Towards a genetic system for the genus *Sulfobacillus*

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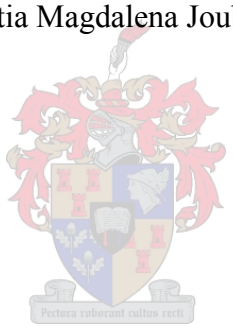

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CERTIFICATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work unless otherwise referenced or acknowledged and that I have not previously, in its entirely or part, submitted it to any university for a degree.

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T.M. Joubert Date

ABSTRACT

Members of the genus *Sulfobacillus* form an important part of the microbial consortia that are active in the biooxidation of sulphide ores in biomining processes, yet very little is known about these industrially important organisms. The study of sulfobacilli, and other biomining organisms, is hampered by the absence of effective gene cloning and inactivation systems. During this study, the groundwork was laid for the development of a genetic system for the genus *Sulfobacillus*.

The plasmid diversity present in industrial and environmental isolates of sulfobacilli was assayed. Plasmids were plentiful in the assayed strains, providing the basis for development of cloning vectors for sulfobacilli. Plasmid DNA isolated from *Sulfobacillus thermosulfidooxidans* strain DSM 9293^T was methylated at *dam* and *dcm* sites. Whether the methylase enzymes responsible for this methylation pattern form part of restriction-methylation systems or only play a regulatory role is unknown, but it does indicate the appropriate methylation state of DNA for the transformation of this strain.

The DNA sequences of three plasmids originating from sulfobacilli were analysed and compared. There was no significant similarity between the three plasmid sequences, indicating diversity in plasmid genetic load and replication mechanisms. Plasmid pSulfBC1 was predicted to replicate via the rolling circle mechanism, while the replication mechanisms of pKara and pTHWX could not be predicted from sequence data.

Two antibiotics, chloramphenicol and tetracyline, were found to be suitable for selection of *Sulfobacillus* transformants. *E. coli – Sulfobacillus* shuttle vectors were constructed using the *Sulfobacillus* plasmid, pKara, as the backbone with a Gram-positive chloramphenicol resistance marker and appropriate elements allowing replication in, and mobilization from, *E. coli.* These shuttle vectors were used in the evaluation of electroporation and conjugation as methods for the delivery of DNA to *Sulfobacillus*.

Transformants of sulfobacilli could not be obtained by either transformation method, although some progress was made towards determining the optimal conditions for both methods. The most promising finding was that cells of *E. coli* and *Sulfobacillus* could be maintained on the same medium for a theoretically sufficient time to allow mating. It is likely that *Sulfobacillus*

transconjugants can be obtained with the right combination of donor, mobilizable vector, selectable marker and treatment to neutralize restriction systems.

OPSOMMING

Lede van die genus Sulfobacillus maak ‘n belangrike deel uit van die mikrobiese gemeenskap wat verantwoordelik is vir die oksidasie van sulfied ertse in die biomynwese, en tog is daar min bekend omtrend hierdie industrieel-belangrike organismes. Die studie van sulfobacilli, en ander biomynwese organismes, word belemmer deur die gebrek aan effektiewe geen-klonering en –inaktivering stelsels. Met hierdie studie is die basis gelê vir die ontwikkeling van ‘n genetiese stelsel vir die genus *Sulfobacillus*.

Die diversiteit van plasmiede teenwoordig in industriële- en omgewings-isolate van sulfobacilli is ondersoek. Plasmiede blyk om algemeen voor te kom in die *Sulfobacillus* stamme wat ondersoek is. Hierdie plasmiede kan dien as basis vir die ontwikkeling van kloneringsvektore vir sulfobacilli. Metielering van *dam* en *dcm* setels is opgemerk op plasmied DNA wat uit *Sulfobacillus* stam DSM 9293^{T} geisoleer is. Dit is onbekend of hierdie metielering deel uit maak van ‘n restriksie-metielering sisteem en of dit bloot betrokke is by regulatoriese funksies. Tog dui dit daarop dat DNA wat gebruik word vir die transformasie van hierdie stam van *Sulfobacillus* ook by hierdie setels gemetieleer moet wees.

Die DNA volgordes van drie *Sulfobacillus* plasmiede is ge-analiseer en vergelyk. Daar is geen beduidende ooreenstemming gevind tussen die DNA volgordes van die drie plasmiede nie, en daaruit het die gevolgtrekking gevloei dat die geen-lading en repliseringsmegansismes van die plasmiede verskil. Plasmied pSulfBC1 maak heel moontlik gebruik van die rollende-sirkel metode van replisering. Die repliseringsmeganismes van plasmiede pKara en pTHWX kon nie uit die DNA volgordes afgelei word nie.

Twee antibiotika, chloramfenikol en tetrasiklien, is geskik bevind vir transformant-seleksie in *Sulfobacillus.* *E. coli* – *Sulfobacillus* pendel-vektore is gemaak deur die *Sulfobacillus* plasmied, pKara, as ‘n basis te gebruik en ‘n Gram-posetiewe chloramfenikol weerstandbiedendheidsmerker, asook DNA elemente wat replikasie in-, en mobilisering vanaf *E. coli* toelaat, daarin te kloneer. Hierdie pendel-vektore is gebruik in die evaluering van elektroporering en konjugasie as metodes vir die DNA oordrag na *Sulfobacillus.*

Transformante van sulfobacilli is nie deur enige van die transformasie metodes verkry nie. Tog is daar vordering gemaak met die bepaling van toestande wat transformasie deur

elektroporering en konjugasie bevorder. Die mees belowende bevinding was dat selle van *E. coli* en *Sulfobacillus* gesamentlik kon oorleef op dieselfde medium vir ‘n tydperk wat teoreties lank genoeg is om konjugasie toe te laat. Transformasie van sulfobacilli mag wel moontlik wees met die korrekte kombinasie van donor, mobiliseerbare plasmied, selekteerbare merker en behandeling om restriksiesisteme te neutraliseer.

