶-methyladenine and the internal cytosine residues in the sequences CCAGG or CCTGG to 5-methylcytosine respectively. Therefore, should the *NotI* sites joining subclones 1 and 3 be methylated, subclone 4 could be the result of two unresolved plasmid bands. Subclone 4 was passaged through *dcm*⁻/*dam*⁻ *E.coli* strain GM2929 in order to remove methylation should it be present. Plasmid DNA was re-isolated and digested with *NotI* to determine whether methylation had been removed. Subclone 4 remained intact. The degree to which *dam*- and *dcm*- methylation differs from that of *mcrA* methylation could be sufficient reason for the lack of de-methylation to the *NotI* site in subclone 4. A *mcrA*⁻ *E.coli* strain was not available and it was decided to not pursue further investigation into the proposed presence of partial plasmid bands.

Digestion with *PacI* and *XbaI* individually yielded a single band of approximately 30.0 kb, whilst *PmeI* yielded a smaller, single band of approximately 27.0 kb (Figure 3.25A). For each of these three digests large amounts of DNA remained in the wells. This could either be the result of partial digestion, or the presence of two plasmids, a ~30.0 kb plasmid with one site each of *PacI* and *XbaI* and a ~27.0 kb plasmid with a single *PmeI* site. Should the unresolved DNA in the wells be the result of partial digestion, plasmid 49879 would have a minimum of two restriction endonuclease sites for each of the enzymes *PacI*, *XbaI*, and *PmeI* to enable the release of the bands mentioned above on digestion. Double restriction endonuclease digest combinations of these restriction enzymes could then generate up to four bands, depending on restriction site positioning. However, should the unresolved DNA in the wells be the result of two plasmids, double restriction endonuclease digests using *XbaI-PmeI* and *PacI-PmeI* would result in two bands per digest, 30.0 kb and 27.0 kb. Double restriction endonuclease digests with *PacI-PmeI* and *XbaI-PmeI* supported this, generating two distinct bands of ~30.0 kb and ~27.0 kb with no residual DNA remaining in the wells (Figure 3.25B). The likelihood of restriction endonuclease site positioning for three enzymes on a single plasmid yielding the exact predicted sizes of 27.0 kb and 30.0 kb is slim. However, this cannot be disregarded as being impossible. Therefore, although results suggested the presence of two plasmids this was not assumed to be correct, unless further evidence was obtained to support this claim. The presence of two plasmids is not inconsistent with the proposal of faint plasmid

bands being the result of partial digestion, since both plasmids appear to have a similar copy number due to comparable band intensities as shown in Figure 3.25A. Regardless of this result, the possibility that plasmid DNA represented a single plasmid was not discarded.

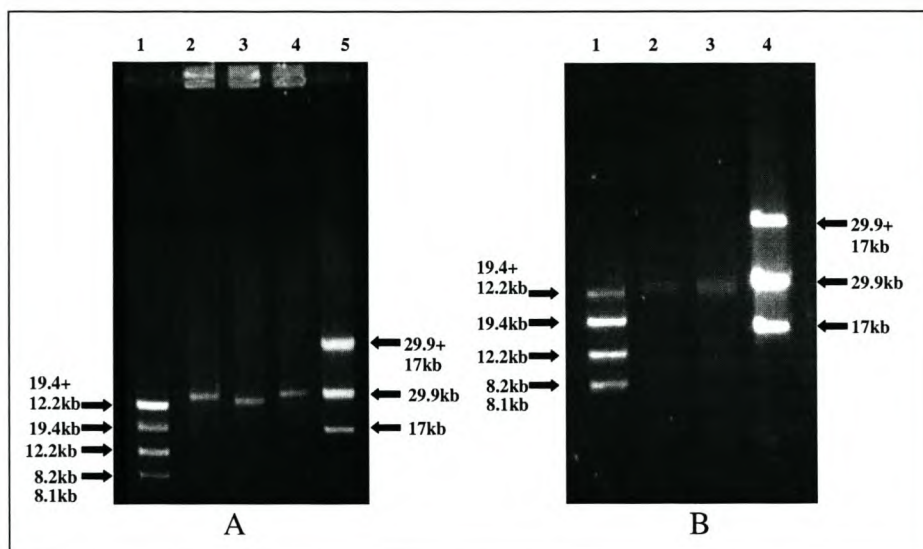


Figure 3.25: Restriction endonuclease analysis of p49879 with *PacI*, *XbaI* and *PmeI*. Lane 1 contains molecular marker λ -*SmaI*. (A) Lane 2, *XbaI*; lane 3, *PmeI*; lane 4, *PacI*; lane 5, molecular marker λ -*KpnI*. (B) Lane 2, *XbaI*-*PmeI*; lane 3, *PacI*-*PmeI*; lane 4, molecular marker λ -*KpnI*.

3.3.4 Mapping of 49879 plasmid DNA

Mapping was achieved through a combination of Southern hybridization, restriction endonuclease analysis and sequencing. Single digests (performed in duplicate) of plasmid DNA with *NotI*, *SalI*, *HindIII*, *SphI* and *SacII*; were separated via standard gel electrophoresis. Gels were blotted and probed with whole-labeled p49879 DNA (Figure 3.26).

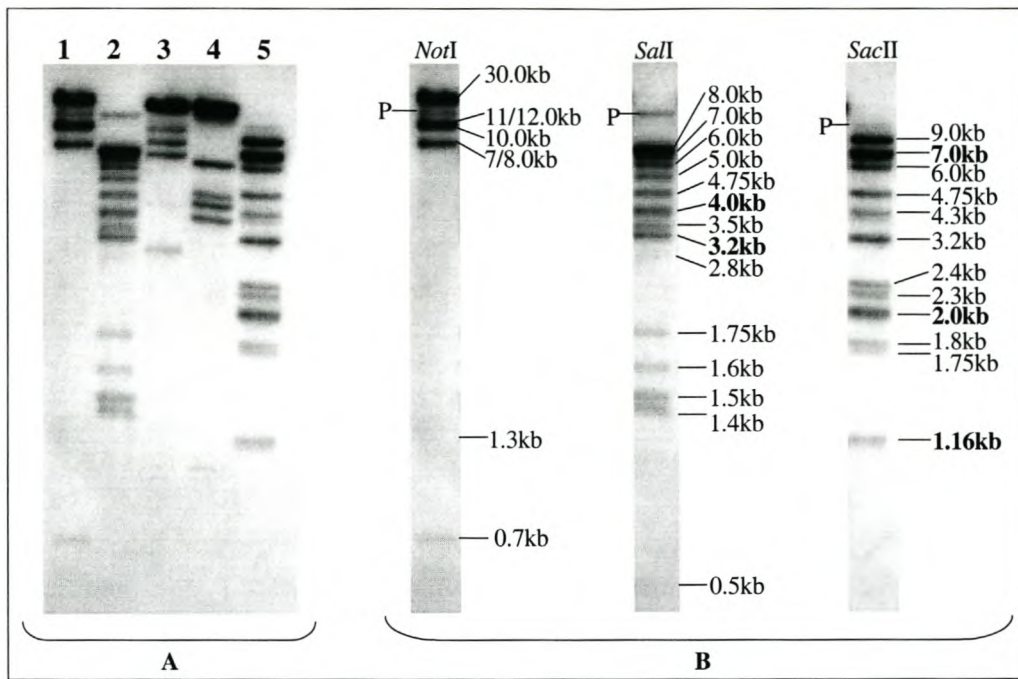


Figure 3.26: Southern blot (template blot) of restriction endonuclease digests of p49879 DNA probed with whole DIG-labeled 49879 plasmid DNA. (A) Lane 1, *NotI*; lane 2, *SalI*; lane 3, *HindIII*; lane 4, *SphI*, and lane 5, *SacII*. (B) Fragment sizes generated by the *NotI*, *SalI* and *SacII* digests of p49879 DNA. The 1.3 kb and 0.7 kb *NotI* fragments, not visible in Figure 3.23 may be seen here. The *SalI* 0.3 kb and 0.14 kb fragments, known to exist, are not visible. P- indicates the presence of partial bands. Fragment sizes in bold print designate the presence of a doublet.

These blots were used as templates to (i) screen large subclone numbers for inserts of different sizes as predicted by the total plasmid digests, and (ii) to link adjacent subclones. The latter was achieved when a probe, generated using different restriction enzymes to those of the target DNA, produced two or more hybridization signals. This principle is illustrated in Figure 3.27.

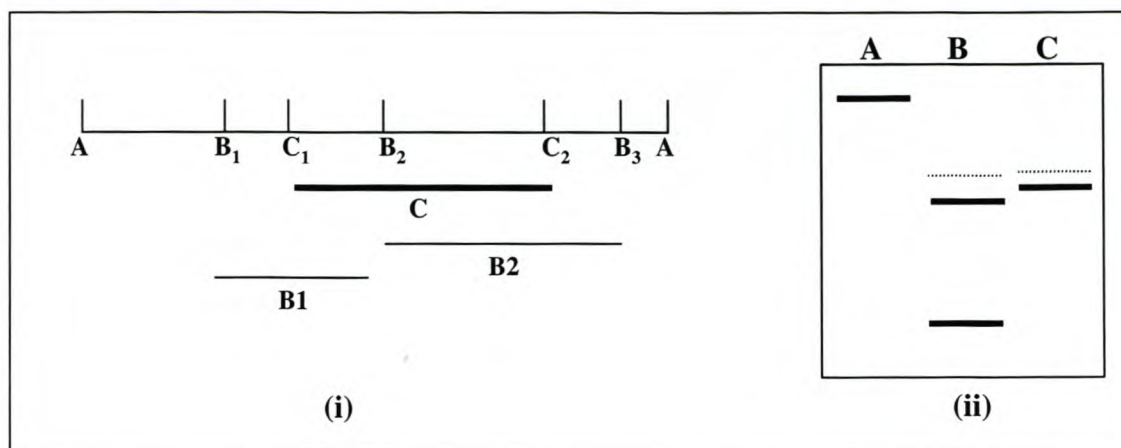


Figure 3.27: Principle of adjacent clone linkage through hybridization. (i) Linear DNA map with restriction endonucleases sites A, B, and C. Bold bar beneath map (C) represents a DIG-labeled probe which overlaps fragments B1 and B2. (ii) The Southern blot generated from digestion of the DNA in (i) probed with C. Hybridizing fragments are in bold. Non-hybridizing fragments are shown as dotted lines. B1 and B2 are both homologous to C and therefore are likely to be linked.

Initially all five restriction enzymes mentioned above were used to generate plasmid subclones, eventually only *NotI*, *SalI*, and *SacII* were used extensively in mapping. The latter yielded a good size spread of fragment sizes, while three of the large fragments generated by *NotI* digestion, were cloned early in this study.

Of the thirty-seven possible clones generated by the single *NotI*, *SalI* and *SacII* digests, thirty-four were eventually captured. Once subclone origin was identified as belonging to plasmid 49879, each clone was mapped and further sub-cloned using enzymes *NotI*, *SalI*, and *SacII*. The ends of each clone were sequenced and processed using DNAMAN v 4.13 (Lynnon Biosoft, 1999). Nucleotide sequence was examined and ORF's were identified using the BLASTX algorithm program (Altschul *et al*, 1990). Although sequence data was able to identify a few ORF's that linked adjacent clones, a large proportion of the amino acid sequence identities obtained at the clone ends were spurious. Map construction therefore relied predominantly on the results generated from Southern hybridization and restriction endonuclease digestion.

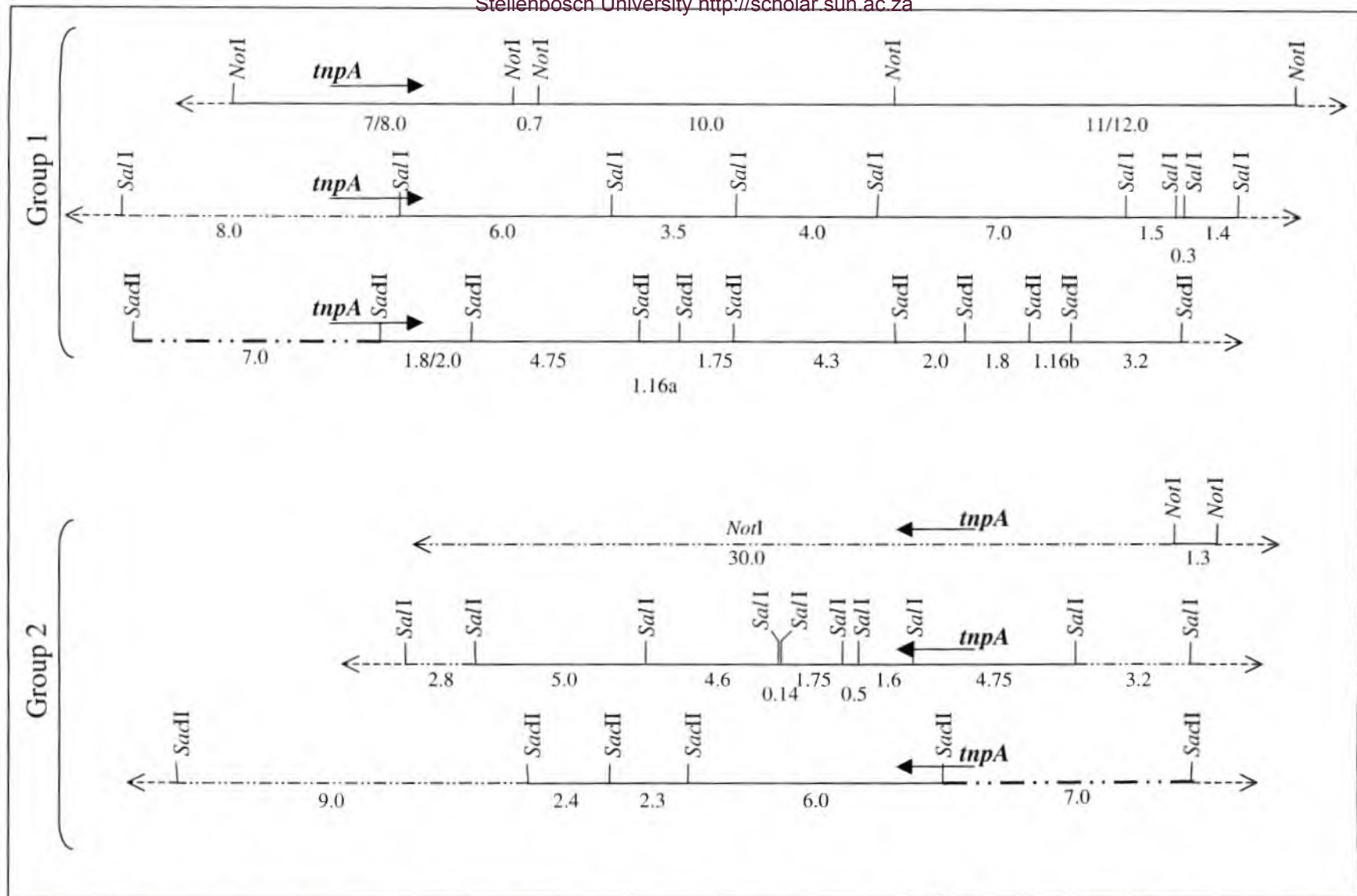


Figure 3.28: Preliminary two-part map of plasmid 49879 DNA. Dashed arrows represent uncloned flanking regions. Double dot-dashed lines represent fragments not yet cloned, but provisionally placed through Southern hybridization. Bold double dot-dashed line represents the 7.0kb-*SacII* fragment referred to in the text. The 1.3kb-*NotI* fragment of Group2 is cloned, but has been placed only by Southern hybridization. Amino acid identity to the transposase of Tn21 (*tnpA*) is shown by a labeled arrow, representing gene orientation. Fragment sizes are indicated below the maps in kilobases.

Using a combination of the three techniques, subclones were found to separate into two main groups (Figure 3.28, opposite page) with a 7.0 kb-*SacII* fragment, seemingly common to both groups through hybridization homology (Figure 3.29) and amino acid sequence identity (TnpA) obtained through sequence analysis of the ends of adjacent and overlapping fragments. This fragment was thought to bridge the two groups, generating a single plasmid map. Additional inter-group homology, in the vicinity of this apparent “join” region, was also obtained and are listed as follows: (the 7.0 kb-*SacII* fragment, seemingly common to both subclone groups is indicated in bold print) (i) 7/8.0 kb-*NotI* hybridized to 30.0 kb-*NotI*, 4.75 kb-*SalI*, 3.2 kb-*SalI*, and **7.0 kb-*SacII***. (ii) 0.7 kb-*NotI* hybridized to 30.0 kb-*NotI*, 5.0 kb-*SalI*, 2.8 kb-*SalI*, and 9.0 kb-*SacII*. (iii) 11/12.0 kb-*NotI* hybridized to 30.0 kb-*NotI*, 4.75 kb-*SalI*, and **7.0 kb-*SacII***. (iv) 1.8/2.0 kb-*SacII* hybridized to 30.0 kb-*NotI*, 4.75 kb-*SalI*, and **7.0 kb-*SacII***. (v) 6.0 kb-*SalI* hybridized to 30.0 kb-*NotI*, 4.75 kb-*SalI*, **7.0 kb-*SacII***, and 6.0 kb-*SacII*. (vi) 6.0 kb-*SacII* hybridized to 7/8.0 kb-*NotI*, 8.0 kb-*SalI*, 6.0 kb-*SalI*, 1.8/2.0 kb-*SacII*, and **7.0 kb-*SacII***. (vii) 4.75 kb-*SalI* hybridized to 7/8.0 kb-*NotI*, 8.0 kb-*SalI*, 1.8/2.0 kb-*SacII*, and **7.0 kb-*SacII***. All Southern hybridization results are recorded in Appendix B. The 7.0 kb-*SacII* fragment was uncloned at this stage, therefore a probe was constructed by excising the appropriate band from an agarose gel. Hybridization results confirmed homology to 30.0 kb-*NotI*, 7/8.0 kb-*NotI*, 8.0 kb-*SalI*, 4.75 kb-*SalI*, and 3.2 kb-*SalI* (Figure 3.29D).

Although hybridization results indicated that homology existed between the two groups at the 7.0 kb-*SacII* region, feasibility of joining the two groups at this fragment did not seem likely. Firstly, the predicted restriction endonuclease maps of *SalI* and *NotI* did not align. The 7.0 kb-*SacII* fragment of Group 1 should have an internal *NotI* site (generating insert fragments of ~3.0 kb and ~4.4 kb on digestion with *NotI* and *SacII*), while the 7.0 kb-*SacII* fragment of Group 2 should have internal *SalI* and *NotI* sites (generating fragments of ~4.0 kb, ~3.1 kb, and ~0.1 kb on digestion with *NotI*, *SalI*, and *SacII*). Secondly, if the extrachromosomal DNA from *L. ferrooxidans* ATCC49879 was from a single plasmid with two fragments (group1 and group2) joined at the transposase gene, the fragments surrounding *tnpA* in each group would be the same. This was not found to be, as the fragments adjacent to the 7.0 kb-*SacII* fragment of Group1 differed to those fragments adjacent to the 7.0 kb-*SacII* fragment of Group2.

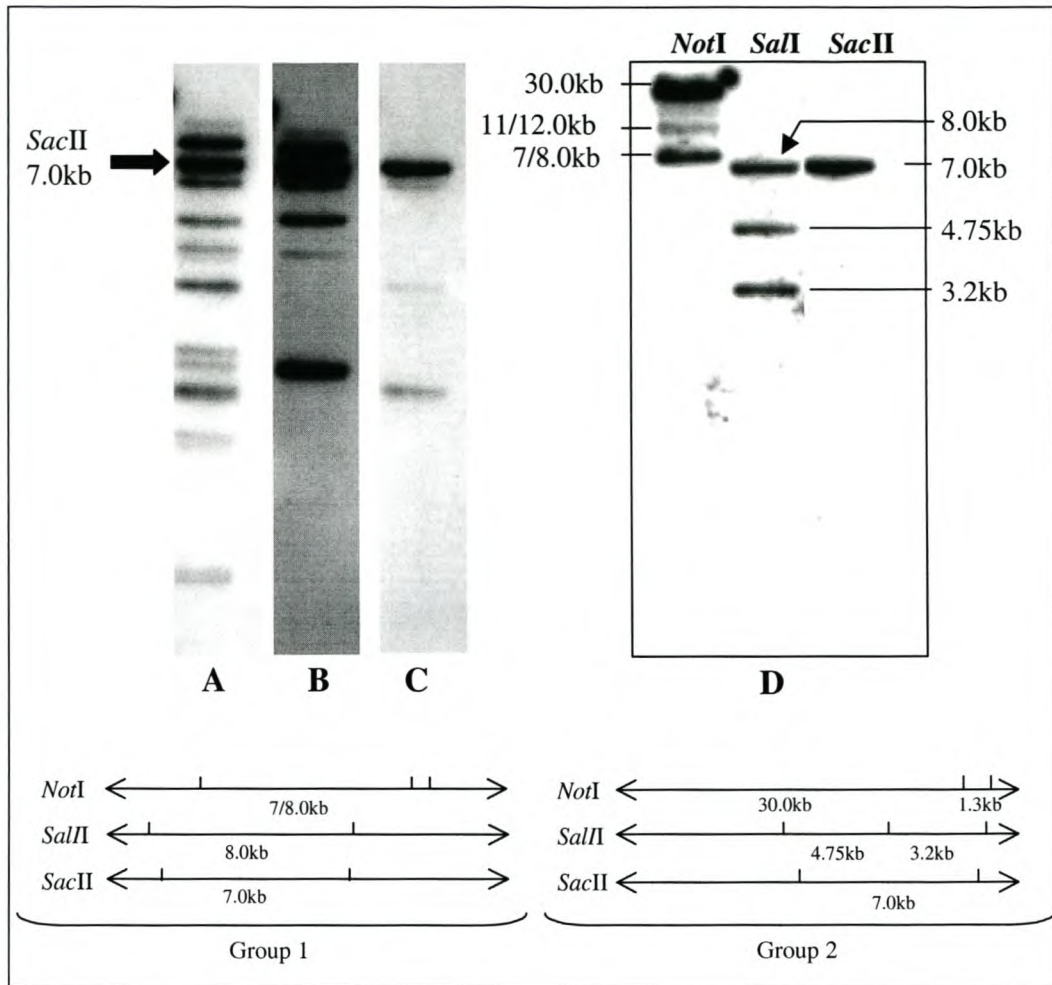


Figure 3.29: Southern blots of whole 49879 plasmid DNA digested with *SacII* only (A-C) as well as *NotI*, *SalI*, and *SacII* (D). Probes: (A) whole-labeled 49879 plasmid DNA, (B) 7/8.0 kb *NotI* fragment from Group1, (C) 4.75 kb *SalI* fragment from Group2, and (D) 7.0kb *SacII* gel excised fragment. The bold arrow indicates the 7.0 kb *SacII* fragment, seemingly common to both groups. Sizes of all bands, which gave homology to the gel-excised 7.0 kb *SacII* probe, are indicated in (D), and are in agreement with the sub-clone layout in Figure 3.28. A partial map of the two groups is given below the hybridization results, indicating the relevant regions.

In order to take a closer look at this region, an attempt at cloning the 7.0 kb-*SacII* fragment was made. The appropriate band was excised from an agarose gel, ligated into pSK and transformed. Several transformants were screened for the presence of a 7.0 kb insert. Digestion of miniprep DNA with *SalI*, *NotI*, and *SacII* revealed the presence of two different 7.0 kb-*SacII* fragments with banding signatures

corresponding to the predicted restriction patterns of each group (Figure 3.30). The Group 1 7.0kb-*Sac*II fragment gave a 3.0 kb and 4.4 kb insert band as well as a 3.0 kb vector band, when digested with *Sac*II, *Sal*I and *Not*I (Figure 3.30, Lane 3). The Group 2 7.0 kb-*Sac*II fragment gave 4.0 kb, 3.1 kb, and 0.1 kb insert bands as well as a 3.0 kb vector band (Figure 3.30, Lane 4).

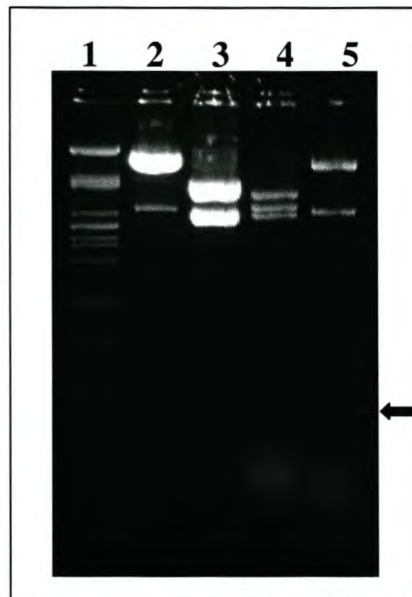


Figure 3.30: Restriction endonuclease patterns of the Group 1 and Group 2 7.0 kb-*Sac*II fragments from plasmid p49879. Lane 1, λ -*Pst*I; lane 2, 7.0 kb-*Sac*II fragment (Group 1) digested with *Sac*II; lane 3, 7.0 kb-*Sac*II fragment (Group 1) digested with *Sac*II/*Sal*I/*Not*I; lane 4, 7.0 kb-*Sac*II fragment (Group 2) digested with *Sac*II/*Sal*I/*Not*I; lane 5, 7.0 kb-*Sac*II fragment (Group 2) digested with *Sac*II. In lane 3 the 3.0 kb doublet appears as a single bright band. The 0.1 kb band generated from digesting the 7.0 kb-*Sac*II fragment of Group 2 with *Sac*II, *Sal*I, and *Not*I (Lane 4) is indicated by a solid arrow.

The existence of two 7.0 kb-*Sac*II fragments went undetected when the 7.0 kb-*Sac*II probe was constructed, as a mixture of both fragments would have been present in the excised gel slice. This hybridization experiment was repeated with the individual, DIG-labeled, 7.0 kb-*Sac*II fragments (Figure 3.31). Results indicated a difference between the two fragments when only one of the probes hybridized to a 3.2 kb-*Sal*I fragment predicted to be present in Group 2 (Figure 3.31B). The 7.0 kb-*Sac*II probe, believed to be of Group 1 origin, also hybridized more strongly to the 7/8.0 kb-*Not*I and 8.0 kb-*Sal*I fragments of Group 1 than to the 30.0 kb-*Not*I and 4.75 kb-*Sal*I fragments of Group 2. Inter-group hybridization homology was still apparent, but

probably due to the regions of homologous sequence (*tnpA*) already shown to be present in both groups. Results at this stage seemed to support the two-group/plasmid interpretation of subclone separation.

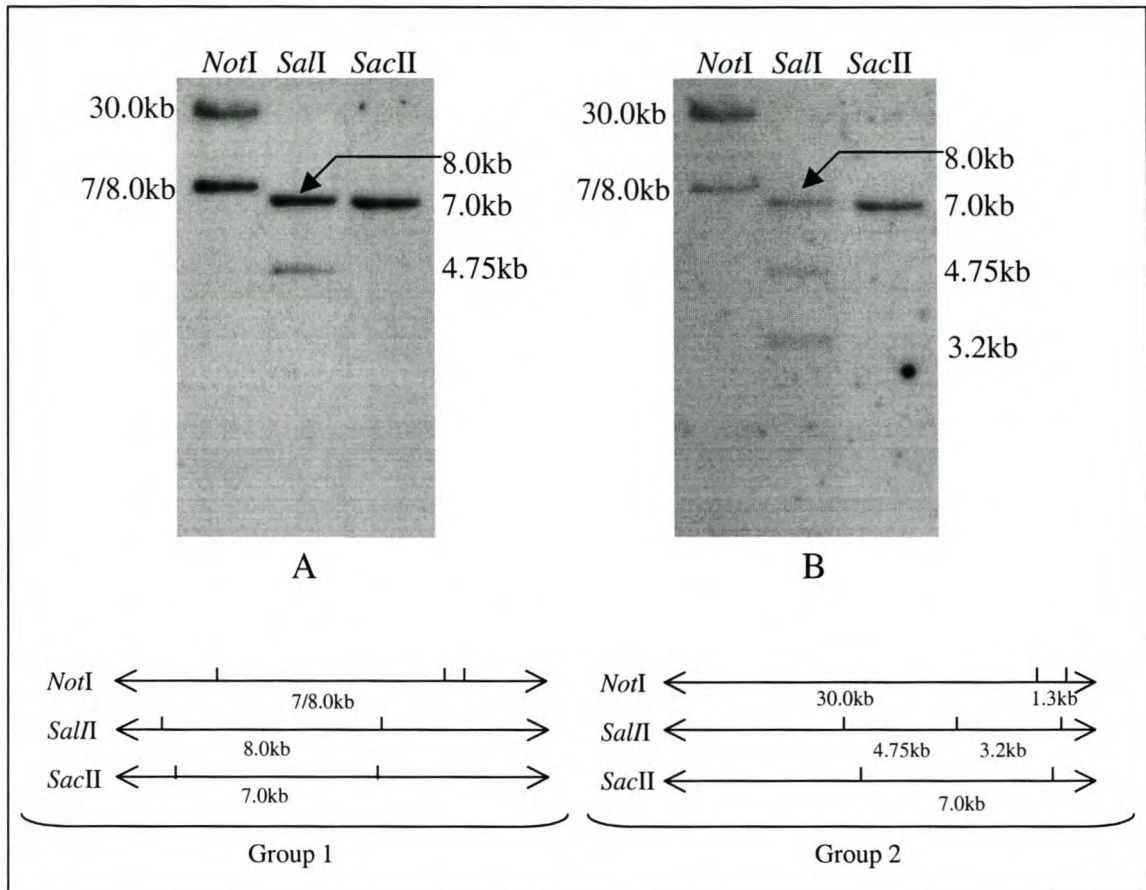


Figure 3.31: Template blots probed with (A) Group 1 7.0 kb-*SacII* fragment, and (B) Group 2 7.0 kb-*SacII* fragment. The 30.0 kb-*NotI* and 7/8.0 kb-*NotI* fragments hybridize to both probes due to sequence homology at the *tnpA* region (refer to Figure 3.28). A difference in hybridization homology between the two probes lies with the 3.2 kb-*SalI* fragment which hybridizes to the Group 2 7.0 kb-*SacII* probe and not the Group 1 7.0 kb-*SacII* probe. A partial map of the two groups is given below the hybridization results, indicating the relevant regions.

An interesting observation was made in an over-exposure of the Southern blot in Figure 3.31A (Figure 3.32). Feint hybridization signals were obtained to the 11/12.0 kb-*NotI* fragment. From the preliminary restriction endonuclease map of Group 1, this result would be inconsistent unless Group 1 fragments were circularized through

an association of the 7/8.0 kb and 11/12.0 kb *NotI* fragments at the *NotI* site internal to the 7.0 kb-*SacII* subclone.

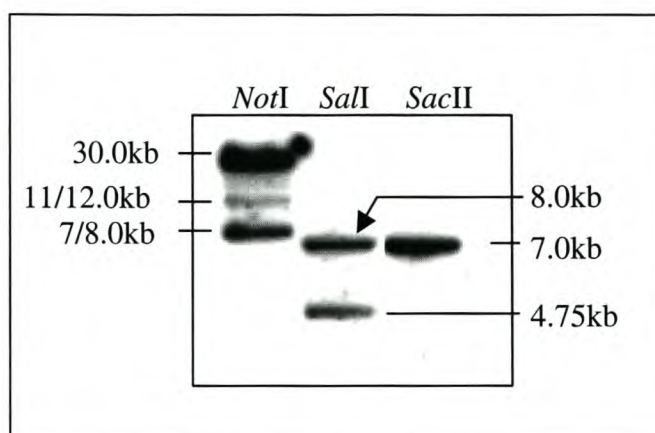


Figure 3.32: Overexposed Template blot probed with the 7.0 kb-*SacII* fragment ex Group 1. 11/12 kb-*NotI* fragment (p1N4) is giving a positive hybridization signal.

Analysis of the sequence data derived from the outer ends of the 7/8.0 kb and 11/12.0 kb *NotI* fragments of Group 1 identified an ORF with amino acid sequence identity to a hypothetical protein of *Sphingomonas aromaticivorans*, which was found to straddle both fragments, circularizing the clones of Group 1 to form a ~ 30.0 kb plasmid, p49879.1 (Group 1). Although the homology to this hypothetical protein was low (therefore not included in Table 3.2), a weak relationship was found on both the 7/8.0 kb and 11/12.0 kb *NotI* fragments. Results from the digestion of plasmid DNA in section 3.3.3 with *PacI*, *XbaI*, and *PmeI*, did indeed represent two different plasmids of approximately 30.0 kb (p49879.1-Group 1) and 27.0 kb (p49879.2- Group 2) respectively. Inter-group homology detected via Southern hybridization was a result of similar genes being present on clones from within both groups. Already mentioned is the transposase (*tnpA*) of Tn21, responsible for hybridization homology amongst clones: 30.0 kb-*NotI*, 7/8.0 kb-*NotI*, 8.0 kb-*SalI*, 6.0 kb-*SalI*, 4.75 kb-*SalI*, 7.0 kb-*SacII*, 7.0 kb-*SacII*, 6.0 kb-*SacII*, and 1.8/2.0 kb-*SacII* (Group 1 fragments are underlined). The ORF responsible for hybridization homology amongst clones: 6.0 kb-*SalI*, 5.0 kb-*SalI*, 2.8 kb-*SalI*, 30.0 kb-*NotI*, and 0.7 kb-*NotI*, is the MobL from pTF1 of *Acidithiobacillus ferrooxidans*.

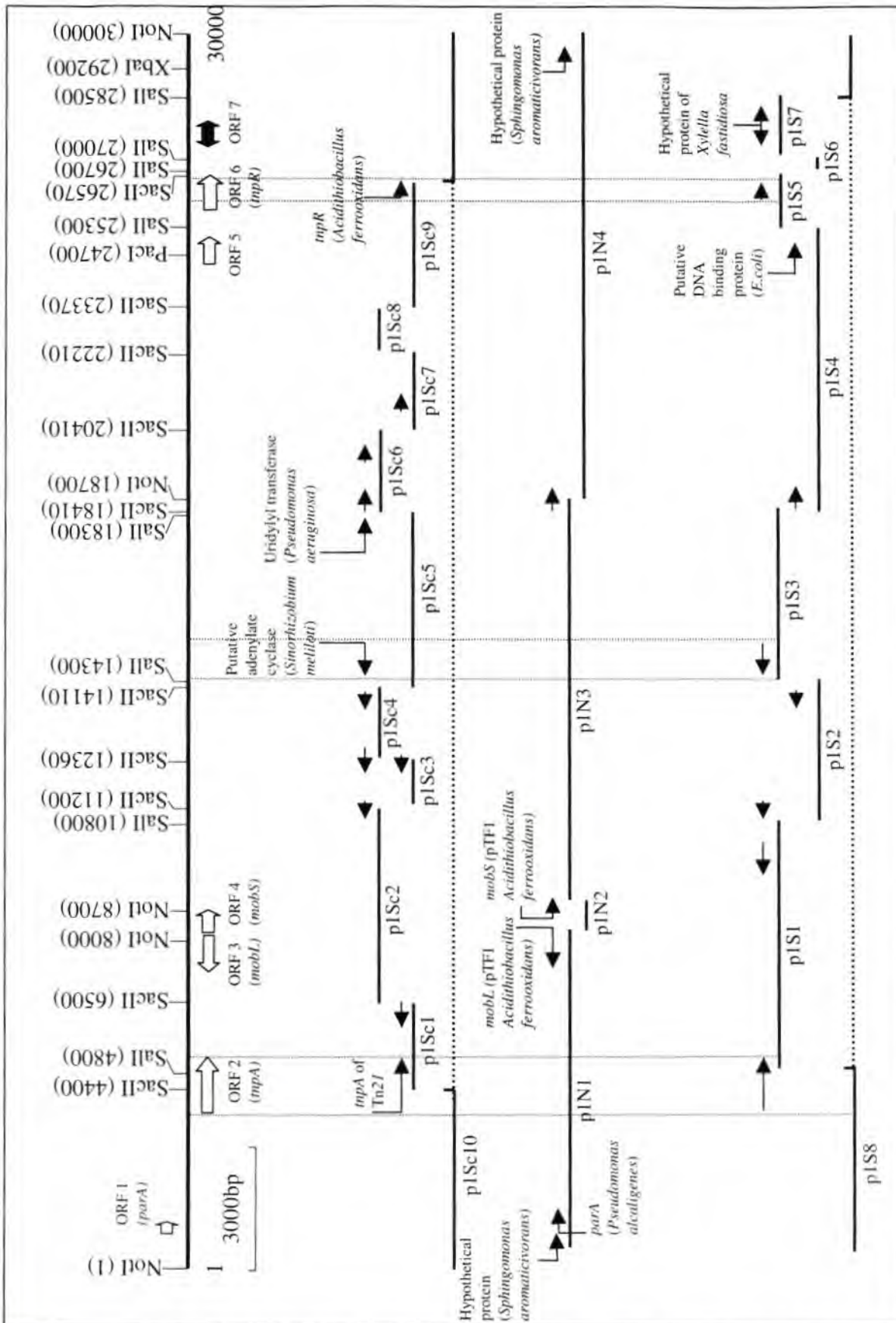


Figure 3.33: Physical map of p49879.1. Subclones are represented as thick black bars beneath the map. Sc= *SacII*, S=*SalI*, N=*NotI*. Thin arrows represent areas which have been sequenced and the closest relationships to known proteins from the database. Block arrows represent ORF's with high amino acid identity to know proteins (an e-value of no greater than 0.001 over at least 20% of the protein). Open block arrows indicate where only part of the ORF is present, filled block arrows indicate the presence of a complete ORF. More information concerning these ORF's is given in Table 3.2. Only maps and subclone are drawn to scale.

The extrachromosomal DNA from *L. ferrooxidans* ATCC49879 therefore constitutes two plasmids, p49879.1 (~ 30.0 kb) and p49879.2 (~ 27.0 kb), which although they share a certain degree of homology, are not identical.

3.3.5 Partial sequence analysis of p49879.1 and p49879.2

The sequence information available for both plasmids is incomplete as only the ends of each clone were sequenced in either the forward or reverse directions. Nucleotide sequence data was processed and ORF's were identified using the BLAST algorithm program (Altschul *et al*, 1990). Not all ORF's could be identified, with some giving very low amino acid identity and similarity percentages to known proteins in the database. Physical maps of the individual plasmids p49879.1 and p49879.2, including all positive identities obtained from predicted amino acid sequence analysis regardless of the degree identity /similarity, are represented in Figures 3.33 (opposite, page) and 3.37 respectively. However, ORF's displaying a maximum e-value of 0.001, over no less than 20% of the target protein, are reported in more detail. In addition to these criteria, ORF identification was also based on selection from the top five most closely related proteins. The location and identity of "significant" ORF's present on p49879.1 and p49879.2 are given in Table 3.4 and Table 3.5 respectively. More than one homologue is given in certain instances to emphasize relationship or function.

3.3.5.1 p49879.1

ORF 1 (ParA) shows high amino acid (aa) identity (84%) and similarity (90%), albeit over a small region (46 aa), to the partition protein, ParA (212aa), of *P. alcaligenes*. Partitioning systems are associated with plasmids, and provide stability to the replicon during cell division. The function of ParA is that of an ATPase, whilst some ParA's also act as site-specific DNA binding proteins (Gerdes *et al*, 2000).

ORF 2 (TnpA) displayed an 81% aa sequence identity to 45.5% of the C-terminal region of the transposase (*tnpA*) for Tn21 (Plasmid R100). ORF 6 (TnpR) displayed a 67% aa identity over 98.4% of the resolvase from the mercury resistant transposon, Tn5037, found in a mercury-resistant *At. ferrooxidans* isolate. The *At. ferrooxidans* resolvase has an 88% aa sequence identity to 85% the N-terminal region of the Tn21 resolvase (*tnpR*) and is considered Tn21-like (Rawlings and Kusano, 1994). Multiple sequence alignments of the deduced TnpR (Figure 3.34) and TnpA (Figure 3.35)

Table 3.4: Location and size of ORF's present in p49879.1 and closest relationship to known proteins

ORF	Approximate position	No. of aa	Total predicted aa no. from ^a	Proportion of protein ^b (%)	Homologue ^a	Organism	E-value ^c	% Identity/ % similarity	Accession number
ORF 1 (ParA)	440-580	46	212	21.7	Partition protein ParA	<i>Pseudomonas alcaligenes</i>	2e ⁻¹⁵	84/90	U88088
ORF 2 (TnpA)	3670-5050	450	988	45.5	Transposase for Tn21	Plasmid R100	e ⁻¹³⁸	81/87	NC_002134
ORF 3 (MobL)	7350-6750	207	378	54.8	Mobilization protein: MobL (ORF ₂) of pTF1	<i>Acidithiobacillus ferrooxidans</i>	4e ⁻²⁴	35/49	X52699
(MobA)		156	709	22.0	Mobilization protein (MobA)	Plasmid RSF1010	2e ⁻⁰⁸	31/45	NC_001740
ORF 4 (MobS)	8360-8510	54	98	55.1	Mobilization protein: MobS (ORF ₆) of pTF1	<i>Acidithiobacillus ferrooxidans</i>	5e ⁻⁰⁷	50/74	X52699
(MobC)		64	94	68.1	Mobilization protein (MobC)	Plasmid RSF1010	4e ⁻⁰⁶	37/63	M28829
ORF 5	24880-25140	83	107	77.6	Putative DNA binding protein	<i>E.coli</i> 0157:H7	8e ⁻⁰⁸	46/65	BAB38021
ORF 6 (TnpR)	25300-25810	186	183	98.4	Resolvase (Tn5037)	<i>Acidithiobacillus ferrooxidans</i>	7e ⁻⁶⁵	67/80	AJ251743
(TnpR)		186	183	98.4	Resolvase (Tn21)	Plasmid R100	8e ⁻⁶¹	63/77	NC_002134
ORF 7	27530-27800 ^d	93	93	100	Hypothetical protein	<i>Xylella fastidiosa</i>	5e ⁻¹⁹	67/80	AE004023

^a Criteria for ORF identification: maximum e-value of 0.0 over no less than 20% of the target protein, selected from within the top 10 closest protein relationships.

^b Indicates % of known protein that query protein from p49879.1 was homologous to.

^c An E (Expect) value of one represents the chance that in the available database you might expect to see one match with a similar score simply by chance (Kirby *et al.*, 2002).

^d The orientation of this ORF is undetermined and could also take the order of position 27700-27970.

sequences to other transposase and resolvase proteins showed 75.7% and 75.1% homology respectively; this correlates with the 70% homology which members of the Tn21 family have been reported to share (Grinsted *et al*, 1990). The transposase and resolvase genes are also transcribed in the same direction, which is in agreement with those of the Tn21 transposon subgroup (Figure 3.19). Whether these are functional or pseudogenes is still to be determined.

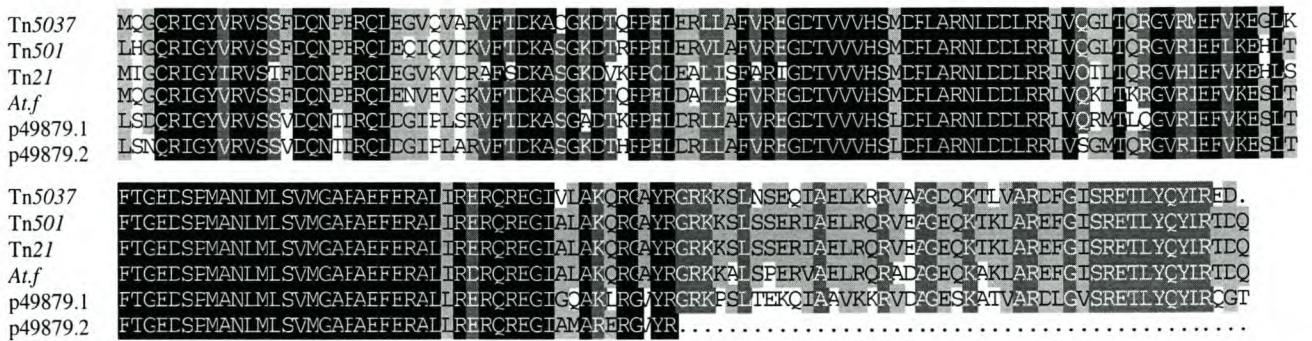


Figure 3.34: Multiple sequence alignment of TnpR proteins from *L. ferrooxidans* ATCC49879 plasmids against TnpR proteins from Tn21-like transposons. Shading black = 100% homology; dark grey = 75% homology; light grey = 50% homology. Accession numbers: Tn5037-CAA70238; Tn501-NP085427; Tn21-BAA78804; *Acidithiobacillus ferrooxidans* (*At.f*) Tn5037-CAC69253/AJ251743. Amino acid codes are given in Appendix E.

ORF 3 and ORF 4 showed homology to products of mobilization genes. ORF 3 displayed a 35% aa sequence identity over 55% of the MobL of plasmid pTF1 from *At. ferrooxidans*. A 31% aa sequence identity to the partly related MobA/RepB protein of RSF1010 was also obtained. Both proteins function as relaxases responsible for DNA nicking at the *oriT* site before conjugal transfer takes place. ORF 4 had a 50% aa sequence identity to 55% of the C-terminal region of the *At. ferrooxidans* pTF1 MobS protein, and 37% aa sequence identity to 68% of the MobC protein of pRSF1010. MobS of pTF1 and MobC of RSF1010 are accessory proteins involved in plasmid mobilization (Rawlings and Tietze, 2001). As a result of incomplete sequence it is uncertain to which protein the sequence of ORF 4 is most

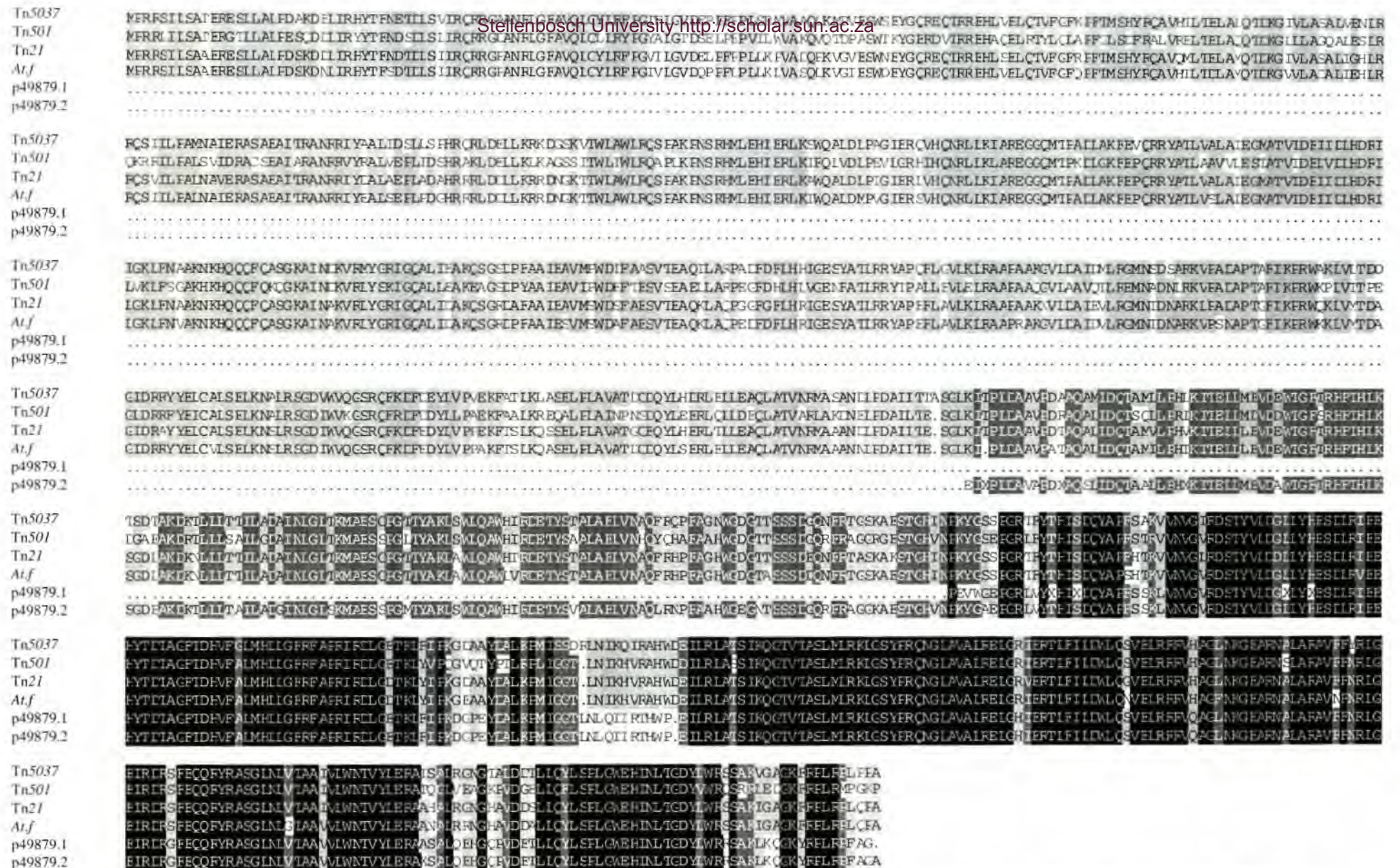


Figure 3.35: Multiple sequence alignment of TnpA proteins from *L. ferrooxidans* 49879 plasmids against TnpA proteins from Tn21-like transposons. Shading: black = 100% homology; dark grey = 75% homology; light grey = 50% homology. Accession numbers: Tn5037-CAA70236; Tn501-NP085428; Tn21-BAA78805; *Acidithiobacillus ferrooxidans* (At.f)-CAC69254.

similar. The *mobS/C*-like and *mobL/A*-like genes of p49879.1 are also divergently transcribed, which is in agreement with the IncQ-like plasmid systems (Figure 3.5). Owing to the identification of both a MobL and MobS, the only other outstanding factor in a mobilizable plasmid system is an origin of transfer (*oriT*). Thus the region between ORF 3 and ORF 4, suspected of accommodating the *oriT* (based on other systems), was examined. The conserved consensus sequence of the RSF1010/pTF1 *oriT* family was identified and is shown in Figure 3.36. Although the presence of a complete, functional mobilization system had not yet been determined, the p1S1 subclone of p49879.1 carrying ORF's 3 and 4 (Figure 3.33) was tested for the ability to be mobilized. p1S1 was excised, cloned into the non-mobilizable vector PUC19, and transformed into *E. coli* S17.1 cells containing the self-transmissible plasmid RP4 (IncP) on the chromosome. The RP4 system is able to provide the genes necessary for mating pair formation, thus establishing cell-to-cell contact with the recipient (CSH56), whilst the genes necessary for DNA transfer and replication should be provided by the p1S1 subclone, if the complete ORF's are present. This system was tested in parallel with a positive plasmid control, *E. coli* S17.1::RP4 (a suitable conjugation donor) containing the complete mobilization gene system of *At. ferrooxidans* plasmid pTF-FC2. The control plasmid with the pTF-FC2 system was mobilized at high frequency (> 1.0 transconjugant/donor) (Rohrer and Rawlings, 1992), but no mobilization was detected for *E. coli* S17.1::RP4 containing p1S1. This result is not conclusive as the presence of a complete system has not yet been determined.

```

p49879.1  TTGCATTAAGTGCGCCCTTC
pTF1     TACTCTAAGTGCGCCCTTG
RSF1010  ACCGGTAAGTGCGCCCTCC

```

Figure 3.36: Alignment of p49879.1 *oriT* region. Nucleotide positions that are conserved within the same family of *oriT* sequences are underlined and bolded. GenBank/EMBL accession numbers: RSF1010, M28829; pTF1, X52699.

ORF 5 and especially ORF 7 share extensive similarity with proteins whose functions have not yet been clearly defined (Table 3.2).

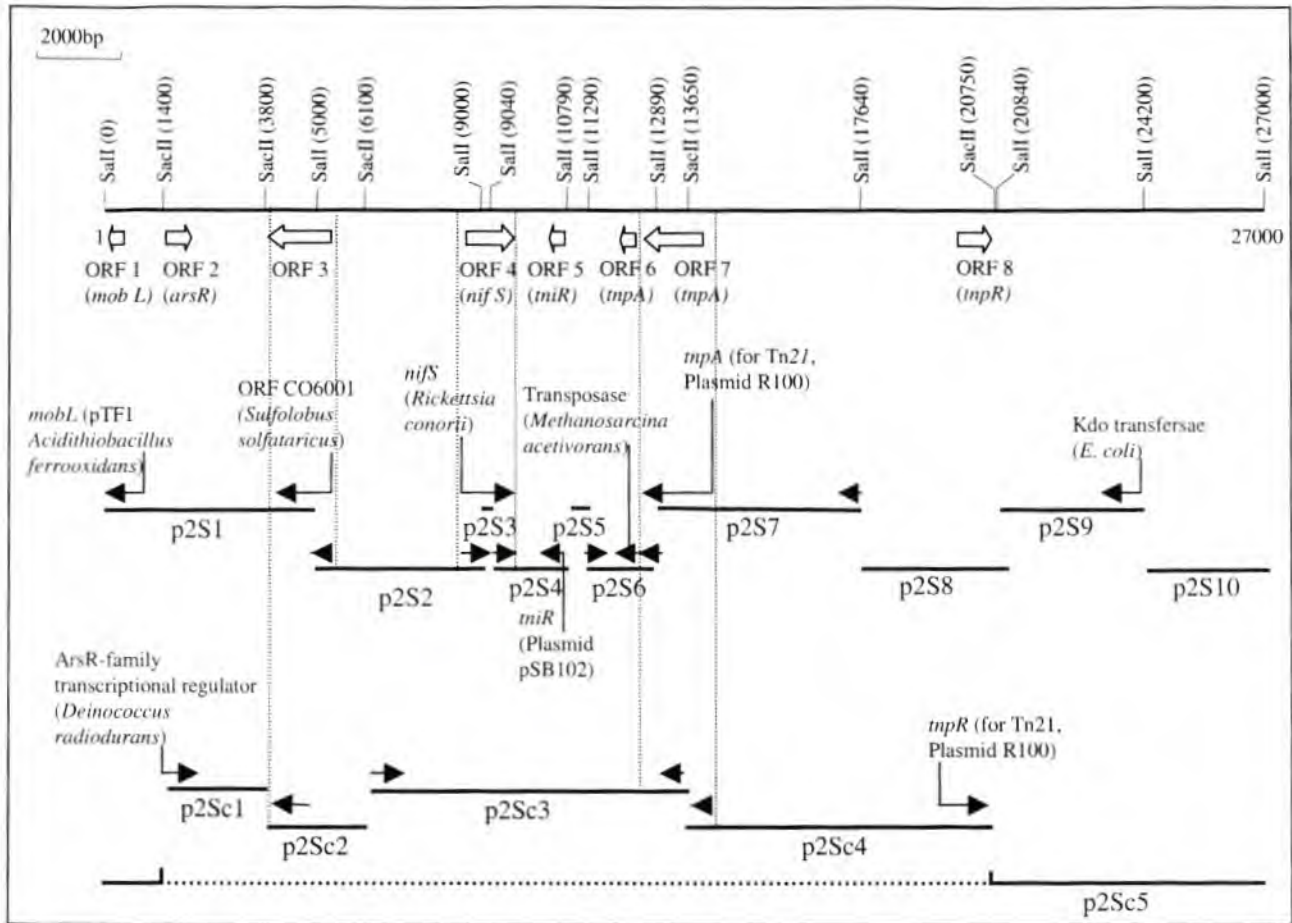


Figure 3.37: Physical map of p49879.2. Subclones are represented as thick black bars beneath the map. Sc= *SacII*, S=*SalI*, N=*NotI*. Thin arrows represent areas which have been sequenced and the closest relationship to known proteins from the database. Block arrows represent ORF's with significant amino acid identity to know proteins (an e-value of no greater than 0.0 over atleast 20% of the protein). Open block arrows indicate were only part of the ORF is present. More information concerning these ORF's is given in Table 3.3. Subclones p2Sc5 and p2S10 are uncloned. The *NotI* and *PmeI* sites, known to be present on this plasmid must lie within this region. Only maps and subclones are drawn to scale.

3.3.5.2 p49879.2

ORF 1 (MobL) (Figure 3.37, opposite page) had a 35% aa identity to 30% of the N-terminal region of the *At. ferrooxidans* pTF1 MobL protein. pTF1 appears to have only two mobilization proteins, MobL, which has an aa sequence related to the first 386 aa of the N-terminal relaxase region of RSF1010 MobA, and MobS, which is related to RSF1010 MobC (Rawlings and Tietze, 2001). Whether there is an equivalent to MobS or MobC requires additional sequencing.

ORF 2 shared extensive aa homology to an ArsR-family transcriptional regulator of *Deinococcus radiodurans*. The *arsR* gene forms part of an *ars* operon that drives an arsenic resistance mechanism, and encodes a repressor that controls the basal levels of *ars* operon expression. Since *L. ferrooxidans* is used in commercial biooxidation processes to recover gold from arsenopyrite ores (Rawlings and Silver, 1995), and total arsenic levels greater than 13 g l^{-1} have been reported in arsenopyrite biooxidation tanks (Dew *et al.*, 1997), the presence of an arsenic resistance mechanism would not be unexpected. Plasmid-associated arsenic efflux resistance mechanisms have been well documented (Silver and Walderhaug, 1992; Cervantes *et al.*, 1994; Silver, 1996); a transposon-based system has also been reported in Tn2502 of plasmid pYV of *Yersinia enterocolitica* (Neyt *et al.*, 1997). Two families of ArsR-like proteins have been shown to exist (Figure 3.38) (B.Butcher, PhD thesis, personal communication). The CLUSTALW program was used for multiple sequence alignments. ORF 2 of p49879.2 has similarity to the second group, of which only one member has been shown to function as an ArsR, *At. ferrooxidans* ArsR. A multiple aa sequence alignment of ORF 2 against the second group of ArsR-like proteins confirmed the presence of conserved homology regions known to exist in ArsR-like proteins (Figure 3.39). A 'helix-turn-helix motif', which members of this family of regulatory proteins (ArsR-like) are reported to contain, is present in p49879.2. This indicates the existence of a DNA binding domain and possibly a regulatory function of some description.

Examples of other bacteria found in arsenopyrite biooxidation tanks, known to carry arsenic resistance mechanisms include *At. ferrooxidans* (Butcher *et al.*, 2000), and *At. caldus* (de Groot, *et al.*, 2001; Dopson *et al.*, 2001). Genes for resistance to the inorganic salts of metals, such as cadmium, mercury, and arsenic are found both on

Table 3.5: Location and size of ORF's present in p49879.2 and closest relationship to known proteins

ORF	Approximate position	No. of aa	Total predicted aa no. from ^a	Proportion of protein ^b (%)	Homologue ^a	Organism	E-value ^c	% Identity/ % similarity	Accession number
ORF 1 (MobL)	340-1	114	378	30.2	Mobilization protein: MobL (ORF ₂) of pTFI	<i>Acidithiobacillus ferrooxidans</i>	1e ⁻⁰¹	35/53	X52699
ORF 2 (ArsR)	1510-1780	90	109	82.6	ArsR-family transcriptional regulator	<i>Deinococcus radiodurans</i>	2e ⁻⁰⁶	35/51	AE002005
ORF 3	5450-4720	246	247	99.6	Hypothetical protein (ORF C06001)	<i>Sulfolobus solfataricus</i>	5e ⁻⁵⁰	45/60	YO8256
ORF 4 (NifS)	8760-9630	292	410	71.2	NifS protein homologue	<i>Rickettsia conorii</i>	6e ⁻²²	46/70	AAL03268
ORF 5 (TniR)	10680-10400	93	204	45.6	TniR protein	Plasmid pSB102	6e ⁻³⁵	61/70	AJ304453
ORF 6 (TnpA)	12770-12250	172	477	36.1	Transposase	<i>Methanosarcina acetivorans</i>	1e ⁻²⁶	40/54	AE010812
ORF 7 (TnpA)	14330-13020	439	988	44.4	Transposase for Tn21	Plasmid R100	0.0	83/88	NC_002134
ORF 8 (TnpR)	20330-20750	140	186	75.3	Resolvase (Tn5037)	<i>At. ferrooxidans</i>	7e ⁻⁵¹	71/81	AJ251743
		140	186	75.3	Resolvase for Tn21	Plasmid R100	1e ⁻⁴⁶	65/78	NC_002134

^a Criteria for ORF identification: maximum e-value of 0.0 over no less than 20% of the target protein, selected from within the top 10 closest protein relationships.

^b Indicates % of known protein that query protein from p49879.2 was homologous to.

^c An E (Expect) value of one represents the chance that in the available database you might expect to see one match with a similar score simply by chance (Kirby *et al.*, 2002).

plasmids and in chromosomes (Rensing *et al.*, 1999). Although high aa sequence identity was obtained to an arsenical resistance-like repressor family, the presence of arsenic resistance genes themselves in p49879.2 has not yet been found.