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GLOSSARY AND LIST OF ABBREVIATIONS

α	alpha
A	adenosine
A	alanine
aa	amino acids
ARDREA	amplified ribosomal DNA restriction enzyme analysis??
ATCC	American Type Culture Collection
ATP	adenosine 5'-triphosphate
β	beta
bioleaching	solublization of metals by microbial oxidation of ores
biooxidation	microbial oxidation of ores to improve the accessibility of metals for recovery by other processes.
bp	base pair(s)
˚C	degrees Celsius
C	cytosine
C-terminal	carboxyl-terminus
cm	chloramphenicol
Cys	cysteine
D	aspartic acid
Da	Daltons
ddH_20	double distilled water
DIG	dioxigenin-11-dUTP (DIG-dUTP)
DNA	deoxyribonucleic acid

dH_2O	distilled water
dsDNA	double stranded DNA
dso	double strand origin
EDTA	ethylenediaminetetra-acetic acid
g	gram(s)
G	guanine
Glu	glutamic acid
h	hour(s)
H	histidine
His	histidine
Inc	incompatibility group(s)
IR	inverted repeat
jarosite	iron-sulphide mineral
kb	kilobase pair(s) or 1000-bp
kDa	kilodaltons
km	kanamycin
LA	Luria Bertani agar
LB	Luria Bertani broth

M	methionine
M	molar
mA	milli Ampére
mDa	mega Dalton(s)
mg	milligram(s)
MIC	minimal inhibitory concentration
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	milliMolar
M-O	minus origin
mRNA	messenger RNA
MW	molecular weight
µg	microgram
µl	microlitre
NCBI	National Centre for Biotechnology Information
N-terminal	amino-terminus
ng	nanogram
nm	nanometre
OD_{600}	optical density at a wavelength of 600 nanometres
ORF	open reading frame
oriT	origin of transfer
oriV	origin of vegetative replication

p	plasmid
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
pH	potential of hydrogen
RBS	ribosome binding site
RC	rolling circle
RCR	rolling circle replication
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
rRNA	ribosomal RNA
S	serine
s	second(s)
S-layer	surface layer
SD	Shine-Dalgarno
SDS	sodium dodecyl sulphate
SET	sucrose EDTA Tris buffer
SP	signal peptide
spp	species
ssDNA	single stranded DNA
sso	single strand origin
T	thymine
TAFE	Transverse Alternating Field Electrophoresis

TBE	tris-borate EDTA buffer
TE	tris EDTA buffer
TMH	trans membrane helix
Tris	tris (hydroxymethyl) aminomethane
Tyr	tyrosine
UV	ultraviolet
v/v	volume per volume
w/v	weight per volume

CHAPTER ONE
LITERATURE REVIEW

1.1. Biomining

Biomining is the application of microbial activities for the recovery of valuable metals from sulphide ores. A distinction can be made between bioleaching, which refers to the solublization of the metal of interest for later recovery from the leach liquor, and biooxidation, which refers to the pre-treatment of ore to improve the accessibility of the metal of interest (gold) for recovery by cyanidation.

Bioleaching is used in cases where traditional mining practices are not cost effective, for instance where the ore is refractive or of lower grade or where infrastructure elements are limiting. Huge amounts of ores are already being processed via biomining methodologies. For example, in the first year of operation of the whole-ore bio-oxidation facility of Newmont Mining Corporation, 2.4 million tons of gold-containing ore were pre-treated by biooxidation, resulting in gold recovery of 55-60% (Bhakta and Arthur, 2002). Biomining is set to become an even more significant source of metals as sources of high grade ores become depleted. Further pressure to move away from traditional biomining processes come from ever more stringent environmental legislation, favouring the more environmentally friendly biooxidation processes (Rawlings *et al.*, 2003).

The advantages of biomining over traditional mining processes lie mainly in three areas: operational gains, extension of the mineable ore body and environmental friendliness.

Operational gains include the greater simplicity and robustness of operation, coupled with lower operating and capital costs compared to other methods of metal recovery (van Aswegen *et al.*, 2007).

High grade ore bodies that can be exploited with traditional mining methods are being quickly depleted but biomining enables economically feasible use of lower grade or inaccessible ores. The dumps left behind after traditional mining processes can be reprocessed to extract residual metal, ore can be leached *in situ* (Rawlings, 2002) and very low grade ore can be processed.

Biomining processes are in some ways more environmentally friendly than conventional mining methods. Waste streams are liquid rather than gas or dust, and are thus easier to control. Biooxidation does not produce environmentally harmful gaseous emissions such as sulphur dioxide. Because tailings have already been biologically leached, the potential for production of harmful acid mine drainage is reduced. However, the liquid effluent needs to be controlled and / or neutralized. Biomining processed also require less energy than roasting or smelting.

There are three major types of commercial processes involving bacterial leaching of ores: dump or heap leaching; *in situ* leaching; and leaching in stirred tank bioreactors. For dump or heap leaching, ores are crushed and stacked in heaps. Liquid is allowed to percolate through the heap and metals are recovered from the leach liquid. This process is often used for lower grade ores. One example is the, now closed, Lo Aguirre mine near Santiago in Chile (Bustos *et al.,* 1993). For *in situ* leaching, water is injected into fractured ore bodies and extracted from another point. This process is used for lower grade ores or inaccessible sites. Examples include the Elliot Lake sites of Denison mines in Ontario (McCready and Gould, 1989). Leaching in bioreactors is practiced with higher grade ores. Ore is crushed and taken to a fermentation vessel where control of important parameters allows faster and more efficient leaching of metals. However, the cost of setup and operation is higher than for heap leaching and there is a significant cost of cooling since bioleaching reactions are exothermic. An example is the Fairview Mine near Barberton in South Africa (van Aswegen *et al.,* 2007).

1.1.1. The microbiologically facilitated chemistry of biomining

The solublization of metals in biomining processes is due to chemical, rather than enzymatic reactions. However, microbial enzymatic processes regenerate the compounds involved in the chemical attack on the ore.

Two chemical mechanisms for mineral dissolution have been proposed: the thiosulphate mechanism which operates on acid insoluble metal sulphides and the polysulphide mechanism which operates on acid soluble metal sulphides. In the thiosulphate mechanism, ferric (Fe^{3+}) ion attack on the mineral generates thiosulphate as the main intermediate. This is further oxidised to sulphate as the main end-product. Both reactions yield ferrous iron and protons. In the polysulphide mechanism both ferric iron and protons are involved in the

attack on the mineral, generating elemental sulphur (S^0) as the main intermediate. This elemental sulphur may be further oxidised to sulphate through microbial action, also producing ferrous iron (Fe^{2+}) and sulphuric acid (Rawlings, 2005).

The role that biomining bacteria, such as *Sulfobacillus,* have in these processes is to re-oxidise the ferrous iron (Fe^{2+}) that is produced by these reactions to ferric iron (Fe^{3+}) and to produce sulphuric acid (Rawlings, 2005). This ferric iron can then take part in another round of attack on the ore, while the sulphuric acid produced allows the liberated metal ions to stay in solution. The biologically driven reactions, especially oxidation of sulphur, generate considerable heat, accelerating the chemical reactions (du Plessis, 2007).