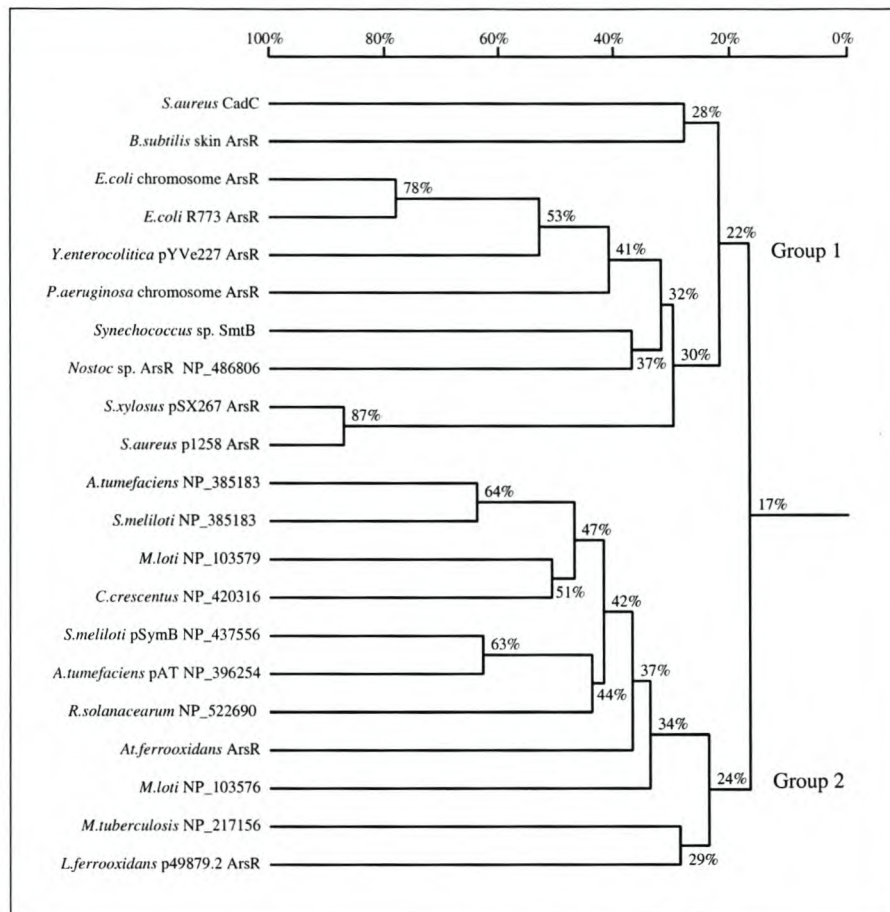


Figure 3.38: Dendrogram of all ArsR-like proteins represented in the database. The division into two groups is evident with the putative ArsR-family transcriptional regulator of ORF 2 (p49879.2), grouping within the second group. Abbreviated names/accession numbers represent the following: Group 1: *S.aureus* CadC, P20047; *B.subtilis* skin ArsR, BAA06967; *E.coli* chromosome ArsR, NP_312400; *E.coli* R773 ArsR, BAB91569; *Y.enterocolitica* pYVe227 ArsR, NP_052440; *P.aeruginosa* chromosome ArsR, AF010234; *Synechococcus* sp. SmtB, P30340; *Nostoc* sp. ArsR, NP_486806; *S.xylosus* pSX267 ArsR, AAA27587; *S.aureus* p1258 ArsR, AAA25636. Group 2: NP_103579, Probable arsenate reductase *M.loti*; NP_354498, *A.tumefaciens* ArsR-like protein; NP_437556, Putative regulation protein *S.meliloti*; NP_522690, Putative arsenical resistance operon transcriptional regulation protein *R.solanacearum*; NP_385183, Hypothetical transcriptional regulation protein *S.meliloti*; NP_217156, Hypothetical protein *M.tuberculosis*; NP_103576, Hypothetical protein *M.loti*; NP_396254, ArsR-like protein *A.tumefaciens*; NP_420316, Transcriptional regulator ArsR-family *C.crescentus*. *At.f* ArsR, AF69241.

p49879.2 ArsR-likeMNTVRFVLVSEMFSAIISHPKRLEPIISHIIGLSNRG...SSEETAEELTRLKSKANQSEHINVLKAGLWVC	64
NP_103579MDKATALQALAAALGOETRLLEAFRIILVRAGAKGVPAEEIATRLSTVQNTMSAHIKITLGGAGLVHA	64
NP_354498MDNIGALAAALSALAOITRELETFRRIVQHEPEEPIPAEEIARLTHVPQNTMSAHILATLARAGLVKS	64
NP_437556MEERQARSSFAALSOETRLGIVRTLVVAGPVGMAAAEAEKMGVSPSNMSEHFKELERTGLITQ	64
NP_522690MEETDAIRSLAALAHSLRLRFRMLVVAGPAGLTPGATAEQLDVPGATYSEHFKELMNAALVTO	64
NP_385183MDTVDI IAAPFAALAOPTRLDAFRFIVKHEPDGLPAEETARLLNVPHNTMSEHFKELQKAGLVTT	64
NP_217156	MPKSLPVIDISAPVCCAPVAAGPMSDGDALAVALRLKALADPARVIMSYLFFSSPAGEQVSSQIAALSLSDGTSEHFKELQKAGLVIS	90
NP_103576MKLEQAALQLEPALGNPTRELIYRILVVRAGEAGRPVVEIQESLGLIAASTYSEHFKELHRLILTGLVSO	64
NP_396254MDEKQALTSFAALSQETRLHIVRIILVVRAGEAGRPVVEIQESLGLIAASTYSEHFKELHRLILTGLVSO	64
At.f ArsRMEPLQDPAQIVARLEALASPVRLIEIFRLIVQAGPAGVAAEETARALNVLPNSLEANLNLVSHSGLTAS	68
NP_420316MLLSALSHCEKRLITFRLIVQAGPAGVAAEETARALNVLPNSLEANLNLVSHSGLTAS	56
p49879.2 ArsR-like	ERSCGTACRMLRLTSSKVLKCDLIRQMILEQLETTSEKRSLSANVTFFLREKKEQS.....	119
NP_103579	ERDGRSIRMTADMTGFRLLLAYIMETCCNGAPELCQPILOAVTKCK.....	110
NP_354498	EROSRSITMRADLEGLRAITLFLKICCGGATELCAPIAELTPCCCQEAEL.....	117
NP_437556	ERESRSITMSASYDALAIVKFLIMETCCVGHASVRA...SLNQNPICRVASSTPAEGNG.....	121
NP_522690	ERDGRHITMRAAFDHMNGILGHITENCCQGEACPEFSAAGCK.....	107
NP_385183	EROSRSITMRADLDGLRHVVSFLKICGAGHPDVCAPLVADLTCCSPMDRPAASRSHRSEGGMDGEADRKN	137
NP_217156	DRRCMHVFRVHPEALQALCTVLPNPNCA.....	119
NP_103576	ERCAITLTCRANYPMNNITIGFLADECCADAACNP..AAGDAAA.....	106
NP_396254	ERESRSITMTANYHCLGALLIRFLIMETCCAGHPEICA...QAVEVAACGVQTAEAARVV.....	119
At.f ArsR	QRECRYORVRAAMPVVRALVAVLTENCCHGTRDCALSGETRSPSVQEGNQ.....	118
NP_420316	ERDGRSITMTADYDAMTGLGFLIMETCCAGAPQICAPLSAIVSGMACGAKA.....	109

Figure 3.39: Multiple aa sequence alignment of ORF 2 (ArsR) from p49879.2 against ArsR-like proteins from the database. Open boxes indicate conserved regions. Underlined aa represent ‘helix-turn-helix’ motifs. Accession numbers represent the following: NP_103579, Probable arsenate reductase *M. loti*; NP_354498, *A. tumefaciens* ArsR-like proetin; NP_437556, Putative regulation protein *S. meliloti*; NP_522690, Putative arsenical resistance operon transcriptional regulation protein *R. solanacearum*; NP_385183, Hypothetical transcriptional regulation protein *S. meliloti*; NP_217156, Hypothetical protein *M. tuberculosis*; NP_103576, Hypothetical protein *M. loti*; NP_396254, ArsR-like protein *A. tumefaciens*; NP_420316, Transcriptional regulator ArsR-family *C. crecentus*. At.f ArsR, AF69241.

ORF 3 shared identity/similarity (45%/ 60%) with a hypothetical protein from *S. solfataricus* of unknown function. *S. solfataricus* has been reported to share some physiological properties with *L. ferrooxidans*, they are both acidophiles and have the ability to oxidize Fe (Rawlings and Woods, 1995). The presence of this common unresolved protein in both bacteria is interesting as it could contribute to the ability to function/survive in the extreme environments in which they exist. However, it is more likely to simply be fortuitous.

ORF 4 gave a 46% aa sequence identity to the NifS protein homologue of *Rickettsia conorii*. The *nifS* gene product (NifS) is a pyridoxal phosphate-binding enzyme that catalyzes the desulfurization of L-cysteine to yield L-alanine and sulphur (Zheng *et al*, 1994). This activity, in the context of nitrogen fixation, is thought to be required for full activation of the nitrogenase component proteins encoded by the *nifHDK* genes. The nitrogenase component proteins (Fe- and MoFe-protein) both contain metalloclusters required for their respective activities, the proposed model for NifS

interaction is the biosynthesis of the metalloclusters by providing the inorganic sulfur required for Fe-core formation (Zheng *et al*, 1994). The nitrogenase enzyme, encoded by the *nif HDK* genes, is responsible for N₂ fixation and is important for any organism inhabiting environments that lack fixed nitrogen. Nitrogen fixation genes can be either chromosomal or plasmid-borne (Finan *et al*, 2001), and have been reported to exist in the biomining bacteria *At. ferrooxidans* (Pretorius *et al*, 1987) and *L. ferrooxidans* (Norris *et al.*, 1995). NifS, and NifS-like proteins (IscC, CSd and CsdB from *E. coli*), are often not associated with nitrogen fixation systems and are still classified members of the homodimeric pyridoxal 5'-phosphate (PLP)-dependent family of enzymes (Lima, 2002). These enzymes are proposed to function as sulphur-delivery proteins for iron-sulphur clusters, thiamin, 4-thiouridine, biotin, and molybdopterin. Owing to the original identification of the *nifS* sequence in the *nif* cluster of *Azotobacter vinelandii* and *Azotobacter chroococcum* it was named as a *nif* gene.

ORF 5 is similar (61% aa identity/ 70% aa similarity) to the TniR protein of plasmid pSB102. Plasmid pSB102 carries a mercury resistant transposon, designated Tn5718, which is 10 414 bp in size (Schneiker *et al*, 2001). It is delimited by 25 bp inverted repeats (IR) and flanked by 5 bp direct repeats, the 25 bp IRs show significant similarity to integron repeats (Brown *et al*, 1996). Tn5718 contains 14 putative ORF's, five of which are named *tniA*, *tniB*, *tniQ*, *tniR*, and *tniM*. *Tni* genes are integron-based transposition genes, which mediate transposition via cointegrate formation followed by site-specific cointegrate resolution. Cointegrate resolution is catalyzed by the product of the *tniR* gene at the *res* region (Kholodii *et al*, 1995). TniR is the integron equivalent of TnpR.

ORF 6 is similar (40% aa identity/ 54% aa similarity) to the transposase of *M. acetivorans* and to the putative transposase (36% aa identity/ 51% aa similarity) of plasmid CP1 from *D. radiodurans* R1. These transposases share a 28.5% aa identity to each other, and a 27.1% aa identity to the C-terminal region of the Tn21-like transposases. Thus the transposase of ORF 6 is related, but not identical to that of ORF 7.

ORF 7 (TnpA) had high aa sequence identity (83%) to 44% of the C-terminal region of both the transposase for Tn21 of plasmid R100, and the transposase of Tn5037 from *At. ferrooxidans* (82%). All three aa sequences were included in the multiple alignment in Figure 3.31; the TnpA of p49879.2 displayed an overall aa homology of 78.3% to the aa sequences of the other TnpA proteins. ORF 8 (TnpR) also showed high aa identity over 76% of the N-terminal region to the resolvases in both the *At. ferrooxidans* Tn5037 (71%) and Tn21 of Plasmid R100 (65%). Both of which belong to the Tn21 family of resolvases. A multiple aa sequence alignment of Tn21-like resolvases including ORF 8 of p49879.2 gave an overall aa homology of 84.7% (Table 3.3). Since it has been suggested 70% aa homology is required to group resolvases into the Tn21 family (Liebert *et al.*, 1999), it would seem that ORF 8 of p49879.2 is a resolvase of this type. ORF's 7 and 8 are also divergently transcribed, which is characteristic of the Tn3-like family of transposable elements (Merlin *et al.*, 2000). Transposase and resolvase genes transcribed in this manner surround a central *res* site (Figure 3.19), however, insufficient sequence data was available to detect the presence of a *res* site.

3.3.6 Plasmid homology in *L. ferrooxidans* isolates

To determine whether the plasmids found in ATCC49879 had sequences, which were also present in other strains, 10 *L. ferrooxidans* and 6 *L. ferriphilum* isolates were screened using whole, DIG-labeled, p49879 DNA as a probe. Total DNA was digested with *Sall*, separated via standard gel electrophoresis, and transferred to a nylon membrane. All *L. ferrooxidans* isolates gave hybridization signals of varying intensities (Figure 3.40). Very weak, or no, hybridization signals were obtained for *L. ferriphilum* isolates. This indicated that only *L. ferrooxidans* isolates housed DNA with sufficient homology to p49879.1 and p49879.2 to give a positive signal. In order to determine the identity of the homologous region/s each DIG-labeled plasmid sub-clone was hybridized against the above-mentioned blot.

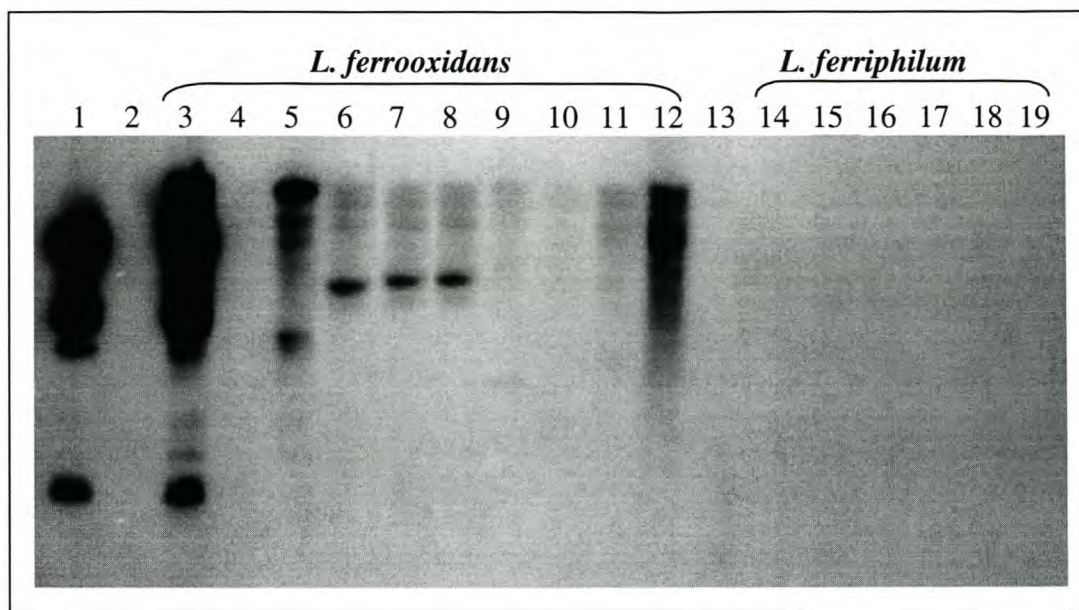


Figure 3.40: Southern blot of all sixteen *Leptospirillum* strains probed with whole DIG-labeled p49879.1/p49879.2. Definite bands and/or smears are apparent for all *L. ferrooxidans* isolates (lanes 3-12), weak or no hybridization signals were apparent for *L. ferriphilum* isolates (lanes 14-19). Feint smears in lanes 9, 10, and 11 are the result of a low DNA concentration. Lane 4 appears to be empty; a separate blot was prepared for this isolate where a positive signal was obtained (results not shown). Lanes 2 and 13 have no DNA, lane 1 p49879.1/p49879.2, lane 3 ATCC49879, lane 4 P3a, lane 5 N₂5, lane 6 DSM2705, lane 7 CF12, lane 8 Chil-Lf₂, lane 9 Parys, lane 10 Crys13, lane 11 BCT2, lane 12 SY, lane 14 Fairview, lane 15 Warwick, lane 16 ATCC49880, lane 17 ATCC49881, lane 18 Bionic 3.1 and lane 19 Mont 4. Species separation is further indicated through brackets.

The region of homology that gave a positive hybridization signal for all *L. ferrooxidans* isolates was localized to an area on p49879.2 only, which constituted subclones p2S5 and p2S6 (Figure 3.41). Subclones on either side of these fragments were also tested for hybridization. Probes p2S2 and p2S4 gave little or no homology to any isolates, while probes p2S1 and p2S7 hybridized to only a few selected isolates from both *L. ferriphilum* and *L. ferrooxidans* species. Homology in these cases could possibly be ascribed to the presence of MobL and TnpA respectively. Since hybridization with p2S7, which carries the predominant portion of the TnpA for Tn21 (Plasmid R100), was poor for all *Leptospirillum* isolates, it can be concluded that the TnpA sequence on p2S6 does not account for the positive hybridization signal received for all *L. ferrooxidans* isolates.

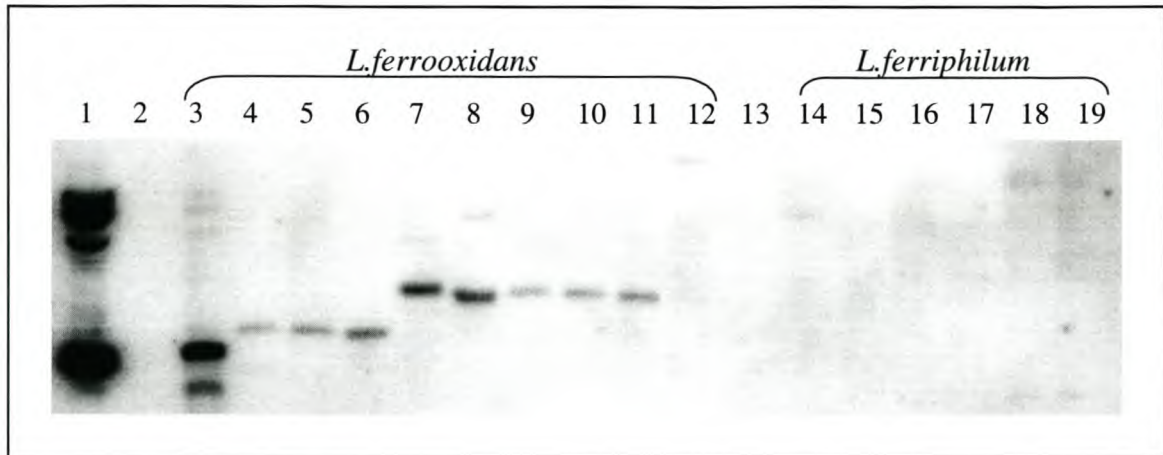


Figure 3.41: Southern blot of all sixteen *Leptospirillum* strains probed with DIG-labeled sub-clone p2S6 containing aa identity to a putative transposase. Definite bands are apparent in all *L. ferrooxidans* isolates (lanes 3-12), weak or no hybridization signals are apparent in *L. ferriphilum* isolates (lanes 14-19). The band in lane 12 appears feint due to overexposure of the membrane in this region; in other blots this region is not affected (results not shown). Lanes 2 and 13 have no DNA, lane 1 p49879.1/p49879.2, lane 3 ATCC49879, lane 4 P3a, lane 5 N₂5, lane 6 DSM2705, lane 7 CF12, lane 8 Chil-Lf₂, lane 9 Parys, lane 10 Crys13, lane 11 BCT2, lane 12 SY, lane 14 Fairview, lane 15 Warwick, lane 16 ATCC49880, lane 17 ATCC49881, lane 18 Bionic 3.1 and lane 19 Mont 4. Species separation is further indicated through brackets.

Results from the sequence analysis of p2S5 gave weak homology to a hypothetical protein of *P. aeruginosa* ($E=0.89$, 46% aa identity/ 57% aa similarity over 3.3% of the protein). Approximate nucleotide positions 11500-11800 of p49879.2, within p2S6, gave low aa identity (26%) /aa similarity (40%) values to a conserved hypothetical protein of unknown function from *Sulfolobus solfataricus* ($E=0.005$). The region within p2S5, which gave highest homology to a known protein in the database, occurred at position 12.7-12.3 kb. Amino acid sequence identity was to a transposase of *M. acetivorans* (ORF 6, Table 3.3), which is localized to the chromosome (Galagan *et al*, 2002), and a putative transposase of plasmid CP1 from *D. radiodurans* R1. The area of homology shared between all *L. ferrooxidans* isolates is therefore a transposase/putative transposase, the origin of which can either be plasmid or chromosome based.

Chapter 4

General Discussion

This study includes both a largely genome-based taxonomic- (Chapter 2), and plasmid- based (Chapter 3) investigation of members of the genus *Leptospirillum*. Evidence for the importance of '*Leptospirillum*' in biomining has been obtained through a number of studies (Rawlings *et al.*, 1999a; Rawlings *et al.*, 1999b; Vásquez and Espejo, 1997; Espejo and Romero, 1997; De Wulf-Durand *et al.*, 1997; Pizarro *et al.*, 1996; Goebel and Stackebrandt, 1995; Rawlings, 1995; Goebel and Stackebrandt, 1994). However, little is known of the taxonomy, biology and genetic make up of this genus. Members of the genus *Leptospirillum* have a limited range of physiological characteristics that can be used in their identification (Johnson, 2001). One objective of the present study was therefore to determine the diversity of leptospirilla isolates from different geographical locations using a variety of molecular techniques to establish whether there were sufficient differences to warrant sub-division at a species level. These studies provide an extended description of a number of characteristics that can be used in the identification of the more commonly encountered leptospirilla. Genetic and phenotypic tools, specified by the currently accepted approach for the speciation of prokaryotes (Rosselló-Mora and Amann, 2001), were applied to a sample group of sixteen bacteria previously described as *L. ferrooxidans* or *L. ferrooxidans*-like. During the course of this study an additional three isolates were included in this sample group (Adapt, BN Mod, and 617). These strains were isolated out of pilot plant tanks from the Billiton Process Research laboratories in South Africa, where the biooxidation of a range of ores at different temperatures (45-55°C) was being tested. The inclusion of strains that have the ability to grow at elevated temperatures improves the diversity of the study. Results indicate that two distinct groups were represented within the sample selection, and that the differences were sufficient to warrant a species level division. Based on the evidence obtained, we proposed that the leptospirilla investigated in this study represent two different species, *L. ferrooxidans* (type strain DSM2705) and *L. ferriphilum* (type strain ATCC49881). This work was published in Applied and Environmental Microbiology

(Appendix G); the name *L. ferriphilum* was validated by the International Committee on Systematic Bacteriology (Appendix H).

Prior to this work, limited phylogenetic studies on a relatively small number of members of the genus *Leptospirillum* had been reported. Harrison and Norris (1985) obtained evidence to suggest that there was considerable variation between isolates belonging to the genus *Leptospirillum*. One group of isolates had a mol% G+C of ca. 51% and another a mol% G+C of 55-56%. This result was further supported by DNA-DNA hybridization studies, in which two isolates had a DNA sequence similarity of 71-73% while all other isolates had > 6-31% DNA-DNA similarity. Unfortunately, all strains but the *L. ferrooxidans* type strain (DSM2705) from this early study were lost (Norris personal communication). Hallmann *et al.* (1993) carried out DNA-DNA hybridization studies with six isolates of leptospirilla. Two pairs of strains were 100% related to each other and there was 38-50% relatedness between these pairs and 31-50% relatedness between all other isolates. No attempts to classify these groups of isolates into different species have been made; collectively they are referred to as *L. ferrooxidans* or *L. ferrooxidans*-like bacteria. A moderately thermophilic *Leptospirillum* with an optimum temperature of 45-50°C (maximum 55-60°C), a mol% G+C of 56% and a DNA similarity of 27% with a mesophilic strain was reported (Golovacheva *et al.*, 1993). This strain was named *L. thermoferrooxidans*, but has also been lost and so was unavailable for comparative studies (Johnson, 2001). The genus name *Leptospirillum*, and species names *ferrooxidans* and *thermoferrooxidans* have been validated (Hippe, 2000). Recently 16S rDNA belonging to a third group of leptospirilla was amplified from DNA isolated directly from slime streamers of an acid mine drainage site, however, bacteria belonging to the third group have not been isolated in pure culture (Bond *et al.*, 2000a).

Therefore, from past and present studies, the current status of the genus *Leptospirillum* is that it contains three different species, which have been described and published, with the likelihood of the existence of a fourth species from the work of Bond *et al.* (2000a). Current understanding is that leptospirilla are one of the most metabolically restricted bacteria known with only ferrous iron serving as the electron donor, and oxygen as the electron acceptor. They are also obligately acidophilic,

relying on a low pH (< 2) to maintain iron (Fe^{3+}) solubility and facilitate energy production. These severe physiological restrictions may also place restrictions on genomic diversity. One may have expected evolutionary pressure to expand the ecological niche of *Leptospirillum*, resulting in new metabolic capabilities. This appears not to have happened, and leptospirilla remain highly competitive within this restricted niche. *Leptospirillum* have a highly optimized ability to oxidize iron ($K_m = 0.25 \text{ mM Fe}^{2+}$), and are not subject to ferric iron inhibition. Since iron oxidation is so highly optimized, any genetic drift that may occur, would probably take place in functions unrelated to aerobic iron oxidation. This would lend support to the finding that few phenotypic differences exist between all three described species, which are in fact separated on a predominantly genomic level.

What forces could drive the occurrence of genomic variation, of up to 95% non-hybridization, between such metabolically restricted, and seemingly closely related species as *L. ferrooxidans* and *L. ferriphilum*? Possibly, adaptation to environmental pressures such as temperature or geographical separation. Although we were unable to report vast differences in temperature optima between species, growth of *L. ferriphilum* was marginally faster than that of *L. ferrooxidans* at 37°C. This tolerance of higher temperature was supported by the observation that some *L. ferriphilum* isolates were able to grow at 45°C. This could however be the result of previous exposure to higher temperatures. The fact that *L. ferriphilum* were found to be the dominant *Leptospirillum* species in South African biooxidation tanks that operate at 40°C is all supportive, though not definitive proof of tolerance of higher temperatures by *L. ferriphilum*. This is not to say that *L. ferrooxidans* are not present in other commercial processes, but merely an observation that *L. ferriphilum* is more tolerant of these particular conditions. Inability of *L. ferrooxidans* isolates to grow at 45°C could also be the result of lack of evolutionary pre-exposure to high temperatures.

The isolates in this study were indeed representative of a wide range of geographical locations, and geographical separation has been known to drive speciation. However it is unlikely that this factor played a role in the differences between *L. ferrooxidans* and *L. ferriphilum*, since not only were isolates from both species found in different

locations, but also in one instance ATCC49880 (*L. ferriphilum*) and ATCC49879 (*L. ferrooxidans*) were isolated from the same mine in Romania.

The understanding of taxonomic diversity within the genus *Leptospirillum* is in its relative infancy. Information obtained from this study is by no means a complete interpretation of leptospirilla variation, but it has contributed to discerning the most prominent species present in some biomining processes. Genetic tools can make a considerable contribution to an investigation of the physiology of the organism. For example, to resolve the function of a gene, it can be mutated or disrupted. Introduction of the mutated gene into the wild type organism, followed by homologous recombination, results in the disruption of the wild type gene. The study of mutants in which a protein of interest is no longer synthesized can help to establish its function. The construction of null mutants by marker exchange mutagenesis would be possible if a reliable genetic transfer system for *Leptospirillum* was available. Owing to the extensive knowledge base available on *E. coli*, it is used as a “micro-laboratory” in which gene constructs are manufactured, after which they are introduced into the host of interest. Currently no genetic system exists for *L. ferrooxidans* or *L. ferriphilum*. However, genetic transfer systems have been described for the acidithiobacilli, *At. ferrooxidans* (Liu *et al.*, 2000; Peng *et al.*, 1994a; Peng *et al.*, 1994b; Kusano *et al.*, 1992), *At. thiooxidans* (Jin *et al.*, 1992) and *At. caldus* (unpublished data, personal communication, L. van Zyl), which share the same extreme ecological niche. In order to develop such a system, all the parameters necessary for the genetic engineering of the bacterium must be available. These include a suitable transfer system, plasmid vectors, and selectable genetic markers.

Potential **transfer systems** include conjugation, electrotransformation and transformation. In conjugation, the plasmid is transferred from the donor to recipient cell through a mating process. In order for conjugation-type transfer to be possible a few parameters need to be met. The vector must be mobilizable, and able to replicate in both donor and recipient cells. A medium that provides suitable conditions for both donor and recipient to mate is required. Originally it was thought that due to the vast physiological differences between the sulfur oxidizing organisms and *E. coli*, the development of a direct conjugation-type transfer system would be challenging. This was mainly due to the low pH levels (< 2) required by the biomining organisms, which could affect *E. coli* during mating. A suitable mating medium, which supports

both sulfur oxidizers and *E. coli*, has been developed (Peng *et al.*, 1994c). This medium, referred to as 2:2 medium, contains 0.5% yeast extract, and has been pH optimised to within a range of 4.6-4.8 (Liu *et al.*, 2000). *At. ferrooxidans*, *At. thiooxidans*, *At. caldus*, and *E. coli* (lower pH limit of 4.4) are able to tolerate this pH range, and sulfur remains in solution. The development of a conjugative-type transfer system in *Leptospirillum* poses a more formidable task. Unlike the acidithiobacilli, leptospirilla do not utilize sulfur, and have an obligatory requirement for iron. A requirement for iron places restrictions on the maximum pH of the media because ferric iron rapidly forms a jarosite precipitate at pH values above 2. Not only is iron affected at pH values > 2, but growth of the bacteria is inhibited. Organic substrates also hinder leptospirilla growth. Therefore the development of a suitable medium for the direct transfer of plasmids from *E. coli* to *Leptospirillum* by conjugation may not be possible. There is however the possibility of transferring the plasmid vector through an intermediate-, or series of intermediate- hosts, with growth requirements compatible to those of *E. coli* and *Leptospirillum*. For instance, a facultative autotroph that has the ability to grow at both low and high pH values (e.g. *Halothiobacillus neopolitanus* or *Acidithiobacillus albertensis*). In doing this, metabolic induced incompatibility between the two organisms is ruled out.

Transformation is a method for inserting naked DNA into a cell. Some cells are naturally transformation competent, although this has not been shown for any biomining organisms. Treating cells with chemical substances (i.e. calcium) in order to make them competent can artificially induce a state of transformation competence. Application of this method to acidophilic bacteria (*At. ferrooxidans*) has proven difficult, and no means of making cells competent using chemicals has been reported. Electrotransformation, also known as electroporation, is the transfer of naked DNA into bacterial cells by the application of a high voltage electrical discharge, and is a transformation procedure that is finding increasing application to a wide range of bacteria, often resistant to routine methods (Kusano, *et al.*, 1992). Electrotransformation has been used to transfer a plasmid into a strain of *At. ferrooxidans* (Kusano *et al.*, 1992). However, this method could not be generally applied to *At. ferrooxidans* as only one out of thirty strains was transformed.

Electroporation may be suitable for *Leptospirillum* as the need for a medium in which both donor and recipient cells can mate is overcome.

Suitable **vectors** include broad host range plasmids and/or endogenous plasmids with the ability to replicate in the host of interest. Shuttle vectors, capable of being used in *E. coli* and the organism of interest, may be made from well-characterized *E. coli* vectors with a multiple cloning site for easy gene sub-cloning, plus an origin of replication for the required host and at least one selectable marker that can be expressed in both organisms. Broad host range plasmids of the IncQ (RSF1010) and IncP (RP4) plasmid families have been transferred successfully in the conjugation-type systems of the acidithiobacilli. Although indigenous plasmids have been isolated from each of these sulfur-oxidizing organisms, they have not been used as vectors. No plasmids had yet been isolated from *Leptospirillum*. This is not to say that the IncQ and IncP broad host range plasmids could not be used as shuttle vectors with this genus. However, *Leptospirillum* belong to the Nitrospira group, which is far removed from the Proteobacteria like *E. coli* and the acidithiobacilli. Whether current broad host range plasmid vectors would be broad host range enough to be able to replicate in *Leptospirillum* is unknown.

A suitable **selectable marker** completes the requirements for a functional genetic system. Antibiotic resistance markers have been widely used as selectable genes in heterotrophic bacteria. However, antibiotics are unstable in low pH and high metal iron-containing media. In the case of the acidithiobacilli, the pH of the 2:2 mating medium is high enough (pH 4.6-4.8) for kanamycin to remain active. Kanamycin is stable at relatively low pH levels (~ 4), and has been the selective marker of choice for the *At. ferrooxidans*, *At. thiooxidans*, and *At. caldus* genetic systems. Although kanamycin has been successfully used as a selectable marker with the acidithiobacilli, the probability of the survival of antibiotic resistance markers in low pH (< 2), iron-containing selection medium, such as that required for leptospirilla, is highly unlikely. Markers conveying metal ion tolerance could possibly be used. Arsenic (Peng *et al.*, 1994a) and mercury (Kusano *et al.*, 1992) resistance genes are likely candidates as they have the potential for conferring industrially significant characteristics. Mercury

is however not a stringent selectable marker as it volatilises from plates during long incubation periods required for leptospirilla to grow. As a result of its instability, *At. ferrooxidans* colonies isolated on mercury containing media often do not carry the plasmid of interest (Kusano *et al.*, 1992).

This study reports the first isolation of plasmids from *Leptospirillum*. In a previous study, a plasmid was identified, though not isolated in the Fairview culture (*L. ferriphilum*) (unreported, personal communication, C. Dominy). The presence of this plasmid was not confirmed in this study. Although pulsed field gel evidence for plasmid presence in *L. ferrooxidans* (Parys and CF12) was obtained in this work, plasmid DNA could only be isolated from *L. ferrooxidans* (ATCC49879). Plasmid DNA from ATCC49879 although initially thought to be a single ~ 60.0 kb plasmid, consisted of not one but two plasmids of approximately 30.0 kb and 27.0 kb, for p49879.1 and p49879.2 respectively.

Sequence analysis of p49879.1 and p49879.2 indicated that the plasmids were not identical but shared regions of homology. This included amino acid sequence identity to the TnpA and TnpR of the Tn21-like transposon family, and the mobilization regions of IncQ-like plasmids (particularly that of pTF1 from *At. ferrooxidans*). Both regions are known to play important roles in the survival/spread of plasmids within a bacterial community via horizontal transfer. Whether either of these systems is functional still remains to be determined. The mere presence of a mobilization region suggests the existence of a mating system in leptospirilla. Another interesting ORF was identified in p49879.2 with high aa sequence identity to an ArsR-like protein that belongs to a second atypical family of ArsR transcriptional regulators (Butcher and Rawlings, 2002). No conserved CVC motif within the *ars* binding site is present within this group of regulators, however some members have still been shown to function as regulators of the *ars* system. Although the regions flanking this ORF have not yet been sequenced, sufficient space is available to allow for the existence of the other arsenic resistance genes (*arsBC*), reported to be associated with this family of transcriptional regulators (addressed in Butcher *et al.*, 2000). Should additional arsenic resistance genes be present and functional, being plasmid-based, they could serve as selective markers in a plasmid transfer system. An ArsR has however been reported to occur on the Inc11 plasmid R64 without additional arsenic resistance genes

and was found not to be involved in arsenic resistance but rather alleviated type I restriction endonuclease digestion (Rastorguev *et al.*, 1998).

Although this is the first conclusive report of plasmid presence in *Leptospirillum*, plasmids are widely associated with bacteria involved in metal leaching from ores. The occurrence of more than one plasmid within a strain is also not unique. From as early as 1980, chemoorganotrophic *Acidithiobacillus ferrooxidans* (TFG-0) and *Acidiphilium acidophilus* (AFG-1) were shown to contain plasmids of different molecular weights (Mao, 1980), whilst 13 different strains (TF1-TF13) within the same species of *At. ferrooxidans* were shown to carry many different sizes of plasmid DNA (Martin *et al.*, 1981). Often plasmids originating from different bacterial strains, within the same species, would share high sequence homology (Shiratori, 1991). When high homology between two resident plasmids exists, an unstable situation could result as the plasmids could occur in either a cointegrated- or separated- state. This did not however seem to be the case with plasmids p49879.1 and p49879.2. Although p49879.1 and p49879.2 were always isolated together, Southern hybridization results indicated that they existed in a separated state as individual plasmids. Plasmids are believed not to encode functions crucial to bacterial survival, such as ferrous iron oxidizing functions in iron-oxidizing leptospirilla. Instead plasmids are believed to encode inessential functions that are likely to contribute to bacterial competitiveness.

The current plasmid study amongst *Leptospirillum* isolates, although removed from that of a taxonomic approach seems to lend support to the presence of two species of *Leptospirillum* in a way that was not expected. A region was identified on plasmid p49879.2, which when used as a probe against whole DNA obtained from all isolates, was present in only one species and not the other. The region on p49879.2 (ORF 6, Figure 3.37), with aa sequence identity to a putative transposase, was found to share homology with all *L. ferrooxidans* isolates, but no *L. ferriphilum* isolates represented in this study. Why this transposase should be present in *L. ferrooxidans* from four geographical locations (North America, South America, Europe and New Zealand), but not in *L. ferriphilum* is puzzling. One would expect that the transposon should have been able to move readily between species. What makes this even more surprising is the presence of mobilization genes on plasmid p49879.2. Although

preliminary tests showed that the *mob* genes were not functional in *E. coli*, this could be the result of differences in host specificity. The absence of interspecies transfer is surprising when one considers that *L. ferrooxidans* ATCC49879 (which has the transposon) and *L. ferriphilum* ATCC49880 (which does not have the transposon) were both isolated from the same mine in Romania. This transposon appears therefore to have entered *L. ferrooxidans* and has become distributed in *L. ferrooxidans* strains around the world but has not entered *L. ferriphilum*.

Owing to the close association of some *L. ferrooxidans* and *L. ferriphilum* isolates, without indication of interspecies transfer having taken place, the separation of *Leptospirillum* isolates into two different species is further supported. Although evidence for the apparent lack of interspecies transfer is not particularly strong, should this be true, it would imply that a gene transfer barrier of unknown description exists between the two species. It is difficult to imagine what such a barrier might be.

A potential barrier is the possibility of the existence different restriction endonuclease systems in *L. ferriphilum* and *L. ferrooxidans*. The restriction-modification (RM) system in bacteria is a small-scale "immune system" for protection from infection by foreign DNA. This system is composed of a restriction endonuclease enzyme and a methylase enzyme, of which each bacterial species and strain has their own combination. A modification methylase methylates the DNA at the same recognition sequence that the restriction endonuclease uses to bind to the DNA prior to cleavage. This activity discriminates "self" DNA from "foreign" DNA. Incoming DNA would not be protected by methylation, and is subject to degradation by the restriction endonuclease. DNA stabilization is only achieved if the incoming DNA does not harbor a RM system with the same target sequence specificity. Should this occur, stabilization would be blocked, and both native and incoming DNA susceptible to degradation. Therefore, *L. ferriphilum* might have a different RM system to that of *L. ferrooxidans*, which would explain transposon presence in one species and not the other. However, this speculation needs to be tested.

Future work involves the complete sequencing of both plasmids p49879.1 and p49879.2. This would enable the possible identification of origins of replication (*oriV*), not only for the development of vectors or shuttle vectors for use in a genetic

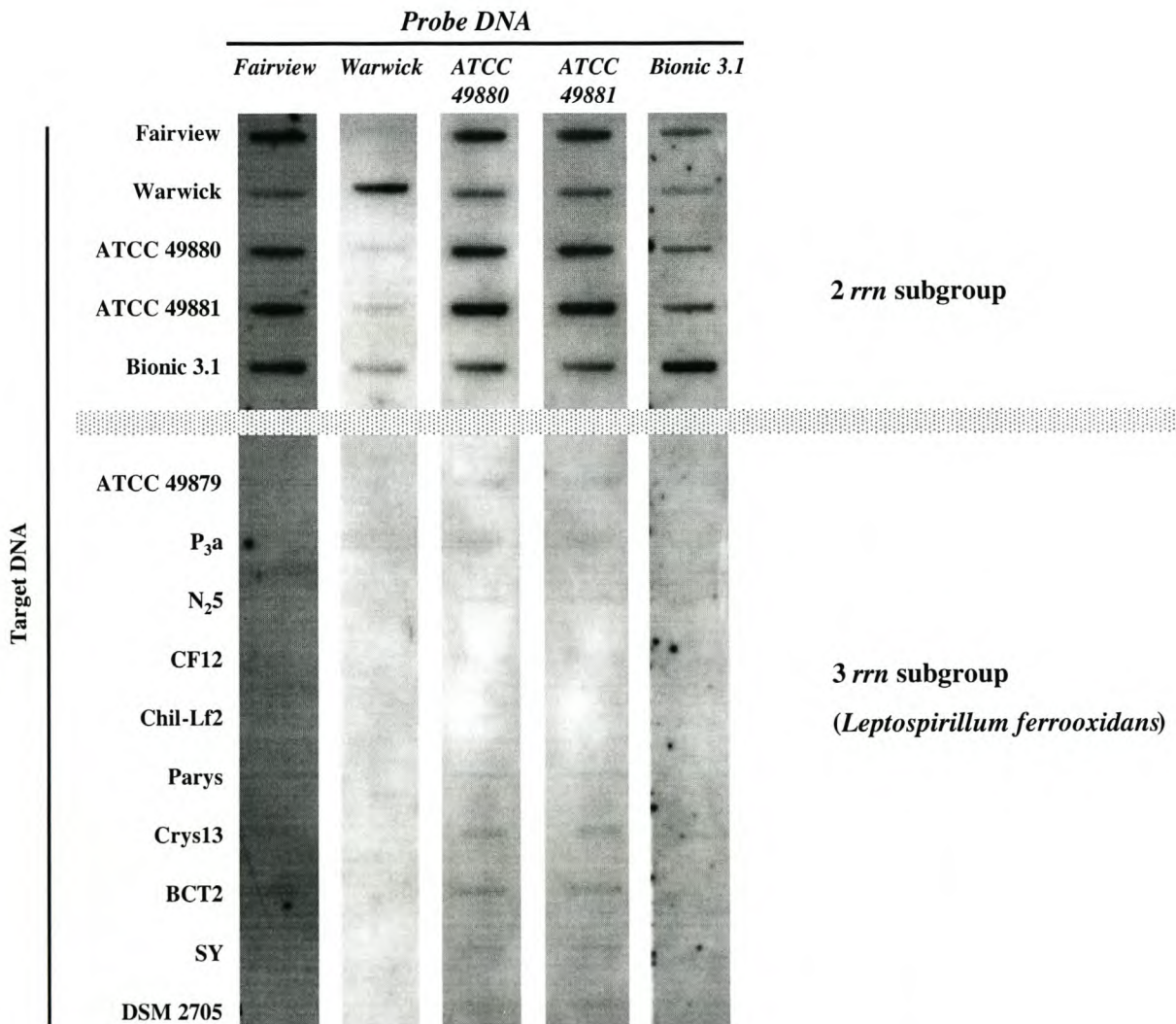
system, but to determine how the replicon relates/compares to those of plasmids in other bacteria outside of this ecological niche. The identification of genes and sites involved in conjugative transfer such as origins of transfer (*oriT*), DNA transfer and replication genes, as well as mating pair formation genes would assist in determining whether the plasmids are self-transmissible or mobilizable. By comparison to currently known mobilization regions, this would in turn establish the nature of an appropriate conjugation helper plasmid for use in a genetic system. Although the possibility of a direct *E. coli* to *Leptospirillum* conjugation-type transfer system seems unlikely, information concerning these regions would add to the knowledge base of plasmid mobilization, should an applied function not be served. Furthermore, the identification of genes that are actively transported within the horizontal gene pool, should they provide a selective/competitive advantage to the host, could possibly be used as selectable markers. This information would address both a fundamental and applied interest. Ultimately, apart from possibly understanding the lack of inter-species transfer between *L. ferrooxidans* and *L. ferriphilum* of p49879.2 or part thereof, the suitability of these plasmids for use in the development of a genetic system for *Leptospirillum* will be determined.

Appendix A

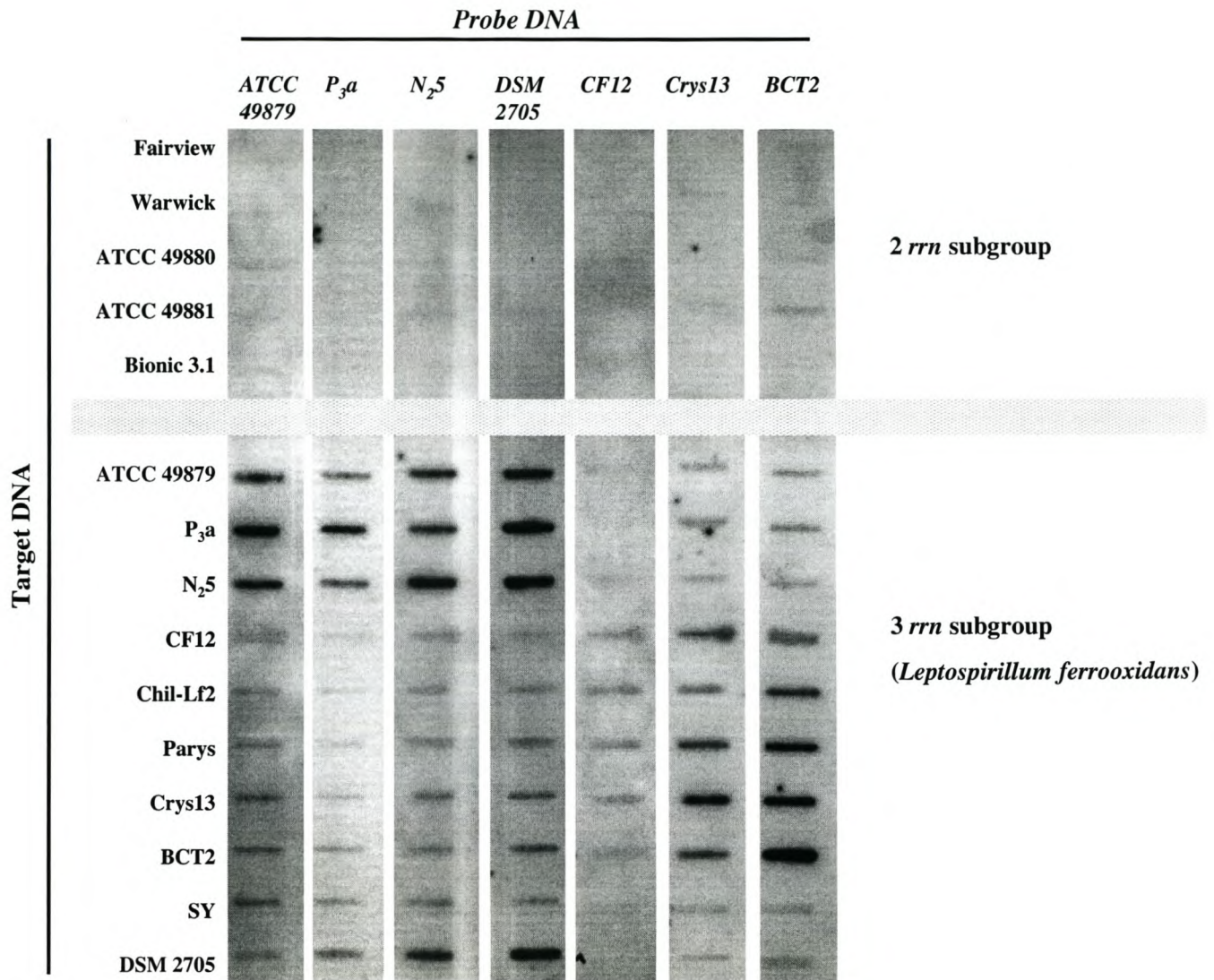
DNA-DNA hybridization (slot blots)

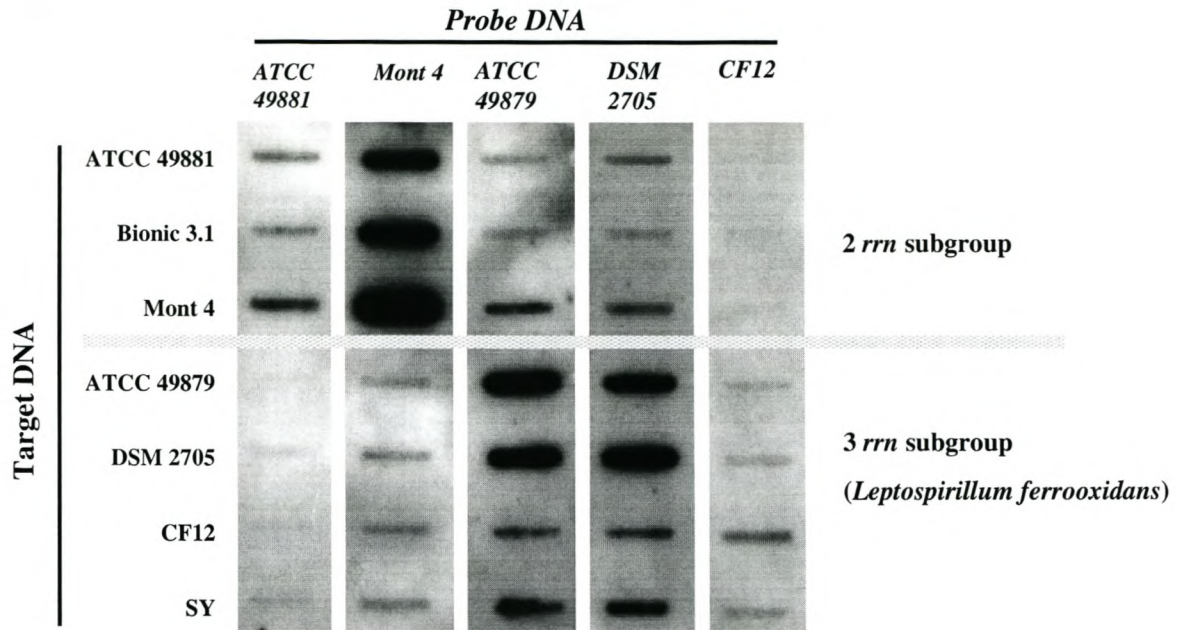
Sections A.1, A.2, and A.3 are slot blots of target DNA from 16 *Leptospirillum* isolates (indicated vertically in bold print), which have been hybridized against 13 different *Leptospirillum* probes (indicated in *italics* along the top horizontal axis of the blots). Sections A.1 and A.2 do not include isolate Mont 4, which is represented in section A.3 along with representative members of each of the two groups. Although blots were performed in triplicate and with different concentrations of DNA, a sample result represented by one blot/DNA concentration per probe is given in each case.

A.1 2 *rrn* subgroup probes



A.2 3 *rrn* subgroup probes

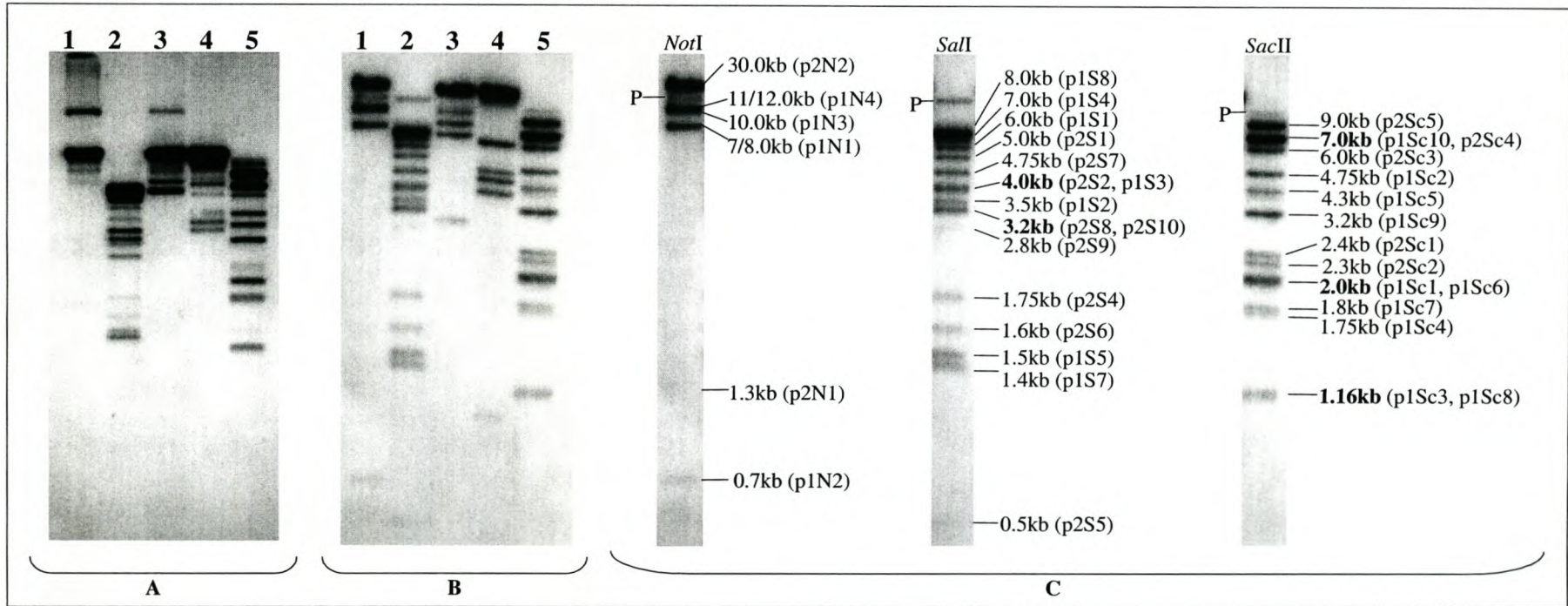


A.3 Mont 4 target DNA and representative *Leptospirillum*

Appendix B

Plasmid p49879.1 and p49879.2 mapping blots

The master/template Southern blots of combined p49879.1 and p49879.2 plasmid DNA digested with *NotI* (1), *SalI* (2), *HindIII* (3), *SphI* (4), and *SacII* (5), are indicated in the window (A and B). Blot B has been dissected (C) in order to indicate fragment sizes and identification according to plasmid maps in Figures 3.33 and 3.37 respectively. DIG-labeled probes were constructed from most of these fragments and hybridized against one of the original blots (A or B). Sections B.1 and B.2 illustrate the hybridization results obtained. Probes used in each hybridization are indicated at the base of each blot, the letter A or B in the bottom right corner of each blot indicates which template blot was used.



A and **B**: Master/template Southern blots of combined p49879.1 and p49879.2 plasmid DNA

C: Dissection of blot **B**, indicating fragment sizes and clone identification. Fragment sizes shown in **bold** print indicate the presence of a doublet.

Appendix C

Media, buffers and solutions

All media, buffers and solutions were sterilized by autoclaving at 121°C for 20 minutes. Heat labile substances were filter sterilized using 0.22 µm membrane filters (Millipore).

C.1 Media

C.1.1 10× Mineral salt solution

(NH ₄) ₂ SO ₄	30.0 g.l ⁻¹
KCl	1.0 g.l ⁻¹
K ₂ HPO ₄	5.0 g.l ⁻¹
MgSO ₄ .7H ₂ O	5.0 g.l ⁻¹
Ca(NO ₃) ₂ .4H ₂ O	0.14 g.l ⁻¹
Na ₂ SO ₄	14.5 g.l ⁻¹

pH to 2.5 with H₂SO₄ and autoclave.

C.1.2 FeSO₄.7H₂O

FeSO ₄ .7H ₂ O	186 g.l ⁻¹
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pH to 1.2 with H₂SO₄ and autoclave.

C.1.3 1000×Trace elements

ZnSO ₄ .7H ₂ O	10.0 g.l ⁻¹
CuSO ₄ .5H ₂ O	1.0 g.l ⁻¹
MnSO ₄ .4H ₂ O	1.0 g.l ⁻¹
CoCl ₂ .6H ₂ O	0.5 g.l ⁻¹
Cr ₂ (SO ₄) ₃ .15H ₂ O	0.5 g.l ⁻¹
Na ₂ B ₄ O ₇ .10H ₂ O	0.5 g.l ⁻¹
NaMoO ₄ .2H ₂ O	0.5 g.l ⁻¹

Add 530 µl.l⁻¹ H₂SO₄ and autoclave.

The mineral salt and trace element solutions were added aseptically to the iron solution, and the pH adjusted to pH 1.6 with H₂SO₄; this is referred to as 9K medium.

C.1.4 Luria-Bertani media

Bactotryptone	10 g.l ⁻¹
NaCl	10 g.l ⁻¹
Yeast extract	5 g.l ⁻¹

Solid media contained 1.5% (w/v) agar

C.2 Media additives

C.2.1 Antibiotics

Antibiotic stock solutions were as follows

Ampicillin (Amp) in water	100 mg/ml
Streptomycin (Strep) in water	50 mg/ml
Naladixic acid (Nal) in 0.1N NaOH	50 mg/ml

All antibiotics were filter sterilized and stored at -20°C.

C.2.2 X-Gal (5-bromo-4-chloro-3-indolyl-β-galactoside)

X-Gal (2% w/v)	0.2 g
Dimethylformamide	10 ml

The solution was stored in aliquots at -80°C.

C.3 Buffers and solutions

C.3.1 DNA loading buffer (6×)

Bromophenol blue	0.25 g
Sucrose	40.0 g
Distilled water to 100 ml	

C.3.2 EDTA (Ethylene diamine tetracetic acid, 0.5M pH 8)

EDTA.2H ₂ O	168.1 g
Distilled water to 1000 ml	
pH was adjusted to 8 with NaOH (10N)	

C.3.3 Ethidium bromide

A solution of 10 mg/ml (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide) was made in distilled water and stored in the dark.

C.3.4 20× SSC

NaCl	175.3 g.l ⁻¹
Sodium citrate	88.2 g.l ⁻¹

Adjust pH to 7.4 with NaOH (10N) and autoclave.

C.3.5 TE buffer

Tris	1.21 g.l ⁻¹
EDTA	0.34 g.l ⁻¹

Adjust pH to 8 and autoclave.

C.3.6 10× TBE

Tris	108 g
Boric acid	55 g
EDTA (0.5M, pH8)	40.0 ml
Distilled water to 1000 ml	

C.3.7 20× PFGE buffer

Tris	24.2 g
EDTA (free salt)	2.9 g
Glacial acetic acid	5.0 ml
Deionized water to 1000 ml	

C.3.8 ES solution

EDTA	16.8 g
Na-lauroyl sarcosine	1.0 g
Distilled water to 100 ml	

Adjust pH to 8 with NaOH (10N) and autoclave.

C.3.9 ESP solution

ES solution containing 1 mg/ml proteinase K

C.3.10 SET buffer

Sucrose	50 g
EDTA (0.5M, pH8)	0.8 ml
Tris (1M, pH8)	10 ml
Distilled water to 200 ml	

C.3.11 TE₅₀ for PFGE

Tris	3.6 g.l ⁻¹
EDTA (Na ₂)	16.8 g.l ⁻¹

Adjust pH to 7.6 and autoclave.

C.3.12 Pefabloc SC (Roche biochemicals)

2 mg/167 µl distilled water (50mM stock), use at final concentration of 1-5 mM

Store at -20°C.

Appendix D

General techniques

General techniques such as agarose gel electrophoresis, and the transformation of *E.coli* were carried out as stated in Sambrook *et al.* (1989). Strains used in transformation experiments are listed in section D.4.

D.1 DNA isolation and restriction endonuclease digestion

E.coli plasmid DNA was isolated from small (5ml) and large (200ml) overnight cultures according to the method of Ish-Horowicz and Burke (1981). Chromosomal and plasmid DNA digestions were carried out according to manufacturer's specifications (Roche Biochemicals). Where necessary DNA was precipitated with 10% (v/v) Na-citrate (1M, pH 5.2), 2 volumes 100% EtOH, and centrifuged at 14 000 rpm for 10 min.

D.2 Cloning and ligation protocols

Digested DNA fragments to be subcloned were electrophoresed in 0.8% agarose gels containing 1× TBE buffer and ethidium bromide. Bands were excized under long UV (365 nm) light. DNA was electroeluted from the gel slices, precipitated and resuspended in TE buffer. Inserts were combined with digested plasmid vector DNA; all ligations were done overnight at 15°C in a 20 µl volume. The total DNA concentration was always in the order of 5-10 pmol/ml reaction volume. Physical maps of plasmids that were used as cloning vectors are represented in Appendix F.

D.3 Southern hybridization

Pulsed field and agarose gels for use in Southern hybridization were placed in 0.25M HCl solution for 30 minutes to facilitate depurination. The DNA was neutralized in 0.4N NaOH (Saarchem), and transferred to nylon Hybond N⁺ membrane (Amersham) by capillary blotting overnight, whereupon excess buffer was removed and the transfer completed by dry blotting the gel for a further 4-6 h. The membrane was pre-hybridized at 40°C with DIG Easy Hyb solution (Roche Biochemicals) for a minimum of 30 min, and hybridized at 40°C overnight with a DIG oligonucleotide 3'-end labeled probe. Washing was for 20 min at room temperature followed by 20 min

at 65°C. Membrane detection performed as per manufacturer's instruction (Roche Biochemicals).