Biomining bacteria further facilitate the dissolution of minerals by providing a favourable reaction space for the abovementioned chemical reactions. When bacteria attach to the mineral, they produce an exopolysaccharide layer. Within this layer, concentrations of Fe^{2+} and H^+ are much higher than in the bulk fluid (Rawlings, 2005).

The chemical reactions that are involved in biomining processes can take place abiotically, but are greatly accelerated by biological action. At a pH of 2-3, the microbial oxidation of ferrous to ferric iron is 10^5 to 10^6 times faster then the chemical oxidation of ferrous iron (Lacey and Lawson, 1970).

1.1.2. Microorganisms involved in metal solublization from sulphide ores.

Biomining habitats are characterized by low concentrations of organic nutrients, low pH and high concentrations of metal ions (Rawlings, 2002; Dopson *et al.,* 2003). The bacteria that thrive in such environments are necessarily autotrophic or oligotrophic and acidophilic and possess elevated tolerance to toxic metal ions. Since bioleaching and biooxidation reactions are more efficient at higher temperatures, there is a move towards the development of high temperature biomining processes, which require moderately or extremely thermophilic microorganisms for the microbial catalysis reactions.

The most commercially significant bacterial species taking part in biomining processes are from the genera *Acidithiobacillus*, *Leptospirillum*, *Sulfobacillus* and *Sulfolobus*, although the total species diversity present can be much greater (Sampson and Phillips, 2001; Johnson and Hallberg, 2003). Bacteria of the genus *Sulfobacillus* are some of the principal participants in

biomining operations operating at higher temperatures – between 45˚C and 50˚C (Rawlings, 2002).

It is not possible nor desirable to have a defined culture in a biomining situation. The different bacterial species present behave synergistically in the attack on the ore. The species present in a typical biomining operation and their relative abundance are influenced by the type of ore; the type of processing employed and the temperature, pH, O_2 and CO_2 supply, heavy metals, surfactants,organic extracts and presence of predatory protozoa (Bosecker, 1997; Johnson, 1998; Sampson and Phillips, 2001). Complex interrelationships exist between members of biomining consortia which may have positive or deleterious effects on biooxidation or bioleaching effectiveness. Frequently a mixed population results in a more effective and robust process (Johnson, 1998; 2001). Therefore it is important to consider other organisms besides sulfobacilli that may occur in the shared habitat. Only a selection of the most often encountered organisms will be discussed.

The bacteria involved in biomining processes have been placed in three main groups, based on their optimum growth temperatures. These groupings are artificial and the growth ranges of the groups will overlap.

The mesophilic group consists mostly of Gram-negative bacteria such as species of *Acidithiobacillus* and *Leptospirillum,* but also includes some strains of the Gram-positive genus *Sulfobacillus*. These bacteria have an optimum growth temperature of 25 – 40˚C.

The most abundant moderately thermophilic organisms are archaea and Gram-positive bacteria. The moderately thermophilic Gram-positive bacteria include species of *Sulfobacillus, Alicyclobacillus* and *Acidimicrobium* (Norris, 2007). The Gram-negative *Acidithiobacillus caldus,* some strains of *Leptospirillum thermoferrooxidans,* and *Hydrogenobacter acidophilus* are also moderate thermophiles.

The thermophilic grouping consists mostly of Archaea such as *Acidianus, Metallosphaera* and *Sulfolobus* that have optimum growth temperatures between 60˚C and 85˚C (Bosecker, 1997; Logan *et al.,* 2007).

1.1.2.1. Acidithiobacillus

Acidithiobacillus species that are active in biooxidation of ores include *Acidithiobacillus caldus, At. thiooxidans* and *At. ferrooxidans.* Bacteria of this genus were originally included in the genus *Thiobacillus,* which was later found to be polyphyletic. The new genus *Acidithiobacillus* incorporates the highly acidophilic thiobacilli; *At. ferrooxidans*, *At. thiooxidans* and *At. caldus* and is placed phylogenetically near the branch of the β and γ subdivisions of the Proteobacteria (Rawlings, 2002).

At. ferrooxidans is obligately autotrophic and may use ferrous iron or reduced inorganic sulphur compounds as electron donors. It can also use ferric iron as an electron acceptor when reduced sulphur compounds are the electron donors (Rawlings, 2002). *At. ferrooxidans* proliferates in environments where a high concentration of ferrous iron is present and is less well adapted to conditions of high redox potential (higher concentration of ferric than ferrous iron). Therefore it tends to be one of the dominant organisms within ores during dump- or heap leaching, while it has a minor role in stirred tank bioreactors (Rawlings, 2002). *At. ferrooxidans* has pH and temperature optima for growth of 20˚C - 35˚C and pH 1.8-2.0 (Rawlings, 2002). *At. thiooxidans* uses reduced inorganic sulphur compounds as electron donors but, unlike *At. ferrooxidans*, it cannot use ferrous iron as an electron donor. It has similar temperature optima to *At. ferrooxidans*, but lower pH optimum (pH 0.5-5.5). *At. caldus* also uses reduced inorganic sulphur compounds as electron donors but cannot use ferrous iron as an electron donor. This species can tolerate lower pH and higher temperatures than *At. ferrooxidans* and *At. thiooxidans*. *At. caldus* is a moderate thermophile with an optimum growth temperature of 45˚C and can grow mixotrophically using yeast extract or glucose. *At. caldus* is more effective at oxidation of minerals when growing in co-culture with *Sulfobacillus thermosulfidooxidans* or *Leptospirillum ferrooxidans* (Rawlings, 2002).

1.1.2.2. Leptospirillum

The genus *Leptospirillum* lies within the division *Nitrospira.* *Leptospirillum* species are Gram-negative, obligately chemoautolithotrophic and obligately aerobic, vibrio or spiral shaped bacteria. Leptospirilli are highly acid tolerant, with pH optima from 1.3 to 2.0. Leptospirilli have a highly specialised physiology, being reliant on ferrous iron as the only electron donor they can to use. Leptospirilli are the dominant iron-oxidising organisms in continuous-flow biooxidation tanks. They can withstand higher concentration of ferrous and

ferric iron and lower pH than *Acidithiobacillus ferrooxidans*. *Leptospirillum ferrooxidans* is mesophilic, while *L. thermoferrooxidans* and *L. ferriphilum* are capable of growth at 45˚C (Rawlings, 2002).