D.4 Bacterial strains used in transformations

Strain	Genotype	Reference/origin
<i>Escherichia coli</i> JM109	<i>endA1 recA1 gyrA96 thi hsdR17 (r_k⁻, m_k⁺) relA1 supE44 Δ(lac-proAB) [F' trad36 proAB lacI^qZΔM15]</i>	Promega Corp. USA
<i>Escherichia coli</i> DH5α	<i>φ80dlacZΔM15 endA1 recA1 gyrA96 thi-1 hsdR17 (r_k⁻, m_k⁺) relA1 supE44 deoR Δ(lacZYA-argF) U169</i>	Promega Corp. USA

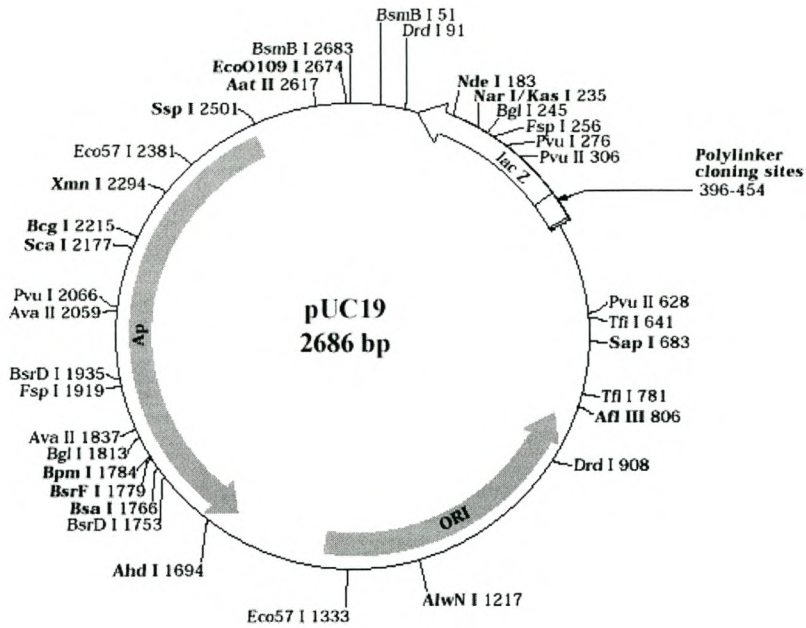
Appendix E

Amino acid codes

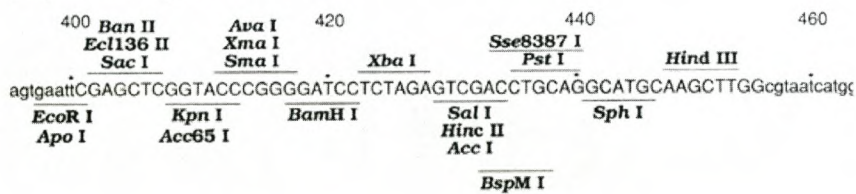
Amino acid	Codes	Abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

Appendix F

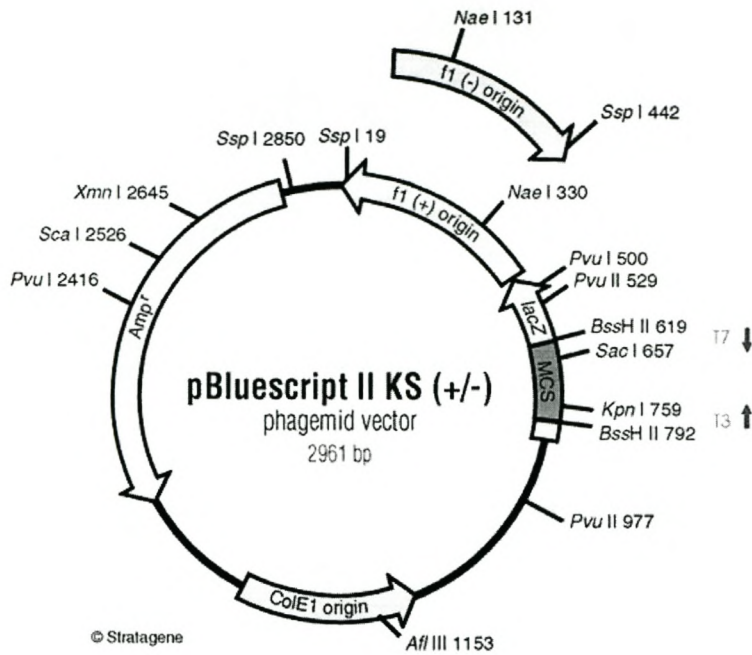
Physical maps of vectors



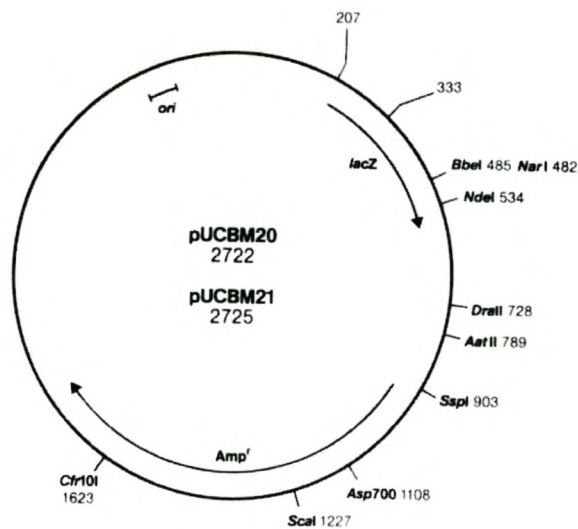
polylinker region



F.1 pUC19 (<http://www.biology.ucsc.edu/classes/bio20L/content/molbio2/puc19.gif>)



F.2 Bluescript KS+ (pBS-KS+). Bluescript SK+ differs in the orientation of the multiple cloning site (MCS) polylinker between the KpnI and SacI sites. Stratagene, San Diego, CA, USA.



F.3 pUCBM21 (Roche biochemicals)

Appendix G

Publication of taxonomic study

Molecular Relationship between Two Groups of the Genus *Leptospirillum* and the Finding that *Leptospirillum ferriphilum* sp. nov. Dominates South African Commercial Biooxidation Tanks That Operate at 40°C

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Received 16 August 2001/Accepted 7 November 2001

Iron-oxidizing bacteria belonging to the genus *Leptospirillum* are of great importance in continuous-flow commercial biooxidation reactors, used for extracting metals from minerals, that operate at 40°C or less. They also form part of the microbial community responsible for the generation of acid mine drainage. More than 16 isolates of leptospirilla were included in this study, and they were clearly divisible into two major groups. Group I leptospirilla had G+C moles percent ratios within the range 49 to 52% and had three copies of *rrn* genes, and based on 16S rRNA sequence data, these isolates clustered together with the *Leptospirillum ferrooxidans* type strain (DSM2705 or L15). Group II leptospirilla had G+C moles percent ratios of 55 to 58% and had two copies of *rrn* genes, and based on 16S rRNA sequence data, they form a separate cluster. Genome DNA-DNA hybridization experiments indicated that three similarity subgroups were present among the leptospirilla tested, with two DNA-DNA hybridization similarity subgroups found within group I. The two groups could also be distinguished based on the sizes of their 16S-23S rRNA gene spacer regions. We propose that the group II leptospirilla should be recognized as a separate species with the name *Leptospirillum ferriphilum* sp. nov. Members of the two species can be rapidly distinguished from each other by amplification of their 16S rRNA genes and by carrying out restriction enzyme digests of the products. Several, but not all, isolates of the group II leptospirilla, but none from group I (*L. ferrooxidans*), were capable of growth at 45°C. All the leptospirilla isolated from commercial biooxidation tanks in South Africa were from group II.

Bioremediation and biooxidation of minerals are industrial processes which involve a consortium of acidophilic iron- and/or sulfur-oxidizing bacteria (21). *Acidithiobacillus ferrooxidans* (previously *Thiobacillus ferrooxidans*) was the first microorganism isolated from an acidic leaching environment, and subsequently, microbial research in this field has centered around the elucidation of the properties of this chemolithoautotrophic bacterium. Although *A. ferrooxidans* was considered to be the primary biological catalyst in biomining processes, leptospirilla have been found to be the dominant iron-oxidizing bacteria in industrial continuous-flow biooxidation tanks, such as those used for the treatment of gold-bearing arsenopyrite concentrates (19, 20). There are several possible reasons for this, probably the most important being that the high ferric-ferrous iron ratio present in biooxidation tanks is less inhibitory to leptospirilla than it is to *A. ferrooxidans* (22). In many environmental samples, *Leptospirillum* has also been shown to outnumber *Acidithiobacillus* at a ratio of 2:1 under appropriate conditions (25). Temperatures above 40°C and pH values below 1.0 are two other conditions more suitable to the growth of leptospirilla than acidithiobacilli. Under these conditions, leptospirilla have been reported to be important contributors to the generation of acid mine drainage and its associated environmental problems (26). Together, these findings have sug-

gested that leptospirilla are more important to both uncontrolled (natural) and deliberate mineral bioremediation and biooxidation processes than has been generally recognized.

Bacteria belonging to the genus *Leptospirillum* are small, gram-negative, vibrio- or spiral-shaped cells (14, 16). They are obligately chemolithotrophic organisms, fixing carbon by the Benson-Calvin cycle, using ferrous iron as their sole electron donor and oxygen as their electron acceptor (11, 14). These obligately acidophilic bacteria grow optimally in inorganic media within the pH range 1.3 to 2.0. Since they use only ferrous iron as an electron donor, they are among the most metabolically restricted organisms known. Possibly as a result of this substrate specificity, they have a high affinity for ferrous iron ($K_m = 0.25$ mM) relative to *A. ferrooxidans* ($K_m = 1.34$ mM) (17). Optimum leaching efficiency is obtained at lower substrate concentrations than have been reported for *A. ferrooxidans* (25).

Limited phylogenetic studies of a relatively small number of members of the genus *Leptospirillum* have been reported. Harrison and Norris (10) obtained evidence to suggest that there was considerable variation among isolates belonging to the genus *Leptospirillum*. One group of isolates had a moles percent G+C content of ca. 51%, and another group had a G+C content of 55 to 56%. This result was further supported by DNA-DNA hybridization studies, in which two isolates had DNA sequence similarity of 71 to 73% while all other isolates had >6 to 31% DNA-DNA similarity. Unfortunately, all but the *L. ferrooxidans* type strain (DSM2705) from this early study were lost (P. R. Norris, personal communication). Hallmann et

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TABLE 1. Strains of *Leptospirillum*

Strain	Type or group	Source	Origin	GenBank accession no. ^a
P ₃ a	I	Barrie Johnson	Coal mine, North Wales, United Kingdom	AF356837
ATCC 49879	I	Wolfgang Sand	Romania	AF356832
SY	I	Barrie Johnson	Sygun Cu mine, North Wales, United Kingdom	AF356839
N ₂ 5	I	Barrie Johnson	New Zealand	ND
Crys13	I	Barrie Johnson	Ag mine, Montana	ND
BCT2	I	Barrie Johnson	Birch Coppie, United Kingdom	AF356833
Parys	I	Barrie Johnson	Parys Mountain, Anglesey Cu mine, Wales	AF356838
CF12	I	Frank Roberto	Idaho Cu mine	AF356834
Chil-Lf2	I	Barrie Johnson	Cu mine, Chile	AF356835
DSM2705	I	DSMZ, Braunschweig, Germany ^b	Markosyan strain (1972), Cu mine, Armenia	X86776
Fairview	II	Ellen Lawson	South Africa	AF356830
Warwick	II	Paul Norris	Warwick, United Kingdom	AF356831
ATCC 49880	II	Wolfgang Sand	Romania	ND
ATCC 49881	II	Wolfgang Sand	Peru	AF356829
Bionic 3.1	II	Shelly Deane	Nickel pilot plant, Billiton, South Africa	ND
Mont 4	II	Peggy Arps	Pyrite column, Montana	ND

^a ND, not determined.^b DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen.

al. (8) carried out DNA-DNA hybridization studies with six isolates of leptospirilla. Two pairs of strains were 100% related to each other, and there was 38 to 50% relatedness between these pairs and 31 to 50% relatedness among all other isolates. A moderately thermophilic *Leptospirillum* isolate with an optimum temperature of 45 to 50°C (maximum, 55 to 60°C), a moles percent G+C of 56%, and a DNA similarity of 27% with a mesophilic strain was reported (5). This strain was named *Leptospirillum thermoferrooxidans*, but it has also been lost and so is unavailable for comparative studies (14). The genus name *Leptospirillum* and the species names *ferrooxidans* and *thermoferrooxidans* have recently been validated (11). Also recently, 16S ribosomal DNA (rDNA) belonging to a third group of leptospirilla was amplified from DNA isolated directly from slime streamers of an acid mine drainage site; however, bacteria belonging to the third group have not been isolated (2).

Members of the genus *Leptospirillum* have a limited range of physiological characteristics that can be used in their identification (14). One objective of the present study, therefore, was to determine the diversity of *Leptospirillum* isolates from different geographical locations using a variety of molecular techniques to establish whether there were sufficient differences to warrant subdivision at a species level. These studies provide an extended description of a number of characteristics that can be used in the identification of the more commonly encountered leptospirilla. A second aim was to determine which *Leptospirillum* type dominated industrial biooxidation tanks. This would help identify which species should be the focus of long-term molecular biology research. From these findings, we propose that two distinct *Leptospirillum* species are represented among these isolates.

MATERIALS AND METHODS

Bacterial strains, media, and growth. The strains used in this study are listed in Table 1. Strains were routinely grown at 30°C in 800 ml of basal medium [(NH₄)₂SO₄, 0.2% (wt/vol); K₂HPO₄, 0.05% (wt/vol); MgSO₄ · 7H₂O, 0.05% (wt/vol); KCl, 0.01% (wt/vol); and Ca(NO₃)₂, 0.001% (wt/vol)] supplemented

with FeSO₄ · 7H₂O (500 mM) and adjusted to pH 1.6 with concentrated H₂SO₄. Strain purity was checked using the overlay technique of Johnson (13). Experiments at 45°C were carried out using the same medium. The ferrous iron concentration was determined by volumetric titration with potassium dichromate using diphenylamine 4-sulfonic acid indicator (28).

DNA preparation. Bacterial cells were harvested by centrifugation at 15,000 × g for 35 min and washed with acid water (pH 1.2) to remove ferric iron precipitate. The cells were either used immediately or stored frozen at -20°C in SET buffer (25% sucrose, 2 mM EDTA, 50 mM Tris; pH 8.0). Prior to lysis, the cells were treated with proteinase K (20 ng/μl) at 37°C for 30 min. Cell lysis was achieved by the addition of 10% sodium dodecyl sulfate. DNA was extracted via spooling and resuspended in Tris-EDTA buffer by overnight shaking at 30°C.

DNA techniques and Southern hybridization. Standard methods as described by Sambrook et al. (24) were used for restriction enzyme digestions and gel electrophoresis. Restriction enzymes and buffers were obtained from Roche Biochemicals and used in accordance with the manufacturer's specifications. For Southern hybridization used in ribotyping, 5 μg of chromosomal DNA was digested with *Bam*HI, and the restriction nuclease fragments were separated by agarose gel electrophoresis. The DNA was denatured in 0.25 M HCl, neutralized in 0.4 M NaOH, and transferred to a nylon Hybond N⁺ membrane (Amersham) by capillary blotting overnight. The 1.5-kb 16S rDNA PCR product of isolate P₃a (chosen randomly from the 15 isolates) was labeled with digoxigenin using the DIG oligonucleotide 3'-end labeling and detection kit (Roche Biochemicals) and used as the hybridization probe. The hybridization temperature was 40°C. Washing was done for 20 min at room temperature, followed by 20 min at 65°C. Membrane detection was performed in accordance with the manufacturer's instructions (Roche Biochemicals).

PCR amplification for restriction enzyme mapping. PCR amplifications of the 16S rRNA gene were routinely carried out to generate a 1.5-kb band on electrophoresis using the primers pDD₂ (5'-CCGGATCCGTCGACAGAGTTGATCITGGCTCAG-3'), which contains *Bam*HI and *Sal*I cloning sites towards the 5' end, and primer prDD₂ (5'-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3'), which has *Hind*III and *Xba*I cloning sites. Approximately 100 ng of chromosomal DNA was subjected to amplification in a total volume of 50 μl containing 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (vol/vol) Tween 20, 3 mM MgCl₂, 2.5 μM each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 0.2 μM each primer, and 2 U of Redhot polymerase (Advanced Biotechnologies). Denaturation was performed at 94°C for 60 s followed by 25 amplification cycles of 30 s at 94°C, 30 s at 52°C, and 90 s at 72°C. An additional 120 s at 72°C and a cooling step at 4°C for 60 s completed the reaction. The reactions were carried out in a Biometra Personal Cycler. PCR product restriction enzyme analysis was performed using *Eco*RV, *Stu*I, *Kpn*I, *Ava*I, *Sma*I, *Age*I, *Mro*I, *Nco*I, *Avr*II, *Bfr*I, *Sac*II, and *Hind*III in order to generate a discriminatory banding pattern on gel electrophoresis.

PCR of 16S rDNA for sequencing. Three different sets of prokaryotic specific primers targeting internal regions of the 16S rRNA gene were used. Forward and reverse sequencing primers from conserved 16S rRNA gene regions were made based on nucleotides 8 to 27, 517 to 536, and 1053 to 1074 in the forward direction and nucleotides 1512 to 1492, 1074 to 1053, and 536 to 515 in the reverse direction (*Escherichia coli* numbering). A maximum of 50 ng of template DNA was used per reaction in a 50- μ l volume combined with 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl (pH 8.8 at 25°C), 0.1% (vol/vol) Tween 20, 0.5 mM MgCl₂, 2.5 μ M each deoxyribonucleotide (dATP, dCTP, dGTP, and dTTP), 10 μ M each primer, and 2.5 U of Redhot polymerase. The amplification protocol was as follows: one cycle of 2 min at 96°C, followed by 25 cycles of 45 s at 96°C, 30 s at 51°C, and 90 s at 72°C, and finally one cycle of 45 s at 96°C, 30 s at 51°C, and 3 min at 72°C. The PCR products were purified using the QIAquick PCR purification kit (Qiagen), following the manufacturer's recommendations. Concentrations were determined by reading at 260 nm in a UV spectrophotometer.

Sequencing and analysis of the 16S rRNA gene. The 16S rDNA was sequenced using the dideoxy chain termination method. Cycle-sequencing reactions (with a maximum of 40 ng of template DNA), using fluorescently labeled Cy5-Far Red primers, were performed with a Thermosequenase cycle-sequencing kit (Amersham Pharmacia Biotech United Kingdom Ltd.). The sequencing reactions were run on an Allexpress automated DNA sequencer (Pharmacia Biotech, Uppsala, Sweden). Each isolate was sequenced in both the forward and reverse directions. PILEUP and CLUSTALW were used for multiple sequence alignments, and phylogenetic dendrogram construction (see Fig. 2) was done with the DNAMAN for Windows program version 4.13. A secondary-structure model of the 16S rRNA molecule transcribed from the primary sequence of isolate Fairview was constructed by Robin Gutell (7), and the file was interpreted using Aladdin Ghostscript version 5.1 graphical interface software.

PCR amplification and analysis of the 16S-23S intergenic region (IR). The conditions used for 16S-23S amplification were the same as those used for 16S rRNA gene amplification, except the annealing step took place at 45°C. The primers used in amplification were G1.2 (5'-GTCGTAACAAGGTAICCG-3') and L1.2 (5'-GCCAAGGCATCCACC-3'), modeled on primers designed by Jensen et al. (12).

Moles percent G+C content. Genomic DNA was treated with RNase A at a final concentration of 50 μ g/ml for 30 min at 37°C. The DNA was then phenol extracted, followed by ethanol precipitation. The purified DNA was dissolved in 0.1 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) at concentrations between 10 and 40 μ g/ml and dialyzed against 0.1 \times SSC overnight. The DNA solutions were stored in 0.1 \times SSC at 4°C. The G+C content of the DNA was determined as described by Harrison (9).

DNA-DNA hybridization. Genomic DNA was prepared as for moles percent G+C content determinations, with the exception of 0.1 \times SSC dialysis. DNA was resuspended in Tris-EDTA buffer. Three twofold dilutions, 125-ng starting concentration, of all genomic DNAs were prepared in a denaturing solution (final concentration, 0.4 M NaOH-10 mM EDTA). Samples were boiled for 10 min, flash cooled, and loaded onto a positively charged nylon membrane using a slot blot manifold as described by Sambrook et al. (24). The membrane was rinsed briefly in 2 \times SSC and air dried. Genomic DNA probes were sonicated for seven 10-s periods with a Biosonik III instrument (Bronwill Scientific Inc., Rochester, N.Y.) at an energy setting of 60% before being labeled with digoxigenin using the DIG oligonucleotide 3'-end labeling and detection kit. Hybridization was in DIG-Easyhyb at 40°C, followed by washing in 1 \times SSC at 25°C and a second washing in 0.1 \times SSC at 65°C. Quantification of hybridization signals was carried out on a Uvidoc gel documentation system using Alphaimager 2000 software.

Nucleotide sequence accession numbers. The *Leptospirillum* sequences determined in this study were assigned the GenBank accession numbers listed in Table 1.

RESULTS

Number of *rrn* genes and ribotyping. Genomic DNA from 16 different *Leptospirillum* isolates was analyzed in Southern hybridization experiments using 16S rDNA from strain P_{3a} as a probe. Each band represented a single copy of an *rrn* operon, as genomic DNA was digested with *Bam*HI and it had been established that none of the *Leptospirillum*-derived 16S rDNA PCR products had an internal *Bam*HI cleavage site. Two main groups of leptospirilla could be distinguished from each other, one with two *rrn* operon copies and the other with three *rrn*

TABLE 2. Some molecular characteristics of the leptospirilla in this study

Isolate	Ribotyping (kb) ^a	Mol% G+C (\pm 1%) ^b	16S-23S IR (kb)	Ability to grow at 45°C ^c
3 <i>rrn</i> subgroup				
P _{3a}	5.10, 2.9, 2.6	51.9	2.3, 1.75, 1.0	-
N ₂₅	5.10, 2.9, 2.6	51.9	2.3, 1.75, 1.0	-
DSM2705	5.10, 2.9, 2.6	51.7	2.3, 1.75, 1.0	-
ATCC 49879	5.08, 2.8, 2.1	51.7	2.3, 1.75, 1.0	-
BCT2	5.08, 2.8, 2.1	51.0	1.9, 0.47	-
Crys13	5.08, 2.8, 2.1	50.7	3.0, 2.84, 1.6	-
SY	5.08, 2.6, 2.4	48.8	3.0, 1.6	-
Parys	5.0, 4.5, 2.7	51.5	2.84	-
CF12	5.0, 4.5, 2.7	51.2	2.84	-
2 <i>rrn</i> subgroup				
Fairview	4.75, 1.97	58.0	0.5	+
Adapt ^d	4.75, 1.97	ND	0.5	+
BN Mod ^d	4.75, 1.97	ND	0.5	+
Warwick	10.5, 1.97	54.9	0.5	-
ATCC 49880	4.6, 1.97	57.8	0.5	+
ATCC 49881	4.6, 1.97	56.6	0.5	+
617 ^d	4.6, 1.97	ND	0.5	+
Bionic 3.1	4.4, 1.97	56.1	0.5	-
Mont 4	4.4, 1.97	ND	0.5	-

^a Sizes of bands of genomic DNA following digestion with *Bam*HI, separation of fragments on an agarose gel, and Southern hybridization with labeled 16S rDNA.

^b ND, not determined.

^c +, growth; -, no growth.

^d Isolate did not form part of the original study but was added during the course of the investigation.

copies. This result was confirmed by digestion of *Leptospirillum* genomic DNA with *Sal*I, which also has no internal 16S rDNA cleavage site (results not shown). A further subdivision of the two main groups into ribotype subgroups can be made from a comparison of hybridization fragment sizes (Table 2). These subgroups provide an indication of the positioning of *Bam*HI restriction endonuclease sites flanking the 16S rRNA genes. Four subgroups within each *rrn* group were identified. Interestingly, some members that belonged to the same subgroup were isolated from very different geographical locations. For example the group with three *rrn* gene copies has a 5.08-, 2.8-, 2.1-kb ribotype subgroup containing leptospirilla isolated from Romania, Montana, and England, while the 5.0-, 4.5-, and 2.7-kb ribotype subgroup has leptospirilla isolated from Wales, Idaho, and Chile.

Sequence analysis of the 16S rDNA PCR products. The 16S rRNA genes of 10 of the 16 *Leptospirillum* isolates were sequenced directly from the PCR-amplified products in both forward and reverse directions. A homology matrix (not shown) between these sequences and five other *Leptospirillum* sequences previously deposited in GenBank, EMBL, and Ribosomal Database Project databases was constructed. Isolates within the group with two *rrn* gene copies had 16S rDNA sequences which were 97.2 to 100% identical, whereas those within the group with three *rrn* gene copies were 98.2 to 99.9% identical. Sequence identity between the members of the two groups was 91.0 to 93.4%. A dendrogram of all strains of *Leptospirillum* for which sequences are available illustrates the clustering of the two *rrn* groups (Fig. 1). With the assistance of Robin Gutell (7), a secondary-structure diagram of the *Leptospirillum* strain Fairview 16S rRNA was drawn (not shown).

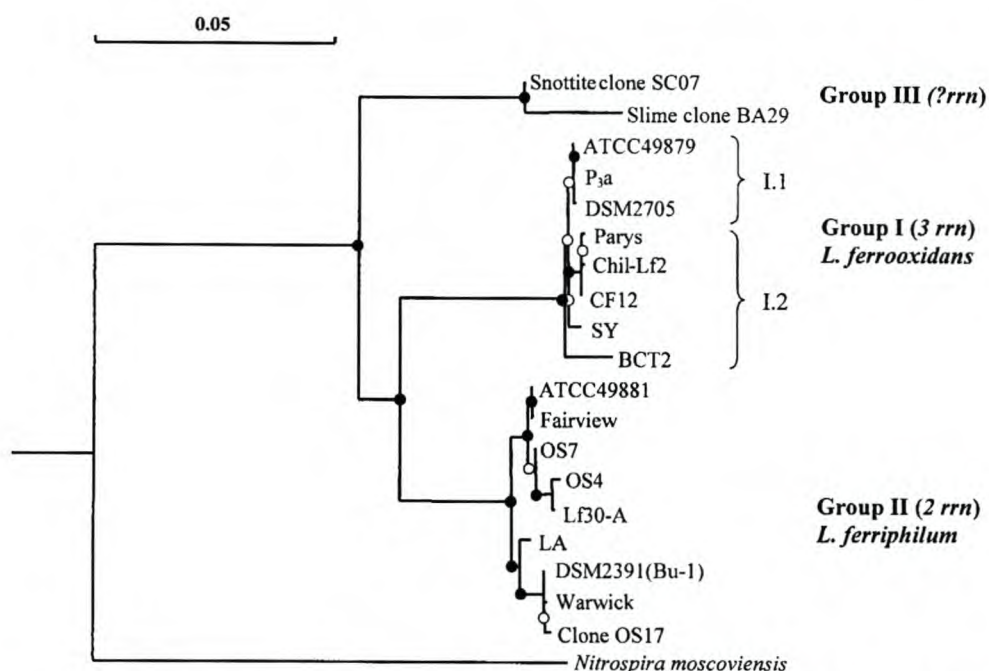


FIG. 1. Evolutionary-distance dendrogram of leptospirilla based on approximately 1,450 bp of 16S rDNA sequence. Branch points supported by bootstrap values of $>75\%$ are shown by solid circles, and those supported by bootstrap values between 50 and 75% are shown by open circles. The scale bar represents changes per nucleotide. The two DNA-DNA hybridization subgroups (Table 3) within the leptospirilla with three *rrn* gene copies (group I) are indicated with brackets. Based on 16S rDNA sequence data, the genus *Leptospirillum* has been placed within the division *Nitrospira*, and *Nitrospira moscoviensis* has been used as the outgroup. Database accession numbers are as follows: snottite clone SC07, AF225453; slime clone BA29, AF225448; ATCC 49879, AF356832; P_{3a}, AF356837; DSM2705, X86776; Parys, AF356838; Chil-Lf2, AF356835; CF12, AF356834; SY, AF356839; BCT2, AF356833; ATCC 49881, AF356829; Fairview, AF356830; OS7, X86773; OS4, X86770; Lf30-A, X72852; LA, AJ237902; DSM2391(Bu-1), M79383; Warwick, AF356831; clone OS17, X86772; and *N. moscoviensis*, X82558.

Although variations in sequence between groups with two and three *rrn* gene copies occurred in many regions of the 16S rRNA, most variation occurred within variable regions 3 and 6 (not shown). There have been reports of polymorphisms within multiple copies of 16S rRNA genes within the same organism. For example, *Mycoplasma capripneumoniae* subsp. *capripneumoniae* has two copies of 16S rRNA genes, and between 11 and 24 differences in nucleotide sequence between the copies were found in 20 isolates examined (18). The sequencing of the 16S rRNA genes of the leptospirilla in this study was carried out directly from the PCR-amplified products. Assuming that all copies of the 16S rRNA genes were amplified with equal efficiency, polymorphisms between gene copies would have resulted in a mixed population of nonidentical amplification products and ambiguous sequence data in certain positions. No positions with sequence ambiguity were found, and all copies of 16S rRNA genes therefore appeared to be identical.

PCR amplification and restriction enzyme mapping of 16S rDNA. We have routinely used restriction enzyme mapping of amplified 16S rDNA as a convenient method for rapidly identifying isolates of previously isolated iron- and sulfur-oxidizing microorganisms present in biooxidation tanks (19, 20). We wished to determine whether this simple technique could be used as a quick screening method to distinguish between the major groups of *Leptospirillum*. Comparison of the 16S rDNA sequence data from this study and those from previously sequenced leptospirilla deposited in the GenBank and Ribo-

somal Database Project databases enabled us to identify several 6-bp recognition sequence restriction endonucleases which would give different digestion patterns that could be used for this purpose. Based on the view that the presence of a cutting site has more value than the absence of a site, four endonucleases (*AgeI*, *MroI*, *NcoI*, and *SmaI*) were identified that allow for specific identification of the group of leptospirilla with two *rrn* gene copies and six endonucleases (*AgeI*, *AvrII*, *BfrI*, *EcoRV*, *SspI*, and *StuI*) were identified for specific identification of the group with three *rrn* gene copies (Fig. 2). The *AgeI* cutting site was present with the 16S rDNAs of both groups but in sufficiently different positions to allow specific identification. Although *ApaI*, *HindIII*, *KpnI*, and *SacII* cannot be used to distinguish among leptospirilla, these restriction enzymes can be used as diagnostic tools in distinguishing between *Leptospirillum*, *Acidithiobacillus caldus*, *A. ferrooxidans*, and *Acidithiobacillus thiooxidans*. To confirm the usefulness of this approach, 16S rDNAs of *Leptospirillum* strains for which the 16S rDNA had not been sequenced but for which the number of copies of *rrn* had been determined were amplified by PCR. Restriction enzyme digests for *MroI*, *NcoI*, *SmaI*, *BfrI*, *EcoRV*, *SspI*, and *StuI* were carried out, and in each case the *Leptospirillum* isolate could be correctly placed in the group with two or three *rrn* gene copies based on the restriction enzyme digests.

Amplification product profiles of the 16S-23S IRs. The IRs between the 16S and 23S rRNA genes were amplified in all 16

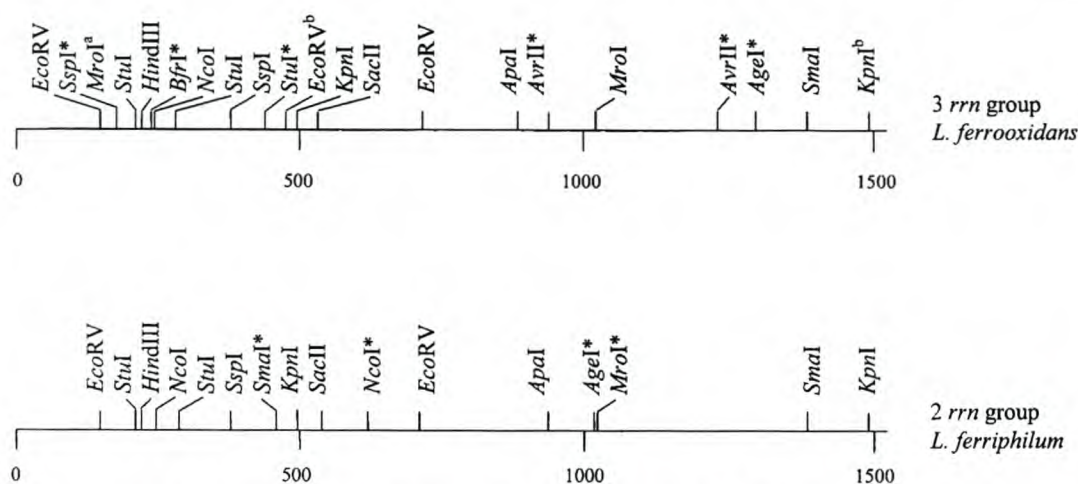


FIG. 2. Map of 6-bp restriction endonuclease cutting sites within the 16S rRNA genes of *L. ferrooxidans* and *L. ferriphilum*. Sites which enable *L. ferrooxidans* to be distinguished from *L. ferriphilum* and which were consistent among all isolates used in this study or for which sequence information is available are marked with asterisks. Restriction endonuclease sites present in all *L. ferriphilum* isolates examined except BCT2 are marked with an a, and those present in all isolates examined except BCT2 and SY are marked with a b.

Leptospirillum isolates. Both single and multiple banding patterns ranging in size from 3.0 to 0.47 kb were obtained (Table 2). PCR product profiles consisted of both intense, highly reproducible fragments (primary products) and weaker fragments, the presence of which varied depending on amplification purposes (secondary products). As secondary products are not used for classification purposes, they were ignored. A single 0.5-kb IR spacer was amplified from leptospirilla of the group with two *rrn* gene copies, whereas IR spacers of a variety of sizes were amplified from leptospirilla of the group with three *rrn* gene copies. Isolates P₃a, N₂5, DSM2705, ATCC 49879, and Crys13 produced three different primary IR products, presumably a different-size product from each of the three *rrn* gene copies. These results are in agreement with existing evidence that multiple IRs of various sizes may be present within a single species (6).

DNA-DNA hybridization. Although sequence analysis of 16S rRNA is a valuable tool in investigating phylogenetic relationships, it has been shown in several cases that almost identical 16S rRNA sequences have yielded DNA-DNA hybridization values of less than 70%, indicating separate species (27). For this reason, DNA-DNA hybridization was used in conjunction with 16S rRNA sequence analysis. DNA-DNA hybridization percentages were obtained for 16 isolates using genomic DNAs from 13 leptospirilla as hybridization probes. The results are given in Table 3. The group I leptospirilla could be divided into two DNA-DNA hybridization subgroups with 94 to 100% and 93 to 100% similarity within a subgroup and 60 to 79% similarity between the two subgroups. We have named the subgroups I.1 and I.2. Group II leptospirilla formed a single DNA-DNA hybridization subgroup with 81 to 100% similarity. However, there was only <5 to 11% similarity between subgroups I.1 and I.2 of the group I leptospirilla and the group II leptospirilla.

Leptospirilla capable of growth at 45°C. One of the few physiological differences reported among leptospirilla is the fact that some isolates are capable of growth at temperatures

of >40°C (5, 26). We have previously investigated the bacteria present in pilot plants operating at 45°C and found that large numbers of leptospirilla were present (20). Furthermore, there is a report of a *Leptospirillum* isolate that is capable of iron oxidation at 55°C and that is considered to constitute a separate species, *L. thermoferrooxidans*. We wished to determine to which group the leptospirilla adapted in pilot plants to grow at 45°C belonged and whether any of the nonadapted *Leptospirillum* isolates were also capable of growth at 45°C. Each of the 16 original isolates was tested for the ability to oxidize ferrous iron at 30 and 45°C. In addition, three new leptospirilla (Adapt, BN Mod, and 617) isolated from bioreactors operating at 45 to 55°C were introduced into the study at this stage. Several members of the *Leptospirillum* group with two copies of *rrn* genes, including those not previously exposed to bioreactors operating at 40°C or above, were able to oxidize iron at 45°C (Table 2). However, the rate of iron oxidation was lower than at 30 or 40°C, and no leptospirilla from the group with three *rrn* gene copies were able to oxidize iron at 45°C.

Lack of marked physiological or physical differences between the two groups of leptospirilla. We examined the type strain of *L. ferrooxidans* (DSM2705) and the proposed type strain of *Leptospirillum ferriphilum* (ATCC 49881) for physiological and physical differences besides temperature tolerance. Both species had properties similar to those reported for *L. ferrooxidans* (11, 14). They were of similar size (0.3 to 0.5 μ m wide and 0.9 to 3.0 μ m long), with *L. ferriphilum* at the narrower end of the width range. Both species were vibrio shaped in young cultures (up to 4 days), helical (two to five turns) in older cultures, and motile by means of a single polar flagellum. They oxidized iron at similar rates (at 37°C), with a doubling time of 12 to 15 h, and could grow autotrophically at the expense of pyrite mineral (data not shown). In addition, both grew optimally on ferrous iron medium within similar pH ranges (pH 1.4 to 1.8 for *L. ferriphilum* and pH 1.6 to 2.0 for *L. ferrooxidans*). Both leptospirilla were catalase negative and peroxidase positive. These physical and physiological observa-

TABLE 3. DNA-DNA hybridization values between *Leptospirillum* groups I and II and between subgroups^a

Probe isolate	Hybridization (%)														
	Group I (3 <i>rm</i>) <i>L. ferrooxidans</i>										Group II (2 <i>rm</i>) <i>L. ferriphilum</i>				
	Subgroup I.1					Subgroup I.2					Fairview	ATCC 49880	ATCC 49881	Warwick	Bionic 3.1
	ATCC 49879	P _{3a}	DSM2705	N ₂ 5	CF12	Chil-Lf ₂	Parys	Crys13	BCT2	SY					
ATCC 49879	100	95	96	94	63	64	67	75	74	72	<5	<5	<5	<5	<5
P _{3a}	96	100	100	97	65	63	65	65	65	65	<5	<5	<5	<5	<5
DSM2705	100	100	100	100	68	64	64	72	78	64	8	5	11	8	<5
N ₂ 5	98	100	100	100	75	76	75	76	76	79	<5	<5	<5	<5	<5
CF12	62	68	75	76	100	98	100	96	98	93	<5	<5	<5	<5	<5
Crys13	69	70	66	69	97	98	98	100	94	90	<5	<5	6	6	8
BCT2	69	68	60	70	94	97	94	97	100	95	7	7	10	6	10
Fairview	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	100	97	99	81	100
ATCC 49880	5	<5	<5	<5	<5	<5	7	9	9	6	100	100	100	89	100
ATCC 49881	<5	7	<5	<5	<5	<5	<5	<5	<5	<5	95	99	100	82	100
Warwick	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	84	84	87	100	97
Bionic 3.1	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	96	97	97	95	100
Mont 4	<5	<5	<5	<5	<5	<5	<5	<5	<5	<5	98	97	97	97	100

^a Labeled genomic DNA from each probe isolate was hybridized against genomic DNA from all target isolates. Each hybridization was carried out at least three times. Mont 4 DNA was used as a probe against all target DNA samples shown but served as a target for only a few of the probes shown. These results were the reciprocal of what is shown in this table, that is, DNA hybridization was 98 to 100% with isolates Bionic 3.1 and ATCC 49881 and <5% with ATCC 49879, DSM2705, and CF12 (not shown). With one exception, standard deviations of DNA-DNA hybridization values ranged from 0 to 8%.

tions are in close agreement with those reported for *L. ferriphilum* strain P_{3a} (now called ATCC 49881), which was a gift from Wolfgang Sand (25).

DISCUSSION

Studies of mesophilic leptospirilla by several workers (3, 4, 8, 10, 15) have indicated that more than one species of *Leptospirillum* exists. Nevertheless, all mesophilic leptospirilla have been generally referred to as *L. ferrooxidans* or *Leptospirillum*-like bacteria, as there have been insufficient physiological grounds or molecular information to decide whether they represented more than one species. Criteria commonly used to identify two bacteria as belonging to the same species are G+C moles percent ratios that differ by 5% or less and genome DNA-DNA hybridization of about 70% or greater (27). Comparison of 16S rRNA sequence data has been reported to be a somewhat less reliable criterion for separation of organisms into species. As a result of the compilation of data carried out by Stackebrandt and Goebel (27), it was suggested that organisms with 16S rRNA sequence identities of less than 97% are unlikely to have DNA-DNA hybridization values above 60%. Similar comparisons have been carried out by Rosselló-Mora and Amann (23), and they suggested a slightly more relaxed interpretation, that genomes should have less than 50 to 70% DNA-DNA hybridization before being considered as belonging to different species.

We suggest that the mesophilic leptospirilla investigated in this study may be subdivided into two groups and that the differences between these groups are sufficient for them to be regarded as separate species. Differences in G+C moles percent ratios of 49 to 52 versus 55 to 58% and 16S rRNA sequence identities of 91 to 93% suggest that division into two species is warranted. In addition, the groups differ in that one group has two copies of *rm* genes while the other group has

three copies. The DNA-DNA hybridization results support separation into two species, as there was a low level of hybridization between the group I and group II leptospirilla. The differences in hybridization between subgroups I.1 and I.2 fall within the suggested guidelines for organisms to be considered as a single species. The differences in size of the IRs between the 16S-23S rRNA genes support separation into two species. Based on the above evidence, we propose that the leptospirilla used in this study should be divided into two species, one of which consists of two distinct subgroups, or genomovars (23). The name *L. ferrooxidans* should be used for group I because the *L. ferrooxidans* type strain (DSM2705) belongs to this group, and we propose that a new species name is required for group II. In the absence of a distinguishing physiological property for all members of both species, we suggest that the name *L. ferriphilum* (*ferri*, iron; *philum*, loving) could be used for the group II leptospirilla. This name reflects a common property of all leptospirilla, which is that they use only ferrous iron as their electron donor, and it will have to be validated by the International Committee on Systematic Bacteriology.

PCR amplification of 16S rDNA genes followed by restriction enzyme digestion and separation of the fragments on an agarose gel is a relatively simple procedure compared with DNA-DNA hybridization, 16S rRNA sequencing, and Southern hybridization studies. Since the restriction enzyme digestion maps shown in Fig. 2 were consistent between the two main species of leptospirilla identified in this study, this could be used as a routine identification method. Where identification is uncertain, more comprehensive tests should be carried out.

One aim of this study was to identify which type of *Leptospirillum* was present in samples taken directly from the commercial biooxidation tanks which operate at the Fairview mine (Barberton, South Africa). These tanks are used to oxidize gold-bearing arsenopyrite concentrates and operate at pH 1.6

and 40°C (21). The isolate from the commercial biooxidation tanks at the Fairview mine was from the group II leptospirilla (two *rm* gene copies). Likewise, the 45°C-adapted BN Mod, Adapt, and 617 isolates belonged to the group II leptospirilla and are therefore of a different species than *L. ferrooxidans*. In a continuous culture study on a culture being prepared for a commercial cobaltiferous pyrite ore bioleaching operation, a *Leptospirillum*-like bacterium (strain L8) with an optimum pH of 1.3 to 1.6 and an optimum temperature of 37.5°C, but which could grow at 45°C, was isolated (1). This bacterium had a G+C ratio of about 55.6 mol%, which suggests that it was also a group II leptospirillum rather than *L. ferrooxidans*. It must be pointed out that the commercial processes in which we report that only group II leptospirilla were present operate at temperatures of 35 to 40°C or higher. It would be interesting to determine whether strains of the *L. ferrooxidans* group with three *rm* gene copies are found in industrial heap leaching- or aeration tank-type processes that operate at temperatures lower than 35°C. Since none of the *L. ferrooxidans* strains that we examined were capable of growth at 45°C, it may be that these bacteria are noncompetitive at temperatures of 35 to 45°C but may well be important in industrial processes that operate at lower temperatures.

Although it was hoped that the selection of more than 16 *Leptospirillum* isolates from many geographical locations would give a broad representation of *Leptospirillum* diversity, there are clearly some types of leptospirilla that were not represented in this group. It is unlikely that any of the isolates in this study are the same species as the moderately thermophilic *L. thermoferrooxidans*. *L. thermoferrooxidans* was reported to have an optimum growth temperature of 45°C and was capable of iron oxidation at 55°C, which is considerably higher than any isolate in this study. Since the culture has been lost, it was not possible to compare the leptospirilla in this study with *L. thermoferrooxidans*. The 16S rRNA sequence of this moderate thermophile is also unreported.

During a recent investigation of the microorganisms present in a subaerial slime from the Iron Mountain acid mine drainage site in California, a 16S rRNA sequence for what is proposed to be a third type of leptospirillum was discovered (2). Sequences corresponding to this *Leptospirillum* group III represented the majority of the clones in a clone bank of 16S rDNA genes prepared from the Iron Mountain slime. There are no reports of leptospirilla belonging to group III having been isolated in pure culture. No leptospirilla belonging to group III were present in our studies based on the direct amplification of 16S rDNA from total DNA isolated from biooxidation tanks nor in our collection of cultured environmental samples. However, the existence of group III illustrates the diversity of leptospirilla, some of which may await discovery.

Description of *Leptospirillum ferriphilum* sp. nov. *Leptospirillum ferriphilum* (*ferr*, iron; *philum*, loving). This description is based on this study and that reported by Sand et al. (25). Cells are small curved rods or spirilla, measuring 0.3 to 0.6 µm wide and 0.9 to 3.5 µm long. Young cells are vibrio shaped, but in cultures older than 4 days, cells are mostly spiral shaped with two to five turns. Cells are gram negative, spore forming, and motile by means of a single polar flagellum. Growth is aerobic and chemolithotrophic, with ferrous iron or pyrite but not sulfur serving as the energy source. Optimum pH is 1.4 to 1.8

and temperature 30 to 37°C, with some isolates having the ability to grow at 45°C. Cells are catalase negative and peroxidase positive. G+C content of the DNA is 55 to 58%, there are two copies of *rm* genes, and based on 16S rRNA sequence analysis, the cells form a phylogenetic cluster which is separate from *L. ferrooxidans*. The size of the 16S-23S rRNA intergenic region is conserved among isolates at 500 bp. The type strain is strain ATCC 49881, which is the same as strain P_{3a} provided by Sand and originally isolated in Peru (25).

ACKNOWLEDGMENTS

We thank Wolfgang Sand, Peggy Arps, Frank Roberto, Paul Norris, and especially Barrie Johnson for providing us with many of the *Leptospirillum* isolates used in this study. We also thank Paul Norris for making available the spectrophotometer used to determine DNA melting temperatures.

This work was funded by grants from the National Research Foundation (Pretoria, South Africa), the University of Stellenbosch, and Billiton Process Research (Randburg, South Africa).

REFERENCES

- Battaglia, F., D. Morin, J.-L. Garcia, and P. Olivier. 1994. Isolation and study of *Leptospirillum*-like bacteria from a natural mixed population cultured on a cobaltiferous pyrite substrate. *Antonie van Leeuwenhoek* **66**:295-302.
- Bond, P. L., S. P. Smriga, and J. F. Banfield. 2000. Phylogeny of microorganisms populating a thick, subaerial predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Appl. Environ. Microbiol.* **66**:3842-3849.
- Goebel, B. M., and E. Stackebrandt. 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.* **60**:1614-1621.
- Goebel, B. M., and E. Stackebrandt. 1995. Molecular analysis of microbial diversity in a natural acidic environment, p. 43-52. In C. A. Jerez, T. Vargas, H. Toledo, and J. V. Weirtz (ed.), *Biohydrometallurgical processing*, vol. II. University of Chile Press, Santiago, Chile.
- Golovacheva, R. S., O. V. Golyshina, G. L. Karavaiko, A. G. Dorofeev, T. A. Pivovarova, and N. A. Chernykh. 1993. A new iron-oxidizing bacterium, *Leptospirillum thermoferrooxidans* sp. nov. *Microbiology* **61**:744-750.
- Gürtler, V. 1999. The role of recombination and maturation in 16S-23S rDNA spacer rearrangements. *Gene* **238**:241-252.
- Gutell, R. R., N. Larsen, and C. R. Woese. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* **58**:10-26.
- Hallmann, R., A. Friedrich, H.-P. Koops, A. Pommerening-Röser, K. Rohde, C. Zenneck, and W. Sand. 1993. Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physicochemical factors influence in microbial metal leaching. *Geomicrobiol. J.* **10**:193-206.
- Harrison, A. P., Jr. 1982. Genomic and physiological diversity amongst strains of *Thiobacillus ferrooxidans*, and genomic comparison with *Thiobacillus thiooxidans*. *Arch. Microbiol.* **131**:68-76.
- Harrison, A. P., Jr., and P. R. Norris. 1985. *Leptospirillum ferrooxidans* and similar bacteria: some characteristics and genomic diversity. *FEMS Microbiol. Lett.* **30**:99-102.
- Hippe, H. 2000. *Leptospirillum* gen. nov. (ex Markosyan 1972), nom. rev., including *Leptospirillum ferrooxidans* sp. nov. (ex Markosyan 1972) nom. rev. and *Leptospirillum thermoferrooxidans* sp. nov. (Golovacheva et al. 1992). *Int. J. Syst. Evol. Microbiol.* **50**:501-503.
- Jensen, M. A., J. A. Webster, and N. Straus. 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* **59**:945-952.
- Johnson, D. B. 1995. Selective solid media for isolating and enumerating acidophilic bacteria. *J. Microbiol. Methods* **23**:205-218.
- Johnson, D. B. 2001. Genus II *Leptospirillum* Hippe 2000 (ex Markosyan 1972, 26), p. 453-457. In G. Garrity (ed.), *Bergey's manual of systematic bacteriology*, 2nd ed., vol. I. Springer-Verlag, Berlin, Germany.
- Lane, D. J., A. P. Harrison, Jr., D. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace. 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J. Bacteriol.* **174**:269-278.
- Markosyan, G. E. 1972. A new iron-oxidizing bacterium *Leptospirillum ferrooxidans* nov. gen. nov. sp. *Biol. J. Armenia* **25**:26-29. (In Russian.)
- Norris, P. R., D. W. Barr, and D. Hinson. 1988. Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite, p. 43-59. In P. R. Norris and D. P. Kelly (ed.), *Biohydrometallurgy*. Proceedings of the International Symposium. Science and Technology Letters, Kew, United Kingdom.
- Pettersson, B., G. Bölske, F. Tiaucourt, M. Uhlén, and K.-E. Johansson. 1998. Molecular evolution of *Mycoplasma capricolum* subsp. *capripneum*.

- moniae* strains, based on polymorphisms in the 16S rRNA genes. *J. Bacteriol.* **180**:2350–2358.
19. **Rawlings, D. E.** 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments, p. 9–17. In C. A. Jerez, T. Vargas, H. Toledo, and J. V. Wiertz (ed.), *Biohydrometallurgical processing*, vol. II. University of Chile Press, Santiago, Chile.
 20. **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.
 21. **Rawlings, D. E., and S. Silver.** 1995. Mining with microbes. *Bio/Technology* **13**:773–778.
 22. **Rawlings, D. E., H. Tributsch, and G. S. Hansford.** 1999. Reasons why '*Leptospirillum*'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for biooxidation of pyrite and related ores. *Microbiology* **145**:5–13.
 23. **Roselló-Mora, R., and R. Amann.** 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**:39–67.
 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, N.Y.
 25. **Sand, W., K. Rohde, B. Sobotke, and C. Zenneck.** 1992. Evaluation of *Leptospirillum ferrooxidans* for leaching. *Appl. Environ. Microbiol.* **58**:85–92.
 26. **Schrenk, M. O., K. J. Edwards, R. M. Goodman, R. J. Hamers, and J. F. Banfield.** 1998. Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: implications for generation of acid mine drainage. *Science* **279**:1519–1522.
 27. **Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**:846–849.
 28. **Vogel, A. L.** 1961. *A textbook of quantitative inorganic analysis*, 3rd ed. Longmans, London, United Kingdom.

Appendix H

Validation of new bacterial name *Leptospirillum ferriphilum*

VALIDATION LIST NO. 86

Validation of publication of new names and new combinations previously effectively published outside the IJSEM

The purpose of this announcement is to effect the valid publication of the following new names and new combinations under the procedure described previously [*Int J Syst Bacteriol* 27(3), iv (1977)]. Authors and other individuals wishing to have new names and/or combinations included in future lists should send the pertinent reprint or a photocopy thereof to the IJSEM Editorial Office for confirmation that all of the other requirements for valid publication have been met. It should be noted that the date of valid publication of these new names and combinations is the date of publication of this list, not the date of the original publication of the names and combinations. The authors of the new names and combinations are as given below, and these authors' names will be included in the author index of the present issue and in the volume author index. Inclusion of a name on these lists validates the name and thereby makes it available in bacteriological nomenclature. The inclusion of a name on this list is not to be construed as taxonomic acceptance of the taxon to which the name is applied. Indeed, some of these names may, in time, be shown to be synonyms, or the organisms may be transferred to another genus, thus necessitating the creation of a new combination.

Name	Proposed as:	Author(s) (reference)	Priority*	Nomenclatural type†
<i>Alkalispirillum</i>	gen. nov.	Rijkenberg <i>et al.</i> (5)	2	<i>Alkalispirillum mobile</i>
<i>Alkalispirillum mobile</i>	sp. nov.	Rijkenberg <i>et al.</i> (5)	2	Strain SL-1 (= DSM 12769)
<i>Anaeromyxobacter</i>	gen. nov.	Sanford <i>et al.</i> (6)	7	<i>Anaeromyxobacter dehalogenans</i>
<i>Anaeromyxobacter dehalogenans</i>	sp. nov.	Sanford <i>et al.</i> (6)	7	Strain 2CP-1 (= ATCC BAA-258)
<i>Desulfovibrio vietnamensis</i>	sp. nov.	Dang <i>et al.</i> (2)	3	Strain G3 100 (= DSM 10520)
<i>Enterococcus gilvus</i>	sp. nov.	Tyrrell <i>et al.</i> (8)	4	Strain PQ1 (= ATCC BAA-350 = CCUG 45553)
<i>Enterococcus pallens</i>	sp. nov.	Tyrrell <i>et al.</i> (8)	4	Strain PQ2 (= ATCC BAA-351 = CCUG 45554)
<i>Lactobacillus ferintoshensis</i>	sp. nov.	Simpson <i>et al.</i> (7)	5	Strain R7-84 (= CIP 106749)
<i>Leptospirillum ferriphilum</i>	sp. nov.	Coram and Rawlings (1)	1	Strain P ₃ a (= ATCC 49881 = DSM 14647‡)
<i>Thermaerobacter nagasakiensis</i>	sp. nov.	Nunoura <i>et al.</i> (4)	8	Strain Ts1a (= JCM 11223 = DSM 14512)
<i>Tsukamurella strandjordii</i> corrig.§ (<i>Tsukamurella strandjordae</i> [sic])	sp. nov.	Kattar <i>et al.</i> (3)	6	Strain 32-92 (= ATCC BAA-173 = DSM 44573‡)

For references to Validation Lists 1–71, see *Int J Syst Bacteriol* 49 (1999) 1325. Lists 72–85 were published in *Int J Syst Evol Microbiol* 50 (2000) 3, 423, 949, 1415, 1699, 1953 and 51 (2001) 1, 263, 793, 1229, 1619, 1945 and 52 (2002) 3, 685.

*Priority number assigned according to the date the documentation and request for validation are received.

†Abbreviations: ATCC, American Type Culture Collection, Manassas, VA, USA; CIP, Collection of the Institute Pasteur, Paris, France; CCUG, Culture Collection, University of Göteborg, Göteborg, Sweden; DSM, DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany; JCM, Japan Collection of Microorganisms, RIKEN, Saitama, Japan.

‡According to the DSMZ online catalogue.

§Name has been corrected on validation.

References

- Coram, N. J. & Rawlings, D. E. (2002). Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. nov. dominates South African commercial biooxidation tanks that operate at 40 °C. *Appl Environ Microbiol* 68, 838–845.
- Dang, P. N., Dang, T. C. H., Lai, T. H. & Stan-Lotter, H. (1996). *Desulfovibrio vietnamensis* sp. nov., a halophilic sulfate-reducing bacterium from Vietnamese oil fields. *Anaerobe* 2, 385–392.
- Kattar, M. M., Cookson, B. T., Carlson, L. C., Stiglich, S. K., Schwartz, M. A., Nguyen, T. T., Daza, R., Wallis, C. K., Yarfitz, S. L. & Coyle, M. B. (2001). *Tsukamurella strandjordae* sp. nov., a proposed new species causing sepsis. *J Clin Microbiol* 39, 1467–1476.
- Nunoura, T., Akihara, S., Takai, K. & Sako, Y. (2002). *Thermaerobacter nagasakiensis* sp. nov., a novel aerobic and extremely thermophilic marine bacterium. *Arch Microbiol* 177, 339–344.
- Rijkenberg, M. J. A., Kort, R. & Hellingwerf, K. J. (2001).

Validation List

Alkalispirillum mobile gen. nov., spec. nov., an alkaliphilic non-phototrophic member of the *Ectothiorhodospiraceae*. *Arch Microbiol* **175**, 369–375.

6. Sanford, R. A., Cole, J. R. & Tiedje, J. M. (2002). Characterization and description of *Anaeromyxobacter dehalogenans* gen. nov., sp. nov., an aryl-halorespiring facultative anaerobic myxobacterium. *Appl Environ Microbiol* **68**, 893–900.
7. Simpson, K. L., Pettersson, B. & Priest, F. G. (2001). Characterization of lactobacilli from Scotch malt whisky distilleries and description of *Lactobacillus ferintoshensis* sp. nov., a new species isolated from malt whisky fermentations. *Microbiology* **147**, 1007–1016.
8. Tyrrell, G. J., Turnbull, L., Teixeira, L. M., Lefebvre, J., Carvalho, M. G. S., Facklam, R. R. & Lovgren, M. (2002). *Enterococcus gilvus* sp. nov. and *Enterococcus pallens* sp. nov. isolated from human clinical specimens. *J Clin Microbiol* **40**, 1140–1145.

References

- Abraham WR, C. Strompl, H. Meyer, S. Lindholst, E.R. Moore, R. Christ, M. Vancanneyt, B.J. Tindall, A. Bennasar, J. Smit, and M. Tesar.** 1999. Phylogeny and polyphasic taxonomy of *Caulobacter* species. Proposal of *Maricaulis* gen. nov. with *Maricaulis maris* (Poindexter) comb. nov. as the type species, and emended description of the genera *Brevundimonas* and *Caulobacter*. *Int. J. Syst. Bacteriol.* **49**: 1053-1073.
- Achtman, M., N. Kennedy, and R. Skurray.** 1977. Cell-cell interactions in conjugating *Escherichia coli*: Role of *traT* protein surface exclusion. *Proc. Natl. Acad. Sci. USA.* **74**: 5104-5108.
- Alonso, J.C., H. Leonhardt, and C. Stiege.** 1988. Functional analysis of the leading strand replication origin of plasmid pUB110 in *Bacillus subtilis*. *Nucleic Acids Res.* **16**: 9127-9145.
- Altschul, S.F., W. Gish, W. Miller, E.W. Myers, and D.J. Lipman.** 1990. Basic logical alignment search tool. *J. Mol. Biol.* **215**: 403-410.
- Amann, R.I., C. Lin, R. Key, L. Montgomery, and D.A. Stahl.** 1992. Diversity among fibrobacter isolates: towards a phylogenetic classification. *Syst. Appl. Microbiol.* **15**: 23-31.
- Amikam, D., G. Glaser, and S. Razin.** 1984. Mycoplasmas (*Mollicutes*) have a low number of rRNA genes. *J. Bacteriol.* **158**: 376-378.
- Anthony, K.A., W.A. Klimke, and L.S. Frost.** 1999. Comparison of proteins involved in pilus synthesis and mating pair stabilization from the related plasmids F and R100-1: Insights into the mechanism of conjugation. *J. Bacteriol.* **181**: 5149-5159.
- Battaglia, F., D. Morin, J-L. Garcia, and P. Olivier.** 1994. Isolation and study of *Leptospirillum*-like bacteria from a natural mixed population cultured on a cobaltiferous pyrite substrate. *Antonie van Leeuwenhoek* **66**:295-302.
- Bennet, J.C., and H. Tribusch.** 1978. Bacterial leaching patterns on pyrite crystal surfaces. *J. Bacteriol.* **134**: 310-317.
- Bercovier, H., O. Kafri, and S. Sela.** 1986. Mycobacteria possess asurprisingly small number of ribosomal RNA genes in relation to the size of their genome. *Biochem. Biophys. Res. Commun.* **136**: 1136-1141.
- Blake, II, R.C. and E.A. Shute.** 1997. Purification and characterization of a novel cytochrome from *Leptospirillum ferrooxidans*, pp.PB3.1-PB3.10. *In* International Biohydrometallurgy Symposium, IBS97. Glenside, South Aust.: Aust. Mineral Found.