1.1.2.3. Acidiphilium

Members of the genus *Acidiphilium* are Gram-negative and acid tolerant rather than acidophilic. Most species are heterotrophs, but *Acidiphilium acidophilum* can grow autotrophically, mixotrophically or heterotrophically. Strains of *Acidiphilium* are often found in close association with *Acidithiobacillus ferrooxidans* or strains of *Sulfobacillus* and may promote effective leaching by scavenging organic compounds that are inhibitory to growth of some autotrophs in the bioleaching environment (Rawlings, 2002).

1.1.2.4. Sulfobacillus

Members of the genus *Sulfobacillus* are Gram-positive, spore-forming rods. They can grow autotrophically, heterotrophically or mixotrophically and oxidise iron, sulphur and sulphide compounds. Although most strains of *Sulfobacillus* are moderate thermophiles with temperature optima between 40˚C and 60˚C, strains of *Sulfobacillus montserratensis* have temperature optima in the mesophilic range (Yahya *et al.,* 1999). The sulfobacilli are described in greater detail in section 1.2: "The genus *Sulfobacillus*".

1.1.2.5. Alicyclobacillus

Alicyclobacilli are acidophilic, Gram-positive, spore forming, rod shaped bacteria that share many growth characteristics with sulfobacilli. They form a cluster distinct from species of *Sulfobacillus* when 16S ribosomal DNA sequences or DNA:DNA reassociation studies are considered (Karavaiko *et al*., 2000).

1.1.2.6. Acidimicrobium

Acidimicrobium species are Gram-positive, non-spore-forming moderate thermophiles that are widespread in mineral sulphide mining environments and natural acidic high temperature environments (Atkinson *et al.,* 2000), but are not well studied (Norris, 2007). They tend to be present in higher numbers than sulfobacilli at the lower end of the moderately thermophilic temperature range, while *Sulfobacillus* spp. become more dominant above 55˚C (Brierley,

2003; Cleaver *et al*., 2007; Norris, 2007). They are more effective at fixing CO_2 than *Sulfobacillus* (Clark and Norris, 1996a).

1.1.2.7. Acidianus

Several species of the archaeal genus *Acidianus* oxidise minerals. *Acidianus brierly* can grow autotrophically or heterotrophically and has temperature and pH optima of 70˚C and pH 1.5-2.0. *Ad. infernus* and *Ad. ambivalens* are obligate chemolithotrophs. *Ad. infernus* has an even higher temperature optimum of 90˚C.

1.1.2.8. Metallosphaera

Metallosphaera are aerobic archaea. They can grow chemolithotrophically by oxidising iron or sulphur but also utilise complex organic compounds such as yeast extract. *Metallospaera sedula* can grow at pH 1.0 - 4.5 and at 80-85˚C.

1.1.2.9. Sulfolobus

Sulfolobus metallicus is an acidophilic, thermophilic archaeon, with an optimum growth temperature of about 69˚C and pH optimum between 1.3-1.7. *S. metallicus* can grow autotrophically by oxidising ferrous iron, reduced inorganic sulphur compounds or sulphur minerals (Rawlings, 2002).

1.1.2.10. Ferroplasma

Ferroplasmas are pleomorphic archaea, lacking cell walls. *Ferroplasma acidiphilum* is a mesophilic, acidophilic, obligate aerobe. It oxidises ferrous iron, but not sulphur. *F. acidarmanus* tends to proliferate in environments with very low pH values (Rawlings, 2002).

1.1.3. Improvement of biomining processes

The practice of bioleaching is an ancient technology, having been practiced at Rio Tinto as long ago as the era of the Roman empire but scientific investigation of this phenomenon only started about 50 years ago (Bosecker, 1997). Biomining processes have shown marked improvement since their modern inception. However, there remains considerable potential for improvement by modern science and technology.

In most cases, inoculation of ore is unnecessary as the microflora present on the crushed ore acts as an innate starting culture, except in the case of extremely thermophilic organisms, which may not naturally be present on the ore. Bacteria capable of growing at moderately thermophilic temperatures (up to 50˚C) are randomly distributed in soil and their numbers increase when a rise in temperature occurs (Norris *et al.,* 2002). Adaptation of bacterial cultures to high concentrations of metal ions can be achieved by continuous culturing in the presence of increasing levels of toxic metals such as arsenic, thus selecting for more rapid growers (Sampson and Phillips, 2001; Rawlings, 2005).

Microbes have a remarkable potential for adaptation by selection, however, this potential might be limited by the genetic potential of the starting culture. Therefore, one possible avenue of research has been to investigate the microbial diversity of naturally heated, acidic environments ("bioprospecting"). Such studies might yield starter cultures of bacteria with a higher potential for bioleaching or greater resistance to heavy metals or extremes of pH and temperature.

Establishing optimal conditions for bacterial bioleaching activities is critical, therefore a detailed knowledge of the physiology of biomining organisms is needed (Brierley, 2000). One example was the finding that leaching by *Sulfobacillus*-like organisms caused the pH of the solution to rise, leading to the formation of precipitates which interfere with further leaching. Maintaining the pH at <1.9 led to an increase in soluble copper yield from 22% to 90% (Kinnunen and Puhakka, 2004). Physiological studies have been carried out for many of the microorganisms involved in biomining activities; however, much work remains to be done in this area.

At low mineral concentrations pyrite / arsenopyrite ores are oxidised more rapidly by moderate thermophiles than by mesophiles, and most rapidly by extreme thermophiles. However, commercially viable leaching at even higher temperatures is hampered by the sensitivity of moderate and extreme thermophiles to high mineral concentrations. Therefore, selection of extreme thermophiles capable of withstanding the imposed stresses must be a priority (Clark and Norris, 1996b).

Additionally, molecular biological methods help to extend scientific knowledge about biomining organisms. Unfortunately, genetic tools are mostly absent for biomining organisms. Many research groups are working towards developing molecular methods for the

study and manipulation of organisms involved in biomining processes. Progress to date is summarised in the section 1.3.7 entitled "Progress towards genetic systems for biomining bacteria."

The advent of molecular techniques has accelerated the study and improvement of many industrially important organisms, a notable example being lactic acid bacteria used in dairy fermentations. It would be advantageous to be able to study biomining bacteria using similar molecular techniques. The aim of this study is to further the development of a genetic system for the sulfobacilli, one of the genera of bacteria that play an important role in biomining processes.

1.2. The genus *Sulfobacillus*

Bacteria of the genus *Sulfobacillus* are Gram-positive, rod shaped, spore-forming, moderately thermophilic bacteria that use elemental sulphur, iron and sulphidic minerals as energy sources (Golovacheva and Karavaiko, 1978). Sulfobacilli form an important part of the bacterial consortia involved in biomining processes operating at 45-50˚C (Rawlings, 2002).