- Blake, II, R.C., E.A. Shute, and G.T. Howard.** 1994. Solubilization of minerals by bacteria: electrophoretic mobility of *Thiobacillus ferrooxidans* in the presence of iron, pyrite, and sulfur. *Appl. Environ. Microbiol.* **60**: 3349-3357.
- Blomberg, P., K. Nordstrom, and E.G. Wagner.** 1992. Replication control of plasmid R1: RepA synthesis is regulated by CopA RNA through inhibition of leader peptide translation. *EMBO J.* **11**: 2675-83.
- Boe, L., M.F. Gros, H. te Riele, S.D. Ehrlich, and A. Gruss.** 1989. Replication origins of single-stranded-DNA plasmid pUB110. *J. Bacteriol.* **171**:3366-72
- Bond, P. L., S. P. Smriga, and J. F. Banfield.** 2000a. Phylogeny of microorganisms populating a thick, subaerial predominantly lithotrophic biofilm at an extreme acid mine drainage site. *Appl. Environ. Microbiol.* **66**: 3842-3849.
- Bond, P.L., G.K. Druschell, and J.F. Banfield.** 2000b. Comparison of acid mine drainage microbial communities in physically and geochemically distinct ecosystems. *Appl. Environ. Microbiol.* **66**: 4962-4971.
- Boon, M., G.S. Hansford, and J.J. Heijnen.** 1995. The role of bacterial ferrous iron oxidation in the bio-oxidation of pyrite, pp.153-181. *In* T. Vargas, C.A. Jerez, J.V. Wiertz, and H. Toledo (eds.), *Biohydrometallurgical Processing*, vol I. Santiago: University of Chile Press.
- Brierley, C.L.** 1978. Bacterial leaching. *Crit. Rev. Microbiol.* **6**: 207-262.
- Brierley, C.L.** 1982. Microbiological mining. *Sci. Am.* **247**: 42-51.
- Brierley, C.L.** 1997. Heap leaching of gold-bearing deposits: theory and operational description, pp.103-115. *In* D.E. Rawlings (ed.), *Biomining: Theory, Microbes and Industrial processes*. Berlin: Springer-Verlag.
- Britten, R.J. and D.E. Kohne.** 1968. Repeated sequences in DNA. *Science* **161**: 529-540.
- Brown, D.P., H.W. Stokes, and R.M. Hall.** 1996. The integrons In0, In2, and In5 are defective transposon derivatives. *J. Bacteriol.* **178**: 4429-4437.
- Burggraf, S., N. Larsen, C.R. Woese, and K.O. Stetter.** 1993. An intron within the 16S ribosomal RNA gene of the archeon *Pyrobaculum aerophilum*. *Proc. Nat. Acad. Sci. USA.* **90**: 2547-2550.
- Butcher, B.G. and D.E. Rawlings.** 2002. The divergent chromosomal *ars* operon of *Acidithiobacillus ferrooxidans* is regulated by an atypical ArsR protein. *Microbiology*. (in press).
- 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**: 1826-33.

- Byrd, D.R. and S.W. Matson.** 1997. Nicking by transesterification: the reaction catalysed by a relaxase. *Mol. Microbiol.* **25**: 1011-1022.
- Cervantes, C., G. Ji, J.L. Ramirez, and S. Silver.** 1994. Resistance to arsenic compounds in microorganisms. *FEMS Microbiol. Rev.* **15**: 355-367.
- Cesareni, G., M. Helmer-Citterich, and L. Castagnoli.** 1991. Control of ColE1 plasmid replication by antisense RNA. *Trends Genet.* **7**: 230-5.
- Chattoraj, D.K.** 2000. Control of plasmid DNA replication by iterons: no longer paradoxical. *Mol. Microbiol.* **37**: 467-76.
- Chattoraj, D.K., R.J. Mason, and S.H. Wickner.** 1988. Mini-P1 plasmid replication: the autoregulation-sequestration paradox. *Cell* **52**: 551-557.
- Christie, P.J.** 1997. *Agrobacterium tumefaciens* T-complex transport apparatus: a paradigm for a new family of multifunctional transporters in Eubacteria. *J. Bacteriol.* **179**: 3085-3094.
- Christie, P.J.** 2001. Type IV secretion: intercellular transfer of macromolecules by systems ancestrally related to conjugation machines. *Mol. Microbiol.* **40**: 294-305.
- Claridge, M.F., H.A. Dawah, and M.R. Wilson (eds.).** 1997. Practical approaches to species concepts for living organisms, pp.1-15. *In: Species: the Units of Biodiversity.* Chapman and Hall, London.
- Clennel, A.M., B. Johnston, and D.E. Rawlings.** 1995. Structure and function of Tn5467, a Tn21-like transposon located on the *Thiobacillus ferrooxidans* broad-host-range plasmid pTF-FC2. *App. Environ. Microbiol.* **61**: 4223-9.
- Colmer, A.R., and M.E. Hinkel.** 1947. The role of microorganisms in acid mine drainage: a preliminary report. *Science* **106**: 253-256.
- Colmer, A.R., K.L. Temple, and M.E. Hinkle.** 1950. An iron-oxidizing bacterium from the acid drainage of some bituminous coal mined. *J. Bacteriol.* **59**: 317-328.
- Condon, C., C. Squires, and C.L. Squires.** 1995. Control of rRNA transcription in *Escherichia coli*. *Microbiol. Rev.* **59**: 623-645.
- Coplin, D.L.** 1989. Plasmids and their role in the evolution of plant pathogenic bacteria. *Ann. Rev. Phytopath.* **27**: 187-212.
- Coram, N.J. and D.E. Rawlings.** 2002. Molecular relationship between two groups of the genus *Leptospirillum* and the finding that *Leptospirillum ferriphilum* sp. Nov. dominates South African commercial biooxidation tanks that operate at 40°C. *Appl. Environ. Microbiol.* **68**: 838-845.

- Couturier, M., F. Bex, P.L. Bergquist, and W.K. Maas.** 1988. Identification and classification of bacterial plasmids. *Microbiol. Rev.* **52**: 375-395.
- Crooke, E.** 1995. Regulation of chromosomal replication in *E.coli*: Sequestration and beyond. *Cell* **82**: 877-880.
- Crosa, J.H., D.J. Brenner, and S. Falkow.** 1973. Use of single-strand-specific nuclease for analysis of bacterial and plasmid deoxyribonucleic acid homo- and heteroduplexes. *J. Bacteriol.* **115**: 904-911.
- De Groot, S.M. Deane, and D.E. Rawlings.** 2001. A transposon-located arsenic resistance mechanism within the chromosome of the biomining bacterium, *Acidithiobacillus caldus*, pp.325-335. In V.S.T. Ciminelli and O. Garcia (eds.), *Biohydrometallurgy and the Environment*. Elsevier Press, Amsterdam.
- De Ley, J., H. Cattoir, and A. Reynaerts.** 1970. The quantitative measurements of DNA hybridization from renaturation rates. *Eur. J. Biochem.* **12**: 133-142.
- Del Solar, G., M. Moscoso, and M. Espinosa.** 1993. Rolling circle-replicating plasmids from Gram-positive and Gram-negative bacteria: a wall falls. *Mol. Microbiol.* **8**: 789-796.
- Del Solar, G., R. Giraldo, M.J. Ruiz-Echevarria, M. Espinosa, and R. Diaz-Orejas.** 1998. Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **62**: 434-64.
- De Stasio EA, D. Moazed, H.F. Noller and A.E. Dahlberg.** 1989. Mutations in 16S ribosomal RNA disrupt antibiotic--RNA interactions. *EMBO. J.* **8**: 1213-1216.
- Dew, D.W., E.N. Lawson, and J.L Broadhurst.** 1997. The BIOX[®] Process for Biooxidation of Gold-Bearing Ores or Concentrates, pp.45-80. In D.E. Rawlings (ed.), *Biomining: Theory, Microbes and Industrial processes*. Berlin: Springer-Verlag.
- De Wulf-Durand, P., L.J. Bryant, and L.I. Sly.** 1997. PCR-mediated detection of acidophilic, bioleaching-associated bacteria. *Appl. Environ. Microbiol.* **63**: 2944-2948.
- DiSpirito, A.A., and O.H. Touvinen.** 1982. Uranous ion oxidation and carbon dioxide fixation by *Thiobacillus ferrooxidans*. *Arch. Microbiol.* **133**: 28-32.
- Dopson, M., E.B. Lindstrom, and K. B. Hallberg.** 2001. Chromosomally encoded arsenical resistance of the moderately thermophilic acidophile *Acidithiobacillus caldus*. *Extremophiles.* **5**: 247-55.
- 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**: 1381-91

- Drolet, M. and P.C.K. Lau.** 1992. Mobilization protein-DNA binding and divergent transcription at the transfer origin of the *Thiobacillus ferrooxidans* pTF1 plasmid. *Mol. Microbiol.* **6**: 1061-71.
- Dufresne, S., J. Bousquet, M. Boissinot, and R. Guay.** 1996. *Sulfobacillus disulfidooxidans* sp. nov., a new acidophilic, disulfide-oxidizing, Gram-positive, spore-forming bacterium. *Int. J. System. Bacteriol.* **46**: 1056-1064.
- Edwards, K.J., M.O. Schrenk, R. Hamers, and J.F. Banfield.** 1998. Microbial oxidation of pyrite: Experiments using microorganisms from an extreme acidic environment. *Am. Mineral.* **83**: 1444.
- Edwards, K.J., T.M. Gihring, and J.F. Banfield.** 1999. Seasonal variations in microbial populations and environmental conditions in an extreme acid mine drainage environment. *Appl. Environ. Microbiol.* **65**: 3627-3632.
- Edwards, K.J., P.L. Bond, T.M. Gihring, and J.F. Banfield.** 2000. An Archeal iron-oxidizing extreme acidophile important in acid mine drainage. *Science.* **279**: 1796-1799.
- Ehrlich, H.L.** 1995. *Geomicrobiology*. 3rd edition. New York: Marcel Dekker.
- Ehrlich, H.L., and C.L. Brierley, (eds.)** 1990. *Microbial Mineral Recovery*. New York: McGraw-Hill Publishing Company.
- Ereshefsky, M (ed.)** 1992. *Units of Evolution: Essays on the Nature of Species*. Cambridge, MA/London: MIT Press.
- Espejo, R.T., and J. Romero.** 1997. Bacterial community in copper sulfide ores inoculated and leached with solution from a commercial-scale copper leaching plant. *Appl. Environ. Microbiol.* **63**: 1344-1348.
- Espinosa, M., G. del Solar, F. Rojo, and J.C. Alonso.** 1995. Plasmid rolling circle replication and its control. *FEMS Microbiol. Lett.* **130**: 111-120.
- Espinosa, M., S. Cohen, M. Couturier, G. del Solar, R. Diaz-Orejas, R. Giraldo, L. Jánniere, C. Miller, M. Osborn, and C.M. Thomas.** 2000. Plasmid replication and copy number control, pp.1-48. *In* C.M. Thomas (ed.), *The horizontal gene pool: Bacterial plasmids and gene spread*. Harwood Academic Publishers.
- Finan, T.M., S. Weidner, K. Wong, J. Buhrmester, P. Chain, F.J. Vorholter, I. Hernandez-Lucas, A. Becker, A. Cowie, J. Gouzy, B. Golding, and A. Puhler.** 2001. The complete sequence of the 1,683-kb pSymB megaplasmid from the N₂-fixing endosymbiont *Sinorhizobium meliloti*. *Proc. Natl. Acad. Sci. U S A.* **98**: 9889-94.
- Firpo, M.A. and A.E. Dahlberg.** 1998. The importance of base pairing in the penultimate stem of *Escherichia coli* 16S rRNA for ribosomal subunit association. *Nucleic Acids Res.* **26**: 2156-2160.

- Fowler, T.A., P.R. Holmes, and F.K. Crundwell.** 1999. Mechanism of pyrite dissolution in the presence of *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **65**: 2987-2993.
- Fox, G.E., J.D. Wisotzkey, and P. Jurtshuk Jr.** 1992. How close is close: 16S rRNA sequence identity may not be sufficient to guarantee species identity. *Int. J. Syst. Bacteriol.* **42**: 166-170.
- Furuya, N. and T. Komano.** 1991. Determination of the nick site at *oriT* of Inc11 plasmid R64: global similarity of *oriT* structures of Inc11 and IncP plasmids. *J. Bacteriol.* **173**: 6612-6617.
- Furuya, N. and T. Komano.** 2000. Initiation and termination of DNA transfer during conjugation of Inc11 plasmid R64: roles of two sets of inverted repeat sequences within *oriT* in termination of R64 transfer. *J. Bacteriol.* **182**: 3191-6.
- Galagan, J.E., C. Nusbaum, A. Roy, M.G. Endrizzi, P. Macdonald, W. FitzHugh, S. Calvo, R. Engels, S. Smirnov, D. Atnoor, A. Brown, N. Allen, J. Naylor, N. Stange-Thomann, K. DeArellano, R. Johnson, L. Linton, P. McEwan, K. McKernan, J. Talamas, A. Tirrell, W. Ye, A. Zimmer, R.D. Barber, I. Cann, D.E. Graham, D.A. Grahame, A.M. Guss, R. Hedderich, C. Ingram-Smith, H.C. Kuettnner, J.A. Krzycki, J.A. Leigh, W. Li, J. Liu, B. Mukhopadhyay, J.N. Reeve, K. Smith, T.A. Springer, L.A. Umayam, O. White, R.H. White, E. Conway de Macario, J.G. Ferry, K.F. Jarrell, H. Jing, A.J. Macario, I. Paulsen, M. Pritchett, K.R. Sowers, R.V. Swanson, S.H. Zinder, E. Lander, W.W. Metcalf, and B. Birren.** 2002. The genome of *M. acetivorans* reveals extensive metabolic and physiological diversity. *Genome Res.* **12**: 532-42.
- Gardner, M.N., and D.E. Rawlings.** 2000. Production of rhodanese by bacteria present in bio-oxidation plants used to recover gold from arsenopyrite ores. *J. Appl. Microbiol.* **89**: 185-190.
- 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**: 3303-9.
- Garnier, T., B. Canard, and S.T. Cole.** 1991. Cloning, mapping, and molecular characterization of the rRNA operons of *Clostridium perfringens*. *J. Bacteriol.* **173**: 5431-5438.
- Gehrke, T., J. Telegdi, D. Thierry, and W. Sand.** 1998. Importance of extracellular polymeric substances from *Thiobacillus ferrooxidans* for bioleaching. *Appl. Environ. Microbiol.* **64**: 2743-2747.
- Gehrke, T., R. Hallman, and W. Sand.** 1995. Importance of exopolymers from *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* for bioleaching, pp.1-11. In T. Vargas, C.A. Jerez, J.V. Wiertz, and H. Toledo (eds.), *Biohydrometallurgical processing*. University of Chile, Santiago.

- Gerdes, K., S. Ayora, I. Canosa, P. Ceglowski, R. Diaz-Orejas, T. Franc, A.P. Gulyaev, R. Bugge Jensen, I. Kobayashi, C. Macpherson, D. Summers, C.M. Thomas, and U. Zielenkiewicz.** 2000. Plasmid maintenance systems, pp.49-88. In C.M. Thomas (ed.), *The horizontal gene pool: Bacterial plasmids and gene spread*. Harwood Academic Publishers.
- Giraldo, R. and R. Diaz.** 1992. The differential binding of wild-type and a mutant RepA protein to *oriR* sequence suggest a model for the initiation of plasmid R1 replication. *J. Mol. Biol.* **228**: 787-802.
- Goebel, B. M., and E. Stackebrandt.** 1994. Cultural and phylogenetic analysis of mixed microbial populations found in natural and commercial bioleaching environments. *Appl. Environ. Microbiol.* **60**: 1614-1621.
- Goebel, B. M., and E. Stackebrandt.** 1995. Molecular analysis of microbial diversity in a natural acidic environment. p. 43-52. In C. A. Jerez, T. Vargas, H. Toledo and J. V. Weirtz (eds) *Biohydrometallurgical Processing, Volume II*. University of Chile Press: Santiago.
- Golovacheva, R.S., and G.I. Karavaiko.** 1979. *Sulfobacillus* – a new genus of spore-forming thermophilic bacteria. *Microbiology* **48**: 658-665.
- Golovacheva, R.S.** 1980. Characteristics of *Sulfobacillus thermosulfidooxidans* morphogenesis. *Microbiology* **48**: 863-867.
- Golovacheva, R. S., O. V. Golyshina, G. L. Karavaiko, A. G. Dorofeev, T. A. Pivovarova, and N. A. Chernykh.** 1993. A new iron-oxidizing bacterium, *Leptospirillum thermoferrooxidans* sp. nov. *Microbiology* (English translation of *Mikrobiologiya*) **61**: 744-750.
- Golyshina, O.V., T.A. Pivovarova, G. Karavaiko, T.F. Kondrat'eva, and E.R.B. Moore.** 2000. *Ferroplasma acidiphilum* gen. Nov., sp. nov., an acidophilic, autotrophic, ferrous iron-oxidizing, cell-wall-lacking, mesophilic member of the *Ferroplasmaceae* fam. Nov., comprising a distinct lineage of the Archaea. *Int. J. Syst. Evol. Microbiol.* **50**: 997-1006.
- Goodfellow, M., G.P. Manfio, and J. Chun.** 1997. Towards a practical species concept for cultivable bacteria, pp.25-59. In M.F. Claridge, H.A. Dawah, and M.R. Wilson (eds.), *Species: the Units of Biodiversity*. Chapman and Hall, London.
- Grahn, A.M., J. Haase, D.H. Bamford, and E. Lanka.** 2000. Components of the RP4 conjugative transfer apparatus form an envelope structure bridging inner and outer membranes of donor cells: implications for related macromolecule transport systems. *J. Bacteriol.* **182**: 1564-74.
- Gregory, S.T. and A.E. Dahlberg.** 1995. Nonsense suppressor and antisuppressor mutations at the 1409-1491 base pair in the decoding region of *Escherichia coli* 16S rRNA. *Nucleic Acids Res.* **23**: 4234-4238.

- Grimont, P.** 1988. Use of DNA reassociation in bacterial classification. *Can. J. Microbiol.* **34**: 541-546.
- Grimont, P.A.D., M.Y. Popoff, F. Grimont, C. Coynault, and M. Lemelin.** 1980. Reproducibility and correlation study of three deoxyribonucleic acid hybridization procedures. *Curr. Microbiol.* **4**: 325-330.
- Grinsted, J., F. de la Cruz, and R. Schmitt.** 1990. The Tn21 subgroup of bacteriophage transposable elements. *Plasmid* **24**: 163-189.
- Gruss, A. and S.D. Ehrlich.** 1989. The family of highly interrelated single-stranded deoxyribonucleic acid plasmids. *Microbiol. Rev.* **53**: 231-241.
- Gürtler, V.** 1999. The role of recombination and maturation in 16S-23S rDNA spacer rearrangements. *Gene* **238**: 241-252.
- Gürtler, V., and V.A. Stanisich.** 1996. New approaches to typing and identification of bacteria using the 16S-23S rDNA spacer region. *Microbiology* **142**: 3-16.
- Gutell, R.R., B. Weiser, C.R. Woese and H.F. Noller.** 1985. Comparative anatomy of 16S-like ribosomal RNA. *Prog. Nucleic Acid Res. Mol. Biol.* **32**: 155-216.
- Gutell, R. R., N. Larsen, and C. R. Woese.** 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* **58**:10-26.
- Guzman, L.M. and M. Espinosa.** 1997. The mobilization protein, MobM, of the streptococcal plasmid pMV158 specifically cleaves supercoiled DNA at the plasmid *oriT*. *J. Mol. Biol.* **266**: 688-702
- Haase, J., Lurz R, Grahn AM, Bamford DH, Lanka E**1995. Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. *J. Bacteriol.* **177**:4779-91.
- Hallberg, K.B., and E.B. Lindström.** 1994. Characterization of *Thiobacillus caldus* sp. nov., a moderately thermophilic acidophile. *Microbiology* **140**: 3451-3456.
- Hallmann, R., A. Friedrich, H-P. Koops, A. Pommerening-Röser, K. Rohde, C. Zenneck, and W. Sand.** 1992. Physiological characteristics of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans* and physiochemical factors influence in microbial metal leaching. *Geomicrobiol. J.* **10**:193-206.
- Haring, V. and E. Scherzinger.** 1989. Replication proteins of the IncQ plasmid RSF1010, pp.95-124. In C.M. Thomas (ed.), *Promiscuous plasmids of Gram-negative bacteria*. Academic Press Ltd., London, United Kingdom.
- Harrison, A. P., Jr.** 1982. Genomic and physiological diversity amongst strains of *Thiobacillus ferrooxidans*, and genomic comparison with *Thiobacillus thiooxidans*. *Arch. Microbiol.* **131**: 68-76.

- Harrison, A.P., Jr.** 1984. The acidophilic thiobacilli and other acidophilic bacteria that share their habitat. *Ann. Rev. Microbiol.* **38**: 265-292.
- Harrison, A. P., Jr., and P. R. Norris.** 1985. *Leptospirillum ferrooxidans* and similar bacteria: some characteristics and genomic diversity. *FEMS Microbiol. Lett.* **30**: 99-102.
- Helle, U. and U. Onken.** 1988. Continuous bacterial leaching of a pyretic flotation concentrate by mixed cultures, pp.61-75. *In* P.R. Norris and D.P. Kelly (eds.), *Biohydrometallurgy: Proceedings of the International Symposium Warwick 1987*. Science and Technical Letters, Kew, Surrey.
- Henderson, D. and R.J. Meyer.** 1996. The primase of broad-host-range plasmid R1162 is active in conjugal transfer. *J. Bacteriol.* **178**: 6888-6894.
- Henderson, D. and R. Meyer.** 1999. The MobA-linked promase is the only replication protein of R1162 required for conjugal mobilization. *J. Bacteriol.* **181**: 2973-2978.
- Hippe, H.** 2000. *Leptospirillum* gen. nov. (ex Markosyan 1972), nom. rev., including *Leptospirillum ferrooxidans* sp. nov. (ex Markosyan 1972) nom. rev. and *Leptospirillum thermoferrooxidans* sp. nov. (Golovacheva *et al.*, 1992). *Int. J. Syst. Evol. Microbiol.* **50**: 501-503.
- Holmes, D.S., J.H. Lobos, L.H. Bopp and G.C. Welch.** 1984. Cloning of a *Thiobacillus ferrooxidans* plasmid in *Escherichia coli*. *J. Bacteriol.* **157**: 324-6.
- Holt, J.G. and N.R. Krieg,** (eds). 1984-1989. *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins Company, Baltimore, MD.
- Hull, D.L.** 1997. The ideal species concept- and why we can't get it, pp.357-380. *In* M.F. Claridge, H.A. Dawah, and M.R. Wilson (eds.), *Species: the Units of Biodiversity*. Chapman and Hall, London.
- Hutchins, S.R., M.S. Davidson, J.A. Brierley, and C.L. Brierley.** 1986. Microorganisms in reclamation of metals. *Ann. Rev. Microbiol.* **40**: 311-336.
- Ish-Horowicz, D. and J.F. Burke.** 1981. Rapid and efficient cosmid cloning. *Nucleic Acid Research.* **9**: 2989-2998.
- Hyde, D.R. and C.P. Tu.** 1985. *tnpM*: a novel regulatory gene that enhances Tn21 transposition and suppresses cointegrate resolution. *Cell.* **42**: 629-38.
- Jacob-Dubuisson, F., C. Loch, and R. Antoine.** 2001. Two-partner secretion in Gram-negative bacteria: a thrifty, specific pathway for large virulence proteins. *Mol. Microbiol.* **40**: 306-313.
- Jensen, M. A., J. A. Webster, and N. Straus.** 1993. Rapid identification of bacteria on the basis of polymerase chain reaction-amplified ribosomal DNA spacer polymorphisms. *Appl. Environ. Microbiol.* **59**: 945-952.

- Jin, S.M., W.M. Yan, and Z.N. Wang.** 1992. Transfer of IncP plasmids to extremely acidophilic *Thiobacillus thiooxidans*. *Appl. Environ. Microbiol.* **58**: 429-430.
- Johnson, D. B.** 1995. Selective solid media for isolating and enumerating acidophilic bacteria. *J. Microbiol. Meth.* **23**:205-218.
- Johnson, D.B.** 1998. Biodiversity and ecology of acidophilic microorganisms. *FEMS Microbiol. Ecol.* **27**: 307-317.
- Johnson, D. B.** 2001. Genus II *Leptospirillum* Hippe 2000 (ex Markosyan 1972, 26). *In* G. Garrity (ed) *Bergey's Manual of Comparative Bacteriology*, Vol. I, pp 443-447.
- Johnson, J.L.** 1989. Nucleic acids in bacterial classification, pp.2306-2309. *In* S.T. Williams, M.E. Sharpe and J.G. Holt (eds.), *Bergey's Manual of Systematic Bacteriology*. Williams and Wilkins, Baltimore, MD.
- Johnson, J.L., C.F. Phelps, C.S. Cummins, J. London, and F. Gasser.** 1980. Taxonomy of the *Lactobacillus acidophilus* group. *Int. J. Syst. Bacteriol.* **30**: 53-68.
- Kelly, D.P., and A.P. Harrison.** 1989. Genus *Thiobacillus* Beijerinck, pp.1842-1858. *In* J.T. Staley, M.P. Bryant, N. Pfennig, and J.G. Holt, *Bergey's Manual of Systematic Bacteriology*, vol. 3. Baltimore: Williams and Wilkins.
- Kelly, D.P., and A.P. Wood.** 2000. Re-classification of some species of *Thiobacillus* to the newly designated genera *Acidithiobacillus* gen. Nov., *Halothiobacillus* gen. Nov. and *Thermithiobacillus* gen. Nov. *Int. J. Syst. Evol. Microbiol.* **50**: 511-516.
- Khan, S.A.** 1997. Rolling-circle replication of bacterial plasmids. *Microbiol. Mol. Biol. Rev.* **61**: 442-55.
- Khatri, G.S., T. MacAllister, P.R. Sista, and D. Bastia.** 1989. The replication terminator protein of *E. coli* is a DNA sequence-specific contra-helicase. *Cell.* **59**(4): 667-74.
- Kholodii, G.Y.a, S.Z. Mindlin, I.A. Bass, O.V. Yurieva, S.V. Minakhina, and V.G. Nikiforov.** 1995. Four genes, two ends, and a res region are involved in transposition of Tn5053: a paradigm for a novel family of transposons carrying either a mer operon or an integron. *Mol. Microbiol.* **17**: 1189-200.
- 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.
- Kirby, C, A. Waring, T.J. Griffin IV, J.O. Falkinham III, N.D.F. Grindley, and K.M. Derbyshire.** 2002. Cryptic plasmids of *Mycobacterium avium*: Tn552 to the rescue. *Mol. Microbiol.* **43**: 173-186.

- Kishimoto, N., Y. Kosako, N. Wakao, T. Tano, and A. Hiraishi.** 1995. Transfer of *Acidiphilium facilis* and *Acidiphilium aminolytica* to the genus *Acidocella* gen. nov., and emendation of the genus *Acidiphilium*. *System. Appl. Microbiol.* **18**: 85-91.
- Konieczny, I. and D.R. Helinski.** 1997. Helicase delivery and activation by DnaA and TrfA proteins during the initiation of replication of the broad host range plasmid RK2. *J. Biol. Chem.* **272**: 33312-33318.
- Kornberg, A. and T. Baker.** 1992. DNA replication, 2nd edition. W.H. Freeman and Co. (eds.), New York, NY: Library of Congress Cataloging-in-Publication Data.
- Kumar, C.C., and R.P. Novick.** 1985. Plasmid pT181 replication is regulated by two countertranscripts. *Proc. Natl. Acad. Sci. USA.* **82**: 638-642.
- Kusano, T., K. Sugawara, C. Inoue, T. Takeshima, M. Numata, and T. Shiratori.** 1992. Electrotransformation of *Thiobacillus ferrooxidans* with plasmids containing a *mer* determinant. *J. Bacteriol.* **174**: 6617-6623.
- Lacey, D.T. and F. Lawson.** 1970. Kinetics of the liquid phase oxidation of acid ferrous sulphate by the bacterium *Thiobacillus ferrooxidans*. *Biotechnol. Bioeng.* **174**: 269-278.
- Lane, D.B., B. Pace, G.J. Olsen, D.A. Stahl, M.L. Sogin, and N.R. Pace.** 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analysis. *Proc. Natl. Acad. Sci. USA.* **82**: 6955-6959.
- Lane, D. J., A. P. Harrison, Jr., D. Stahl, B. Pace, S. J. Giovannoni, G. J. Olsen, and N. R. Pace.** 1992. Evolutionary relationships among sulfur- and iron-oxidizing eubacteria. *J. Bacteriol.* **174**:269-278.
- Lanka, E and B.M. Wilkins.** 1995. DNA processing reactions in bacterial conjugation. *Ann. Rev. Biochem.* **64**: 141-169.
- LeBlond-Bourget, N., H. Philippe, I. Mangin, and B. Decaris.** 1996. 16S rRNA and 16S to 23S internal transcribed spacer sequence analyses reveal inter- and intraspecific *Bifidobacterium* phylogeny. *Int. J. Syst. Bacteriol.* **46**: 102-111.
- Lee, I-M., D.E. Gundersen-Rindal, R.E. Davis, and I.M. Bartoszyk.** 1998. Revised classification scheme of phytoplasmata based on RFLP analyses of 16S rRNA and ribosomal protein gene sequences. *Int. J. Syst. Bacteriol.* **48**: 1153-1169.
- Lessl, M. and E. Lanka.** 1994. Common mechanisms in bacterial conjugation and Ti-mediated T-DNA transfer to plant cells. *Cell.* **77**: 321-4.
- Lessl, M., D. Balzer, R. Lurz, V.L. Waters, D.G. Guiney, and E. Lanka** 1992. Dissection of IncP conjugative plasmid transfer: definition of the transfer region Tra2 by mobilization of the Tra1 region in trans. *J. Bacteriol.* **174**: 2493-500.

- Lessl, M., D. Balzer, W. Pansegrau, and E. Lanka.** 1992. Sequence similarities between the RP4 Tra2 and the Ti VirB region strongly support the conjugation model for T-DNA transfer. *J. Biol. Chem.* **267**: 20471-80.
- Liebert, C.A., R.M. Hall, and A.O. Summers.** 1999. Transposon Tn21, flagship of the floating genome. *Microbiol. Mol. Biol. Rev.* **63**: 507-22.
- Lima, C.D.** 2002. Analysis of the *E. coli* NifS CsdB protein at 2.0 Å reveals the structural basis for perselenide and persulfide intermediate formation. *J. Mol. Biol.* **315**: 1199-208.
- 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.
- Lindström, E.B., E. Gunneriusson, and O.H. Tuovinen.** 1992. Bacterial oxidation of refractory ores for gold recovery. *Crit. Rev. Biotechnol.* **12**: 133-155.
- Linton, D., F.E. Dewhirst, J.P. Clewley, R.J. Owen, A.P. Burnens, and J. Stanley.** 1994. Two types of 16S rRNA gene are found in *Campylobacter helveticus*: analysis, applications and characterization of the intervening sequence found in some strains. *Microbiology* **140**: 847-855.
- Linton, K.J. and C.F. Higgins.** 1998. The *Escherichia coli* ATP-binding cassette (ABC) proteins. *Mol. Microbiol.* **28**: 5-13.
- Liu, Z., N. Guiliani, C. Appia-ayme, F. Borne, J. Ratouchniak, and V. Bonnefoy.** 2000. Construction and characterization of a *recA* mutant of *Thiobacillus ferrooxidans* by marker exchange mutagenesis. *J. Bacteriol.* **182**: 2269-2276.
- Livesey-Goldblatt, E., P.F. Norman, and D.R. Livesey-Goldblatt.** 1983. Gold recovery from arsenopyrite/pyrite ore by bacterial leaching and cyanidation, pp.627-641. In G. Rossi and A. E. Torma (eds.), *Recent Progress in Biohydrometallurgy*. Associazione Mineraria Sarda, Iglesias.
- Llosa, M., G. Grandoso, M.A. Hernando, and F. de la Cruz.** 1996. Functional domains in protein TrwC of plasmid R388: dissected DNA strand transferase and DNA helicase activities reconstitute protein function. *J. Mol. Biol.* **264**: 56-67
- Logan, N.A.** 1994. *Bacterial Systematics*. Blackwell Scientific Publications, London.
- Loughney, K., E. Lund, and J.E. Dahlberg.** 1982. tRNA genes are found between the 16S and 23S genes in *Bacillus subtilis*. *Nucleic Acids Res.* **10**: 1607-1624.
- Lovley, D.R.** 1993. Dissimilatory metal reduction. *Ann. Rev. Microbiol.* **47**: 263-290.