1.2.1. Phylogeny

The genus *Sulfobacillus* falls within the low G+C Gram-positive division of the Firmicutes (Baker and Banfield, 2003) and has four recognised species: *Sulfobacillus thermosulfidooxidans* (Golovacheva and Karavaiko, 1978), *S. acidophilus* (Norris *et al.,* 1996), *S. thermotolerans* (Bogdanova *et al.,* 2006) and *S. sibiricus* (Melamud *et al.,* 2003).

S. thermosulfidooxidans originally had two subspecies, namely "*S. thermosulfidooxidans* subsp. *thermotolerans"* (Kovalenko, 1983) and *S. thermosulfidooxidans* subsp. *asporogenes* (Vartanyan *et al.,* 1988). However, *"S. thermosulfidooxidans* subsp. *thermotolerans"* was later reclassified as *Alicyclobacillus tolerans.* Another species that was formerly included in the genus *Sulfobacillus*, "*S. disulfidooxidans,"* was reclassified as *Alicyclobacillus disulfidooxidans* (Karavaiko *et al.,* 2005).

Interestingly, the 5S ribosomal DNA sequence of *S. thermosulfidooxidans* strain VKM B-1269 (synonymous to DSM 9293^T) is very different from the 5S rRNA of all other bacteria (Karavaiko *et al.,* 1990), although the 16S ribosomal DNA sequence of *S. thermosulfidooxidans* DSM 9293^T clusters with that of other proposed sulfobacilli near the root of the low G+C Gram-positive division (Durand, 1996).

A previous, erroneous, 16S rDNA sequence for *S. thermosulfidooxidans* DSM 9293^T obtained from a contaminated culture (Tourova *et al.,* 1994), placed the sulfobacilli and alicyclobacilli in a single cluster. The 16S rDNA sequences determined by Durand (1996) and Tourova and co-workers (1994) differed greatly. However, members of the genera *Sulfobacillus* and *Alicyclobacillus* share many phenotypic characteristics, making them difficult to distinguish by non-molecular methods. Confusion has arisen regarding assignment of isolates to the genus *Sulfobacillus* or *Alicyclobacillus.* A number of researchers and reviewers have commented on the polyphyletic nature of the organisms assigned to the *Sulfobacillus* group

(Norris, 1997; Karavaiko *et al.,* 2000; Baker and Banfield, 2003), but the phylogenetic confusion around the genera of *Sulfobacillus* and *Alicyclobacillus* has now been mostly resolved. The sulfobacilli are grouped in a monophyletic cluster (Karavaiko *et al.,* 2000), although a number of isolates remain to be reclassified. A combination of cultivation techniques and ARDREA (amplified ribosomal DNA restriction enzyme analysis) can be used to distinguish between many groups of acidophilic iron-oxidising microbes and can also be used to divide *Sulfobacillus* species into two groups, the *S. thermosulfidooxidans* / *"montserratensis"* group and the *S. acidophilus* / "*yellowstonensis"* group (Johnson *et al.,* 2005).

1.2.2. Morphology

Cells of *Sulfobacillus* spp. are typically small rods with rounded or tapered ends (Tsaplina *et al.,* 1991) and show a degree of pleomorphism. A range of morphologies may be observed, depending on the growth substrate. *Sulfobacillus* cells may be longer, shorter, thicker or thinner than the average (Tsaplina *et al.,* 1991). Bent forms, branched cells and pairs and chains of cells also occur (Golovacheva and Karavaiko, 1978; Golovacheva, 1979; Tsaplina *et al.,* 1991). Incomplete separation of cells after division leads to the formation of cell aggregates in the form of bent chains, palisades, rings and Y-formations (Golovacheva, 1979). Sometimes, division occurs before the ends of the cells are rounded, giving the appearance of lens shaped apical caps (Golovacheva, 1979). During exponential growth of *S. thermosulfidooxidans* subsp. *asporogenes*, cells divide by binary fission, while during stationary phase, long filament-like cells undergo multiple divisions (Vartanyan *et al.,* 1988).

Spore formation has been demonstrated for most isolates of *Sulfobacillus* (Golovacheva and Karavaiko, 1978; Schutte, 2004), except *S. thermosulfidooxidans* subsp. *asporogenes* (Vartanyan *et al.,* 1988). Spores are round or oval and can be terminal, subterminal or paracentral with swollen sporangia (Golovacheva and Karavaiko, 1978). Spore formation is triggered by nutrient limitation, especially when cells are grown heterotrophically in the presence of yeast extract (Golovacheva, 1979).

Colonies on solid media containing iron appear after two to five days of growth. Colonies are smooth edged and brown on silica-gel media with iron (Vartanyan *et al.,* 1988) and rounded, shining and bright yellow to brown on agar medium (Golovacheva and Karavaiko,

1978) but cream-coloured when tetrathionate is also present (Johnson, 1995). Young colonies are yellow but turn deep reddish brown as the iron is oxidised (Golovacheva and Karavaiko, 1978). Longer incubation leads to a "fried egg morphology" as iron-sulphur precipitates form at the perimeter of the colony.

Sulfobacilli do not have flagella, but some isolates are weakly motile when growing autotrophically on ferrous iron (Norris *et al.,* 1996). *S. thermosulfidooxidans* subsp. *thermotolerans* seems to be non-motile (Kovalenko and Malakhova, 1983).

1.2.3. Cell envelope structures

Sulfobacilli stain Gram-positive or Gram-variable and display a typical smooth Gram-positive cell wall under electron microscopy (Bogdanova *et al.,* 2006). The traditional Gram staining technique gives variable and unreliable results for moderate thermophiles, including sulfobacilli. A lectin-based staining technique can be used to differentiate Gram-positive bacteria (including sulfobacilli) and Archaea from Gram-negative bacteria (Fife *et al.,* 2000). The cell wall is 25-30 nm thick (Suzina *et al.,* 1999) and there is no outer membrane (Golovacheva and Karavaiko, 1978).

Sulfobacillus spp. have an unusual cell membrane composition, characterised by a high proportion of branched chain fatty acids (which are rare in eubacteria) and alicyclic acids (Tsaplina *et al.,* 1994). Other rare fatty acids also occur in the membranes of sulfobacilli. These include ω-cyclohexane acids and ω-cyclohexyl-α-oxyundecanoic acid, linear α-oxyacids and sphingolipids (Tsaplina *et al.,* 1994). The fatty acid profiles of *Sulfobacillus thermosulfidooxidans* differ between strains of the species and depends on the growth conditions (Tsaplina *et al.,* 1994; Karavaiko *et al.,* 2000; Robertson *et al.,* 2002).