- Lu, Y-B., P.V.A.L. Ratnakar, B.K. Mohanty, and D. Bastia.** 1996. Direct physical interaction between DnaG primase and DnaB helicase of *Escherichia coli* is necessary for optimal synthesis of primer RNA. *Proc. Natl. Acad. Sci. USA.* **93**: 12902-12907.
- Ludwig, W., O. Strunk, S. Klugbauer, N. Klugbauer, M. Weizenegger, J. Neumaier, M. Bachleitner, and K-H. Schleifer.** 1998. Bacterial phylogeny base on comparative sequence analysis. *Electrophoresis.* **19**: 554-568.
- Ludwig, W. and K-H. Schleifer.** 1999. Phylogeny of bacteria beyond the 16S rRNA standard. *ASM News.* **65**: 752-757.
- Lundgren, D.G., and M. Silver.** 1980. Ore leaching by bacteria. *Annu. Rev. Microbiol.* **34**: 263-283.
- Lundgren, D.G., M. Valkova-Valchanova, and R. Reed.** 1986. Chemical reactions important in bioleaching and bioaccumulation, pp.7-22. *In* H. L. Ehrlich and D.S. Holmes (eds.), *Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries.* John Wiley and Sons.
- Markosyan, G. E.** 1972. A new iron-oxidizing bacterium *Leptospirillum ferrooxidans* nov. gen. nov. sp. *Biol. J. Armenia* **25**:26-29. (in Russian)
- Marians, K.J.** 1992. Prokaryotic DNA replication. *Annu. Rev. Biochem.* **61**: 673-719.
- Masukata, H. and J. Tomizawa.** 1986. Control of primer formation for ColE1 plasmid replication: conformational change of the primer transcript. *Cell* **44**: 125-136.
- Majdalani, N., D. Moore, S. Maneewannakul, and K. Ippen-Ihler.** 1996. Role of the propilin leader peptide in the maturation of F pilin. *J. Bacteriol.* **178**: 3748-54.
- Manning, P.A., L. Beutin, and M. Achtman.** 1980. Outer membrane of *Escherichia coli*: Properties of the F sex factor *traT* protein which is involved in surface exclusion. *J. Bacteriol.* **142**: 285-294.
- Mayden, R.L.** 1997. A hierarchy of species concepts: the denouement in the saga of the species problem, pp.381-424. *In* M.F. Claridge, H.A. Dawah, and M.R. Wilson (eds.), *Species: the Units of Biodiversity.* Chapman and Hall, London.
- M^cCready, R.G.L.** 1988. Progress in the bacterial leaching of metals in Canada, pp.177-195, *In* P.R. Norris and D.P. Kelly (eds.), *Biohydrometallurgy-1987.* Kew/Surrey, UK: Sci. Technol. Lett.
- McEachern, M.J., M.A. Bott, P.A. Tooker, and D.R. Helinski.** 1989. Negative control of plasmid R6K replication: Possible role of intermolecular coupling of replication origins. *Proc. Natl. Acad. Sci. USA.* **86**: 7942-7946.

- Mehta, A. and Y.B. Rosato.** 2001. Phylogenetic relationships of *Xylella fastidiosa* strains from different hosts, based on 16S rDNA and 16S-23S intergenic spacer sequences. *Int. J. Syst. Ev. Microbiol.* **51**: 311-318.
- Merlin, C., J. Mabillon, J. Nešvera, and A. Toussaint.** 2000. Gene recruiters and transporters: The modular structure of bacterial mobile elements, pp.363-410. *In* C.M. Thomas (ed.), *The horizontal gene pool: Bacterial plasmids and gene spread.* Harwood Academic Publishers.
- Miao, D-M., Y. Homda, 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.
- Miller, P.C.** 1997. The design and operating practice of bacterial oxidation plant using moderate thermophiles (The BacTech Process), pp.81-100. *In* D.E. Rawlings (ed.), *Biomining: Theory, Microbes and Industrial processes.* Berlin: Springer-Verlag.
- Molin, S. and Nordström.** 1980. Control of plasmid R1 replication: functions involved in replication, copy number control, incompatibility, and switch-off of replication. *J. Bacteriol.* **141**: 111-20.
- Morgan, E.A., T. Ikemura, and M. Nomura.** 1977. Identification of spacer tRNA genes in individual ribosomal RNA transcription units of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA.* **74**: 2710-2714.
- Morin, D.** 1995. Bacterial leaching of refractory gold sulphide ores, pp.25-62. *In* C.C. Gaylarde and H.A. Videla (eds.), *Bioextraction and biodeterioration of metals.* Cambridge University Press.
- Moscoso, M., R. Eritja, and M. Espinosa.** 1997. Initiation of replication of plasmid pMV158: mechanisms of DNA strand-transfer reactions mediated by the initiator RepB protein. *J. Mol. Biol.* **268**: 840-56.
- Motallebi-Veshareth, M., D. Balzer, E. Lanka, G. Jagura-Burdzy, and C.M. Thomas.** 1992. Conjugative transfer functions of broad host-range plasmid RK2 are coregulated with vegetative replication. *Mol. Microbiol.* **6**: 907-920.
- Mukherjee, S., H. Erickson, and D. Bastia.** 1988. Enhancer-origin interaction in plasmid R6K involves a DNA loop mediated by initiator protein. *Cell* **52**: 375-383.
- Neefs, J-M., Y. Van De Peer, L. Hendricks, and R. De Wachter.** 1990. Compilation of small ribosomal subunit RNA sequences. *Nucleic Acids Res.* **18**: 2237-2317.
- Neyt, C., M. Iriarte, V.H. Thi, and G.R. Cornelis.** 1997. Virulence and arsenic resistance in *Yersinia*. *J. Bacteriol.* **179**: 612-9.

- Nordström, K.** 1990. Control of plasmid replication- How do DNA iterons set the replication frequency? *Cell* **63**: 1121-1124.
- Norris, P.R.** 1983. Iron and mineral oxidation with *Leptospirillum*-like bacteria, pp.83-96. In G. Rossi and A.E Torma, Recent Progress in Biohydrometallurgy. Iglesias, Italy: Associazione Mineraria Sarda.
- Norris, P.R.** 1997. Thermophiles and bioleaching, pp.247-258. In D.E. Rawlings (ed.), Biomining: Theory, Microbes and Industrial processes. Berlin: Springer-Verlag.
- Norris, P.R., D.A. Clark, J.P. Owen, and S. Waterhouse.** 1996. Characteristics of *Sulfobacillus acidophilus* sp. nov. and other moderately thermophilic mineral-sulphide-oxidizing bacteria. *Microbiology*. **142**: 775-783.
- Norris, P. R., D.W. Barr, and D. Hinson.** 1988. Iron and mineral oxidation by acidophilic bacteria: affinities for iron and attachment to pyrite. p. 43-59. In P. R. Norris and D. P. Kelly (eds). Biohydrometallurgy, Proceedings of the International Symposium. Science and Technology Letters: Kew (UK).
- Norris, P.R., J.C. Murrell, and D. Hinson.** 1995. The potential for diazotrophy in iron- and sulfur- oxidizing acidophilic bacteria. *Arch. Microbiol.* **164**: 294-300.
- Norris, P.R., N.P. Burton, and N.A.M. Foulis.** 2000. Acidophiles in bioreactor mineral processing. *Extremophiles* **4**: 71-76.
- Novick, R.P.** 1987. Plasmid incompatibility. *Microbiol. Rev.* **51**: 381-395.
- Novick, R.P., S. Iordanescu, S.J. Projan, J. Kornblum, and I. Edelman.** 1989. pT181 Plasmid replication is regulated by a countertranscript-driven transcriptional attenuator. *Cell* **59**: 395-404.
- Olsen, G.J., N. Larsen, and C.R. Woese.** 1991. The ribosomal database project. *Nucleic Acid Res.* **19** (suppl.), 2017-2021.
- Pal, S.K. and D.K. Chatteraj.** 1988. P1 Plasmid replication: Initiator sequestration is inadequate to explain control by initiator-binding sites. *J. Bacteriol.* **170**: 3554-3560.
- Pansegrau, W. and E. Lanka.** 1991. Common sequence motifs in DNA relaxases and nick regions from a variety of DNA transfer systems. *Nucl. Acids Res.* **19**: 3455-3455.
- Pansegrau, W. and E. Lanka.** 1996. Mechanisms of initiation and termination reactions in conjugative DNA processing. *J. Bio. Chem.* **271**: 13068-13076.
- Pansegrau, W., D. Balzer, V. Kruft, R. Lurz, and E. Lanka.** 1990. *In vitro* assembly of relaxosomes at the origin of plasmid RP4. *Proc. Natl. Aca. Sci. USA.* **87**: 6555-6559.

- Pansegrau, W., F. Schoumacher, B. Hohn, and E. Lanka.** 1993a. Site-specific cleavage and joining of single-stranded DNA by VirD2 protein of *Agrobacterium tumefaciens* Ti plasmids: Analogy to bacterial conjugation. *Proc. Natl. Acad. Sci. USA.* **90**: 11538-11542.
- Pansegrau, W., W. Schröder, and E. Lanka.** 1993b. Relaxase (TraI) of IncP α plasmid RP4 catalyzes a site-specific cleaving-joining reaction of single-stranded DNA. *Proc. Natl. Acad. Sci. USA.* **90**: 2925-2929.
- Pansegrau, W., W. Schröder, and E. Lanka.** 1994. Concerted action of three distinct domains in the DNA cleaving-joining reaction catalyzed by relaxase (TraI) of conjugative plasmid RP4. *J. Biol. Chem.* **269**: 2782-9.
- Peng, J-B., W-M. Yan, and X-Z. Bao.** 1994a. Expression of heterogenous arsenic resistance genes in the obligately autotrophic biomining bacterium *Thiobacillus ferrooxidans*. *Appl. Environ. Microbiol.* **60**: 2653-2656.
- Peng, J-B., W-M. Yan, and X-Z. Bao.** 1994b. Plasmid and transposon transfer to *Thiobacillus ferrooxidans*. *J. Bacteriol.* **176**: 2892-2897.
- Peng, J-B., W-M. Yan, and X-Z. Bao.** 1994c. Solid medium for the genetic manipulation of *Thiobacillus ferrooxidans*. *J. Gen. Appl. Microbiol.* **40**: 243-253.
- Perez Luz, S., F. Rodriguez-Valera, R. Lan, and P.R. Reeves.** 1998. Variation of the ribosomal operon 16S-23S gene spacer region in representatives of *Salmonella enterica* subspecies. *J. Bacteriol.* **180**: 2144-2151.
- 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.
- Perumal, N.B. and E.G. Minkley Jr.** 1984. The product of the F sex factor *traT* surface exclusion gene is a lipoprotein. *J. Biol. Chem.* **259**: 5357-5360.
- Perwez, T. and R. 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.
- Pettersson, B., G. Bölske, F. Tiaucourt, M. Uhlén, and K-E. Johansson.** 1998. Molecular evolution of *Mycoplasma capricolum* subsp. *capripneumoniae* strains, based on polymorphisms. *J. Bacteriol.* **180**: 2350-2358.
- Pettersson B, M. Uhlen M, and K.E. Johansson.** 1996. Phylogeny of some mycoplasmas from ruminants based on 16S rRNA sequences and definition of a new cluster within the hominis group. *Int. J. Syst. Bacteriol.* **46**: 1093-1098.
- Pivovarova, T.A., G.E. Markosyan, and G.I. Karavaiko.** 1981. Morphogenesis and fine structure of *Leptospirillum ferrooxidans*. *Mikrobiologiya.* **50**: 482-486.

- Pizarro, J., E. Jedlicki, O. Orellana, J. Romero, and R.T. Espejo.** 1996. Bacterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. *Appl. Environ. Microbiol.* **62**: 1323-1328.
- Plano, G.V., J.B. Day, and F. Ferracci.** 2001. Type III export: new uses for an old pathway. *Mol. Microbiol.* **40**: 284-293.
- Polard, P. and M. Chandler.** 1995. Bacterial transposases and retroviral integrases. *Mol. Microbiol.* **15**: 13-23.
- Pretorius, I.M., D.E. Rawlings, E.G. O'Neill, W.A. Jones, R. Kirby, and D.R. Woods.** 1987. Nucleotide sequence of the gene encoding the nitrogenase iron protein of *Thiobacillus ferrooxidans*. *J. Bacteriol.* **169**: 367-70.
- Pronk, J.T., W.M. Meijer, W. Haseu, J.P. van Dijken, P. Bos, and J.G. Kuenen.** 1991. Growth of *Thiobacillus ferrooxidans* on formic acid. *Appl. Environ. Microbiol.* **57**: 2057-2062.
- Radstrom, P., O. Skold, G. Swedberg, J. Flensburg, P.H. Roy, and L. Sundstrom.** 1994. Transposon Tn5090 of plasmid R751, which carries an integron, is related to Tn7, Mu, and the retroelements. *J. Bacteriol.* **176**: 3257-68
- Rasooly, A., S.J. Projan, and R.P. Novick.** 1994. Plasmids of the pT181 family show replication-specific initiator protein modification. *J. Bacteriol.* **176**: 2450-2453.
- Rastorguev, S.M., G.B. Zavilgelsky, and N.A. Tchurikov.** 1998. IncI1 plasmid R64 encodes the ArsR protein that alleviates type I restriction. *FEBS Letters.* **426**: 21-23.
- Ratnaker, P.V.A.L., B.K. Mohanty, M. Lobert, and D. Bastia.** 1996. The replication initiator protein π of the plasmid R6K specifically interacts with the host-encoded helicase DnaB. *Proc. Natl. Acad. Sci. USA.* **93**: 5522-5526.
- Rawlings, D. E.** 1995. Restriction enzyme analysis of 16S rRNA genes for the rapid identification of *Thiobacillus ferrooxidans*, *Thiobacillus thiooxidans* and *Leptospirillum ferrooxidans* strains in leaching environments. p. 9-17. *In* C. A. Jerez, T. Vargas, H. Toledo and J. V. Wiertz (eds). *Biohydrometallurgical Processing*. Volume II University of Chile Press: Santiago.
- Rawlings, D.E.** 1999c. The molecular genetics of mesophilic, acidophilic, chemolithotrophic, iron- or sulfur- oxidizing microorganisms, pp.3-20. *In* R. Amils and A. Ballester (eds.), *Biohydrometallurgy and the environment toward the mining of the 21st century*, part B. Library of Congress Cataloging in Publication Data.
- Rawlings, D.E.** 2001. The molecular genetics of *Thiobacillus ferrooxidans* and other mesophilic, acidophilic, chemolithotrophic, iron- or sulfur- oxidizing bacteria. *Hydrometallurgy* **59**: 187-201.

- Rawlings, D.E.** 2002. Heavy metal mining using microbes. *Annu. Rev. Microbiol.* **56**: 65-91.
- Rawlings, D.E., and D.R. Woods.** 1995. Development of improved biomining bacteria, pp.63-84. *In* C.C. Gaylarde and H.A. Videla (eds.), *Bioextraction and biodeterioration of metals*. Cambridge University Press.
- Rawlings, D.E. and E. Tietze.** 2001. Comparative biology of IncQ and IncQ-like plasmids. *Microbiol. Mol. Biol. Rev.* **65**: 481-96.
- Rawlings, D. E., and S. Silver.** 1995. Mining with microbes. *Bio/Technology* **13**: 773-778.
- Rawlings, D.E. and T. Kusano.** 2001. Molecular genetics of *Thiobacillus ferrooxidans*. *Microbiol. Rev.* **58**: 39-55.
- Rawlings, D.E., D. Dew, and C. du Plessis.** 2002. Biomineralization of metal-containing ores and concentrates. *In press*.
- Rawlings, D. E., H. Tributsch, and G.S. Hansford.** 1999b. Reasons why '*Leptospirillum*'-like species rather than *Thiobacillus ferrooxidans* are the dominant iron-oxidizing bacteria in many commercial processes for biooxidation of pyrite and related ores. *Microbiology* **145**: 5-13.
- Rawlings, D. E., N. J. Coram, M. N. Gardner, and S. M. Deane.** 1999a. *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 (eds). *Biohydrometallurgy and the Environment Toward the Mining of the 21st Century*. Part A. Elsevier: Amsterdam.
- Rawlings, D.E., R.A. Dorrington, J. Rohrer, and A-M. Clennel.** 1993. A molecular analysis of a broad-host-range plasmid isolated from *Thiobacillus ferrooxidans*. *FEMS Microbiol. Rev.* **11**: 3-8.
- Razzel, W.E. and P.C. Trussel.** 1963. Isolation and properties of an iron-oxidizing *Thiobacillus*. *J. Bacteriol.* **85**: 595-603.
- Recchia, G.D. and R.M. Hall,** 1997. Origins of the mobile gene cassettes found in integrons. *Trends Microbiol.* **5**: 389-94.
- Reeder, R.H. and W. Lang.** 1994. The mechanism of transcription termination by RNA polymerase I. *Mol. Microbiol.* **12**: 11-15.
- Rees, C.E.D. and B.M. Wilkins.** 1990. Protein transfer into the recipient cell during bacterial conjugation: studies with F and RP4. *Mol. Microbiol.* **4**: 1199-1205.
- Rensing, C., M. Ghosh, and B.P. Rosen.** 1999. Families of soft-metal-ion-transporting ATPases. *J. Bacteriol.* **181**: 5891-7.

- Rogall, T., J. Wolters, T. Flohr, and E.C. Böttger.** 1990. Towards a phylogeny and definition of species at the molecular level within the genus *Mycobacterium*. *Int. J. Syst. Bacteriol.* **40**: 323-330.
- 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**: a-h.
- Rohrer, J. and D.E. Rawlings.** 1993. Regulation of mobilization of the broad-host-range plasmid pTF-FC2. *Mol. Microbiol.* **9**: 1051-9.
- Rojas-Chapana, J.A., and H. Tributsch.** 2001. Biochemistry of sulfur extraction in bio-corrosion of pyrite by *Thiobacillus ferrooxidans*. *Hydrometallurgy* **59**: 291-300.
- Rojas-Chapana, J.A., C.C. Baertels, L. Pohlmann, and H. Tributsch.** 1998. Co-operative leaching and chemotaxis of thiobacilli studied with spherical sulfur/sulfide substrates. *Process Photochem.* **33**: 239-249.
- Rosselló-Mora, R., and R. Amann.** 2001. The species concept for prokaryotes. *FEMS Microbiol. Rev.* **25**:39-67.
- Rossi, G.** 1990. *Biohydrometallurgy*. Hamburg: McGraw-Hill.
- Sakai, H., and T. Komano.** 1996. DNA replication of IncQ broad-host-range plasmids in Gram-negative bacteria. *Biosci. Biotech. Biochem.* **60**: 377-382.
- Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. *Molecular Cloning: A laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press:New York.
- Samuels, A.L., E. Lanka, and J. E. Davies.** 2000. Conjugative junctions in RP4-mediated mating of *Escherichia coli*. *J. Bacteriol.* **182**: 2709-2715.
- Sand, W., K. Rohde, B. Sobotke, and C. Zenneck.** 1992. Evaluation of *Leptospirillum ferrooxidans* for leaching. *Appl. Environ. Microbiol.* **58**: 85-92.
- Sand, W., T. Gehrke, R. Hallman, and A. Schippers.** 1995. Sulfur chemistry, biofilm, and the (in) direct attack mechanism – critical evaluation of bacterial leaching. *Appl. Microbiol. Biotechnol.* **43**: 961-966.
- Sand, W., T. Gehrke, R. Hallman, K. Rhode, B. Sobotke, and S. Wentzien.** 1993. In situ bioleaching of metal sulfides: the importance of *Leptospirillum ferrooxidans*, pp.15-27. In A.E. Torma, J.E. Wey and V.I. Lakshmanan (eds.), *Biohydrometallurgical Technologies*, vol. I. Warrendale, PA: TMS Press.
- Sandqvist, M.** 2001. Biology of type II secretion. *Mol. Microbiol.* **40**: 271-283.

- Sanmugasunderam, V.** 1986. Bacterial assisted column leaching, pp.73-82. *In* H.L. Ehrlich and D.S. Holmes (ed.), Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries. Biotechnology and Bioengineering Symposium No. 16. John Wiley & Sons, Inc.
- Schippers, A., and W. Sand.** 1999. Bacterial leaching of metal sulfides proceeds by two indirect mechanisms via thiosulfate or via polysulfides and sulfur. *Appl. Environ. Microbiol.* **65**: 319-321.
- Schippers, A., P-G. Jozsa, and W. Sand.** 1996. Sulfur chemistry in bacterial leaching of pyrite. *Appl. Environ. Microbiol.* **65**: 319-321.
- Schneiker, S., M. Keller, M. Droge, E. Lanka, A. Puhler, and W. Selbitschka.** 2001. The genetic organization and evolution of the broad host range mercury resistance plasmid pSB102 isolated from a microbial population residing in the rhizosphere of alfalfa. *Nucleic Acids Res.* **29**: 5169-81.
- Schnell, H.A.** 1997. Bioleaching of copper, pp.21-43. *In* D.E. Rawlings (ed.), *Biomining: Theory, Microbes and Industrial processes.* Berlin: Springer-Verlag.
- Schlöter, M., R. Borlinghaus, W. Bode, and A. Hartmann.** 1993. Direct identification, and localization of *Azospirillum* in the rhizosphere of wheat using fluorescence-labelled monoclonal antibodies and confocal scanning laser microscopy. *J. Microsc.* **171**: 173-177.
- Schrenk, M. O., K. J. Edwards, R. M. Goodman, R. J. Hamers, and J. F. Banfield.** 1998. Distribution of *Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*: implications for generation of acid mine drainage. *Science* **279**: 1519-1522.
- Seidler, R.J. and M. Mandel.** 1971. Quantitative aspects of deoxyribonucleic acid renaturation: Base composition, state of chromosome replication, and polynucleotide homologies. *J. Bacteriol.* **106**: 608-614.
- Sherratt, D.** 1989. Tn3 and other related transposable elements: Site-specific recombination and transposition, pp.163-184. *In* D.E. Berg and M.M. Howe (eds.), *Mobile DNA.* American Society for Microbiology, Washington, D.C
- Silver, S.** 1996. Bacterial resistance to toxic metals- a review. *Gene* **179**: 9-19.
- Silver, S. and L.T. Phung.** 1996. Bacterial heavy metal resistance: new surprises. *Annu. Rev. Microbiol.* **50**: 753-789.
- Silver, S. and M. Walderhaug.** 1992. Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. *Microbiol. Rev.* **56**: 195-228.
- Silverman, P.M.** 1997. Towards a structural biology of bacterial conjugation. *Mol. Microbiol.* **23**: 423-429.

- Simon, R.A., U. Prierer, 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.
- Sohier D, J. Coulon, and A. Lonvaud-Funel.** 1999. Molecular identification of *Lactobacillus hilgardii* and genetic relatedness with *Lactobacillus brevis*. *Int J Syst Bacteriol*. **49**: 1075-81.
- Sokal, R.R., and T.J. Crovello.** 1970. The biological species concept: a critical evaluation. *Am. Nat.* **104**: 127-153.
- Som, T. and J. Tomizawa.** 1983. Regulatory regions of ColE1 that are involved in determination of plasmid copy number. *Proc. Natl. Acad. Sci. USA*. **80**: 2257-2261.
- Soultanas, P., M. Oram, and S.E. Halford.** 1995. Site-specific recombination at res sites containing DNA-binding sequences for both Tn21 and Tn3 resolvases. *J. Mol. Biol.* **245**: 208-18.
- Stackebrandt, E., and B. M. Goebel.** 1994. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**: 846-849.
- Stark, W.M., M.R. Boocock, and D.J. Sherratt.** 1989. Site-specific recombination by Tn3 resolvase. *Trends Genet.* **5**: 304-9.
- Stokes, H.W. and R.M. Hall.** 1989. A novel family of potentially mobile DNA elements encoding site-specific gene-integration functions: integrons. *Mol. Microbiol.* **3**: 1669-83.
- Sundberg, C.D. and W. Ream.** 1999. The *Agrobacterium tumefaciens* chaperone-like protein, VirE1, interacts with VirE2 at domains required for single-stranded DNA binding and cooperative interaction. *J. Bacteriol.* **181**: 6850-6855.
- Takechi, S. H. Matsui, and T. Itoh.** 1995. Primer RNA syntheses by plasmid-specified Rep protein for initiation of ColE2 DNA replication. *EMBO J.* **14**: 5141-5147.
- Tamaoka, J.** 1994. Determination of DNA base composition, pp.463-469. *In* M. Goodfellow and A.G. O'Donnell (eds.), *Chemical methods in Prokaryotic Systematics*. John Wiley and Sons, Chichester.
- Templeton, A.R.** 1989. The meaning of species and speciation: a genetic perspective, pp.3-27. *In* D. Otte and J.A Endler (eds.), *Speciation and its Consequences*. Sinauer Ass. Inc., Sunderland, MA.
- Thomas, C.D., T.T. Nikiforov, B.A. Connolly, and W.V. Shaw.** 1995. Determination of sequence specificity between a plasmid replication initiator protein and the origin of replication. *J. Mol. Biol.* **254**: 381-91.

- Tomizawa, J. and T. Itoh.** 1981. Plasmid ColE1 incompatibilities determined by interaction of RNA I with primer transcript. *Proc. Natl. Acad. Sci. USA.* **78**: 6097-6100.
- Tomizawa, J. and T. Itoh.** 1982. The importance of RNA secondary structure in ColE1 primer formation. *Cell* **31**: 575-583.
- Tomizawa, J. and T. Som.** 1984. Control of ColE1 plasmid replication: enhancement of binding of RNA I to the primer transcript by the Rom protein. *Cell* **38**: 871-878.
- Tributsch, H.** 2001. Direct vs. indirect bioleaching. *Hydrometallurgy* **59**: 177-185.
- 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.
- Unz, R.F., and J.M. Dietz.** 1986. Biological applications in the treatment of acidic mine drainages, pp.163-170. *In* H.L. Ehrlich and D.S. Holmes (ed.), Workshop on biotechnology for the mining, metal-refining and fossil fuel processing industries. Biotechnology and Bioengineering Symposium No. 16. John Wiley & Sons, Inc.
- Van Aswegen, P.C., M.W. Godfrey, D.M. Miller, and A.K. Haines.** 1991. Developments and innovations in bacterial oxidation of refractory ores. *Miner. Metallurg. Process.* **8**: 188-192.
- Vandamme, P., B. Pot, M. Gillis, P. De Vos, and J. Swings.** 1996. Polyphasic taxonomy, a consensus approach to bacterial systematics. *Microbiol. Rev.* **60**: 407-438.
- Vásquez, M, and R.T. Espejo.** 1997. Chemolithotrophic bacteria in copper ores leached at high sulphuric acid concentration. *Appl. Environ. Microbiol.* **63**: 332-334.
- Vogel, A.L.** 1961. A textbook of quantitative inorganic analysis. 3rd Edition. Longmans: London.
- Wagner, E.G.H., and R.W. Simons.** 1994. Antisense RNA control in bacteria, phages, and plasmids. *Annu. Rev. Microbiol.* **48**: 713-742.
- Waksman, S.A. and J.S. Joffe.** 1921. Acid production by a new sulfur-oxidizing bacterium. *Science.* **LIII**: 216.
- Wang, P., S.J. Projan, V. Henriquez, and R.P. Novick.** 1993. Origin recognition specificity in pT181 plasmids is determined by a functionally asymmetric palindrome DNA-element. *EMBO J.* **12**: 45-52.
- Waters, V.L. and D.G. Guiney.** 1993. Processes at the nick region link conjugation, T-DNA transfer and rolling circle replication. *Mol. Microbiol.* **9**: 1123-1130.

- Wayne, I.G., D.J. Brenner, R.R. Colwell, P.A.D. Grimont, O. Kandler, M.I. Krichevsky, L.H. Moore, W.E.C. Moore, R.G.E. Murray, E. Stackebrandt, M.P. Starr, and H.G. Truper.** 1987. Report of the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics. *Int. J. Syst. Bacteriol.* **37**: 463-464.
- Whitlock, J.L., and G.R. Smith.** 1989. Operation of Homestake's cyanide biodegradation wastewater system based on multi-variable trend analysis, pp.613-625. In: *Biohydrometallurgy-1989*. Jackson Hole, Wyoming.
- Wichlacz, P.L., and D.L. Thompson.** 1988. The effect of acidophilic heterotrophic bacteria on the leaching of cobalt by *Thiobacillus ferrooxidans*, pp.77-88. In P.R. Norris and D.P. Kelly (eds.), *Biohydrometallurgy. Proceedings of the International Symposium, Warwick 1987*. Science and Technology Letters, Kew, United Kingdom.
- Woese, C.R.** 1987. Bacterial evolution. *Microbiol. Rev.* **51**: 221-271.
- Woese, C.R., O. Kandler, and M.L. Wheelis.** 1990. Towards a natural system of organisms: proposal for the domains Archaea, Bacteria and Eucarya. *Proc. Natl. Acad. Sci. USA.* **77**: 494-498.
- Wong, E.M. and B. Polisky.** 1985. Alternative conformations of the ColE1 replication primer modulate its interaction with RNA I. *Cell* **42**: 959-966.
- Yoon, J-H., S.T. Lee, S-B. Kim, M. Goodfellow, and Y-H. Park.** 1997. Inter- and intraspecific genetic analysis of the genus *Saccharomonospora* with 16S to 23S ribosomal DNA (rDNA) and 23S to 5S rDNA internally transcribed spacer sequences. *Int. J. Syst. Bacteriol.* **47**: 661-669.
- 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, pp.87-174. In C.M. Thomas (ed.), *The horizontal gene pool: Bacterial plasmids and gene spread*. Harwood Academic Publishers.
- Zhao, A.C. and S.A. Khan.** 1997. Sequence requirements for the termination of rolling-circle replication of plasmid pT181. *Mol. Microbiol.* **24**: 535-544.
- Zhang, Z., T. Kudo, Y. Nakajima, and Y. Wang.** 2001. Clarification of the relationship between the members of the family *Thermomonosporaceae* on the basis of 16S rDNA, 16S-23S rRNA internal transcribed spacer and 23S rDNA sequences and chemotaxonomic analyses. *Int. J. Syst. Ev. Microbiol.* **51**: 373-383.
- Zheng, L., R.H. White, V.L. Cash, and D.R. Dean.** 1994. Mechanism for the desulfurization of L-cysteine catalyzed by the nifS gene product. *Biochemistry.* **33**: 4714-20.

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.