Membranes of *Alicyclobacillus* spp. contain similar fatty acids to those of sulfobacilli. *Alicyclobacillus thermotolerans* (previously named *S. thermosulfidooxidans* subsp. *thermotolerans*) also has ω-cyclohexane acids (Kinnunen *et al.,* 2003).

The membranes of *Sulfobacillus* spp. display unusual structural features. The cytoplasmic membrane has numerous slit-like invaginations and multilayered lamellar membrane structures in the form of flat lamellar intramembranes (Suzina *et al.,* 1999; Duda *et al.,* 2001).

An S-layer has been shown to encase cells of *S. thermosulfidooxidans* VKMB-1269 and *S. thermosulfidooxidans* subsp. *asporogenes*, strain INMI-41 (Severina *et al.,* 1995; 1998), but was absent in *S. thermotolerans* (Bogdanova *et al.,* 2006).

Sulfobacillus cells possess fimbriae and slime sheaths by which they attach to surfaces. A microcapsule was observed around cells of *S. thermosulfidooxidans* (Senyushkin *et al*., 1997) and *S. thermosulfidooxidans* subsp. *asporogenes* (Vartanyan *et al.,* 1988). *S. thermosulfidooxidans* cells adhere closely to minerals by way of slime produced preferentially at the side of the cell closest to the mineral. *Sulfobacillus* cells also produce two types of appendages by which they attach remotely to mineral particles (Golovacheva, 1979). This attachment may improve their effectiveness in oxidising ore (Golovacheva, 1979; Edwards *et al.,* 1999). The degree of attachment to mineral particles is influenced by the type of mineral and the type and concentration of the growth substrate of the liquid medium (Sampson *et al.*, 2000).

1.2.4. Physiology

Most strains of *Sulfobacillus* identified so far are moderately thermophilic, however, mesophilic strains have been isolated, for instance "*Sulfobacillus montserratensis*" (Yahya *et al.*, 1999). Two isolates from natural geothermal sites have even higher optimum growth temperatures of 55˚C to 60˚C (Norris, 2007).

Sulfobacilli have a versatile physiology, utilizing a variety of energy and carbon sources, both organic and inorganic. They are acidophilic, facultatively thermophilic, facultatively autotrophic (Golovacheva and Karavaiko, 1978) and facultatively anaerobic (Bridge and Johnson, 1998). *Sulfobacillus* spp. can grow under chemoorganoheterotrophic, chemolithoautotrophic or mixotrophic conditions (Tsaplina *et al.,* 1991). Autotrophic or heterotrophic growth is relatively weak (Tsaplina *et al.,* 1991; 2000). In general chemoorganoheterotrophic growth is even weaker, while mixotrophic growth with complex organic substrates such as yeast extract is the strongest (Tsaplina *et al.,* 1991; Zakharchuk *et al.,* 1994; Krasil'nikova *et al*., 1998; Tsaplina *et al.* 2000).

Sulfobacilli can oxidise iron, sulphur or sulphide minerals, but growth characteristics may vary between species. *S. acidophilus* can be maintained in an autotrophic mode with sulphide minerals as an energy source for several subcultures without the addition of complex organic

substrates, while *S. thermosulfidooxidans* can only be maintained in this mode for two or three subcultures (Norris *et al*., 1996; Karavaiko *et al*., 2001). *S. sibiricus* strain $N1^T$ can utilise Fe^{2+} as its sole energy source, though this mode of growth is also unstable, with only two to three subcultures successfully growing in such media. Likewise, growth under heterotrophic conditions, with yeast extract as the sole carbon and energy source, is also unstable (Karavaiko *et al*., 2001; Zakharchuck *et al.,* 2003), with only a limited number of subcultures being successful. In contrast, mixotrophic growth with iron and yeast extract is stably maintained (Zakharchuck *et al.,* 2003). Sulfobacilli can use organic compounds for growth, but grow best with Fe^{2+} or sulphide minerals as energy source and an organic carbon source (Zakharchuk *et al.,* 1994).

Sulfobacilli are capable of metabolising various sulphur compounds. *S. thermosulfidooxidans* and *S. acidophilus* can oxidise sulphide minerals and elemental sulphur to sulphuric acid. The activity of various sulphur-metabolising enzymes in *S. sibiricus* was assayed under aerobic and microaerobic conditions with elemental sulphur or sulphide minerals as substrates (Krasil'nikova *et al.,* 2004). Elemental sulphur could be oxidised under aerobic conditions and also under reduced aeration.

Another source of energy for *Sulfobacillus* strains is the oxidation of iron. While autotrophic growth on iron is possible, it is weak (Johnson *et al*., 2005), and can only be maintained for a few subcultures (Zakharchuck *et al.,* 2003). Instead, oxidation of iron is mostly used as an energy source, while organic substrates are used as a carbon source. Ferrous iron oxidation by *S. thermosulfidooxidans* subsp. *asporogenes* is strongest under mixotrophic conditions (Tsaplina *et al.,* 2000).

The reduction of iron can also be employed to generate energy. Some, but not all, strains of sulfobacilli are capable of iron reduction and cycling between iron oxidation and reduction may be observed (Johnson *et al.,* 1993; Bridge and Johnson, 1998). Both organic and inorganic electron donors can be used when reducing ferric iron and under anaerobic conditions, ferric iron can be used as a sole electron acceptor instead of oxygen (Bridge and Johnson, 1998). The reduction of iron may have significant impact on biomining operations. Within biooxidation heaps, local depletion of oxygen could occur, which may result in increased reduction of ferric iron. This has the effect of reducing the local redox potential and slowing bioleaching rates. On the other hand, reduction of ferric iron could alleviate the

formation of "passivation layers" – accumulation of secondary ferric minerals precipitates which interfere with mineral leaching (Johnson, 2001).

Carbon fixation by species of *Sulfobacillus* is possible, but rather limited. This is shown by the instability of autotrophic growth (Zakharchuk *et al.,* 1994; Tsaplina *et al.,* 2000), and enhanced growth and mineral dissolution when provided with an organic carbon source or in co-culture with more efficient CO_2 fixers (Tsaplina *et al*., 2000). Likewise, sulfobacilli use organic molecules as energy sources, but only to a limited degree. Fixation of inorganic carbon by sulfobacilli was demonstrated by the presence of low, but detectable amounts of ribulose bisphosphate carboxylase activity in the *S. thermosulfidooxidans* type strain (Tsaplina *et al.,* 1991) and the fixation of radioactively labelled carbon dioxide by *S. thermosulfidooxidans* subsp. *asporogenes* (Vartanyan *et al.,* 1988). The fixation of CO_2 proceeds via the Calvin cycle (Tsaplina *et al.,* 2000). *S. thermosulfidooxidans* cells were able to fix carbon under autotrophic, heterotrophic and mixotrophic conditions. CO_2 fixation under autotrophic conditions in the presence of iron and thiosulphate is the strongest (Zakharchuk *et al.,* 1994).

Organic substrates may be used as energy source, but this process in not efficient, once again resulting in weak and unstable growth. The basis for this is believed to be the absence of key enzymes of the TCA cycle and glyoxylate shunt. The TCA cycle of *S. thermosulfidooxidans* and *S. thermosulfidooxidans* subsp. *asporogenes* is incomplete and is used mainly in a biosynthetic role (Zakharchuk *et al.,* 1994; Tsaplina *et al.,* 2000; Zakharchuk *et al.,* 2003). The closely related alicyclobacilli have a complete TCA cycle (Karavaiko *et al.,* 2005).

The utilization of sulphide minerals (of importance for the biomining industry) is also affected by the availability of organic carbon sources. Sulfobacilli oxidise pyrite (an iron-sulphur mineral) under autotrophic conditions. Addition of simple carbohydrates does not significantly alter pyrite oxidation rates, while the addition of complex organic substances like yeast extract increases oxidation rates by an order of magnitude (Tsaplina *et al.,* 1991).

1.2.5. Distribution and significance

Sulfobacilli are globally distributed in acidic environments, both natural and man-made. Isolates related to sulfobacilli have been detected in acidic biofilms growing on the walls of the Grotto del Fiume, a natural cave system in Italy. The microorganisms here may play an

important role in speleogenesis (cave formation) by the production of sulphuric acid which dissolves the limestone. The microbial biofilms were also shown by carbon isotope studies to be the primary producers in the cave ecosystem, forming organic matter chemoautotrophically (Vlasceanu *et al.,* 2000). Sulfobacilli isolated from a natural hydrothermal site on the island of Montserrat included a mesophilic species and one with a higher maximum growth temperature (65˚C) (Atkinson *et al.,* 2000).

Sulfobacilli are commonly isolated from biomining operations and ore deposits (Golovacheva and Karavaiko, 1978; Vartanyan *et al.,* 1988; Robertson *et al.,* 2002) and acid mine drainage (Bond *et al.,* 2004; Morales *et al*., 2004; Tyson *et al*., 2004). *Sulfobacillus* spp. form part of complex microbial consortia that are involved in biomining operations (Battaglia-Brunet *et al.,* 2002). The net products of the consortium are sulphuric acid and ferric iron. Within this consortium, the role of the sulfobacilli is to accelerate leaching reactions by creating a reaction space and oxidising iron and sulphur to produce sulphuric acid (Golovacheva and Karavaiko, 1978). Growth on elemental sulphur, sulphidic minerals or sulphidic ores produces rapid acidification (Golovacheva and Karavaiko, 1978; Vartanyan *et al.,* 1988) which is important for leaching of acid-labile ores and the maintenance of metal ions in solution. Interestingly, Kinnunen and Puhakka (2004) observed an increase, rather than decrease, in pH during the oxidation of chalcopyrite by *Sulfobacillus*-like bacteria. Biomining processes are increasingly moving towards high temperature processing, as higher metal leaching rates are attained with the use of moderate or extreme thermophiles as compared to mesophiles (Witne and Phillips, 2001). The ability to form spores may enable sulfobacilli to survive fluctuations in temperature that may occur locally within biooxidation heaps (Robertson *et al.,* 2002).

Bioleaching environments are characterised by high concentrations of metal ions, which are toxic to cells. Gram-positive moderate thermophiles (*S. thermosulfidooxidans* and *S. acidophilus*) are less tolerant than mesophiles (*Thiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*) of toxic metal ions such as nickel (Ni^{2+}), cobalt (Co^{2+}) and copper (Cu^{2+}) (Sampson and Phillips, 2001). *S. thermosulfidooxidans* tolerates copper (Cu^{2+}) concentrations of 6 mM (Vartanyan *et al.,* 1990); zinc (Zn^{2+}) concentrations of 43 mM, and nickel (Ni^{2+}) concentrations of 5 mM. This compares unfavourably with the metal ion tolerance levels of Gram-negative acidophilic bacteria (Dopson *et al.,* 2003).

Sulfobacillus strains YTF1 and THWX were inhibited by arsenic concentrations between 10 and 15 mM (added as sodium arsenate) (Jordan *et al.,* 1996). This sensitivity does impact on the metal leaching efficiency. The presence of arsenopyrite within ores significantly reduced leaching rates of iron and copper by *Sulfobacillus* strains YTF1 and THWX, while leaching rates by a mesophilic mixed culture was not significantly affected. Despite inhibition by arsenic, the leaching rates by moderate thermophiles (sulfobacilli) remained higher than leaching rates by mesophiles (Jordan *et al.,* 1996). Increasing resistance to arsenic and other heavy metal ions might improve leaching rates of moderate thermophiles even more.

One way to mitigate the sensitivity of *Sulfobacillus* strains to high levels of toxic metal ions would be to introduce plasmids carrying metal resistance determinants into sulfobacilli. This could theoretically be accomplished by using recombinant plasmids and techniques such as electroporation. However, in the biomining setup it is not possible to contain genetically modified organisms within the system. Therefore, the ideal situation would be to find naturally occurring, wide host range plasmids that carry such metal resistance determinants and are conjugative or mobilizable. Using a "starter culture" of bacteria that harbour such a suite of adaptive plasmids to serve as an enriched genetic pool on which the bioleaching consortium may draw, could speed up the selection of a more efficient bioleaching community.

1.3. Genetic systems

A genetic system is a collection of tools and methods that allows the study and manipulation of the genome of an organism. When examining the metabolism of an organism, more rapid progress is made when genetic and biochemical studies are used in compliment. For example, creating gene knockouts can help to elucidate enzymatic or regulatory pathways (Salyers *et al.,* 2000). Even when mutants can be created by chemical or UV mutagenesis it is often useful to compliment such a knockout by transforming the mutant with a vector carrying the wild type gene (Guilhabert *et al.,* 2006) or to transfer the mutant gene to a "clean" background to eliminate the complications of multiple mutations. Note that the term transformation is used here to refer to any mechanism of DNA or RNA transfer to living cells, and not only to the specific case of DNA uptake by naturally competent bacteria. The advent of molecular techniques has accelerated scientific progress in almost all branches of biology - and microbiology is no exception. The ability to manipulate the genetic material of organisms has lead to a deeper understanding of many biological processes and often to novel biotechnological applications.

The possibility for strain- or process improvements is greatly enhanced when a genetic system is available. For example, transforming a biomining organism with multiple heavy metal resistance systems might extend the usefulness of that strain for mining operations. Perhaps less controversial are the process optimisations that would result from a better understanding of bacterial physiology, as exemplified by the process optimizations for the commercial production of amino acids by coryneform bacteria (Hermann, 2003).

A genetic system comprises at least the following components: a method for obtaining colonies originating from a single cell of the relevant organism; a selectable marker; a suitable vector; a DNA delivery system and a DNA extraction protocol.

1.3.1. Selectable markers

The selectable marker is a DNA fragment which confers an easily recognised phenotype to the transformed organism. Antibiotic resistance markers are the most commonly used markers for genetic manipulation. There is a good range to choose from and most are easy to work with. The *cat* gene (which encodes a chloramphenicol resistance determinant) from the

Staphylococcus aureus plasmid, pC194, has been used as a selectable marker in the transformation of many Gram-positive bacteria (Aleshin *et al.,* 1999).

Resistance determinants to inorganic ions are considered to be more suitable for selection in acidic media, since most antibiotics are inactivated under the acidic conditions in which biomining bacteria thrive, but inorganic metal ions are stable at low pH (Rawlings and Woods, 1988; Kusano *et al*., 1992; Ghosh *et al*., 1997). Metal resistance vectors have been developed to transform a variety of bacteria, from *Pseudomonas* (Sanchez-Romero *et al.,* 1998) to various plant associated bacteria of the α-, β-, and γ-Proteobacteria (Taghavi *et al.,* 2001) to strains of *Arthrobacter* (Margesin and Schinner, 1997) and *Acidiphilium multivorum* (Ghosh *et al.*, 1997).

Differential markers such as the *lac*Z gene (encoding the β-galactosidase enzyme) or *gfp* (encoding the green fluorescent protein) allow identification of transformants, without inhibiting non-transformed cells.

1.3.2. Vectors

Vectors are natural or constructed DNA molecules that are used as vehicles for the propagation or transfer of genes or DNA fragments. The chosen vector must be able to replicate in the transformed organism, integrate in the chromosome, or allow expression of some gene before being eliminated. The most commonly used vectors in molecular biology are plasmids, transposons and bacteriophages. The simplest vectors to work with, and the most widely used, are plasmids.

1.3.3. Plasmid vectors

Many plasmid vectors have been developed for a variety of uses in molecular biology. Unfortunately most of these plasmids have been developed for a handful of "workhorse" organisms such as *Escherichia coli* or *Bacillus subtilis*, and will not replicate in other microbes - hence the need for development of novel vectors when designing a genetic system for a new bacterial species.

Plasmids are versatile and easy to use vectors. The features that are of importance to a molecular biologist are the mode of replication, host range, stability, copy number,

incompatibility group, selectable markers, size, restriction enzyme sites and conjugative- or mobilizable ability of the plasmid. Plasmids that are stably inherited are preferred above those that are easily lost from a population. The incompatibility group of a plasmid is of importance when two or more types of plasmids must be resident in the same cell. The copy number of the plasmid is of interest when the expression of a cloned gene must be controlled.

The host range of a plasmid is of great importance to a molecular biologist. When designing a shuttle vector, the host range of the plasmid must include at least the two organisms that it must shuttle between. Two different strategies are often employed: using a plasmid with two separate replicons, or using a broad host range replicon that can function in both organisms. An example is plasmid pC194, which can replicate both in *Staphylococcus aureus* and *B. subtilis* (Horinouchi and Weisblum, 1982). On the other hand, when a suicide vector is being constructed, the host range of the plasmid need not include the target organism.

One solution to obtaining an autonomously replicating plasmid vector is to find a plasmid or phage that is indigenous to the target organism and use that as the basis for a cloning vector. The second strategy is to use a broad host range plasmid such as RSF1010.

1.3.3.1. General introduction to plasmids

Plasmids are extrachromosomal DNA species that replicate independently of the chromosome. Most plasmids are circular double stranded DNA molecules, but double stranded linear plasmids also exist. Plasmids can vary in size from a few hundred nucleotides (Harriott *et al.,* 1994) to hundreds of Kilobases (Barnett *et al.,* 2001). The genetic content varies enormously, but generally plasmids encode accessory-, rather than essential functions. Plasmid-encoded genes include plasmid replication and partitioning genes; conjugation or mobilization systems; resistance determinants to antibiotics, metals or phages; virulence determinants; catabolic functions; or other acquired genes (Stillwell *et al.,* 1995). Such plasmid-encoded genes can often confer a considerable selective advantage to the host cell within a specific environmental niche, but are not essential for host survival under all conditions.

Plasmids are often divided into incompatibility (Inc) groups. When two plasmids are incompatible, they cannot be stably maintained in the same host cell, leading to the eventual loss of one of the plasmids. Plasmid incompatibility is caused by the inability of the

replication and / or partitioning machinery of the plasmids to distinguish between the two replicons leading to an inability to correct stochastic fluctuations in copy number (Novick, 1987). Therefore, if the copy number of one plasmid type drops, no compensating increase in replication will take place as the copy number of the other, indistinguishable, plasmid-type is still high. Incompatibility groups are specified by the prefix "Inc" followed by the group name, for example plasmid RSF1010 belongs to the IncQ incompatibility group. When two or more plasmid vectors must be maintained in one cell, for instance when doing complementation assays, they must belong to different incompatibility groups.

Plasmids are important vehicles in the horizontal gene pool. They may acquire DNA sequences from their hosts in a number of ways: through recombination with the chromosome(s), other plasmids or phages, or by integration of transposable elements, phages or insertion elements. As plasmids are often transferred between hosts, these acquired genes may then be further disseminated to other organisms.

Plasmids have varying host ranges. Narrow host range plasmids can only replicate in a single species or a few closely related species, while broad host range plasmids may replicate in a wider range of hosts. Some broad host range bacterial plasmids can successfully replicate in bacteria of different genera, or may even function in representatives of another kingdom of life (Goursot *et al.,* 1982). There are many factors that influence the natural host range of a plasmid, but the most critical is a plasmid's ability to replicate in the host. Other factors that confer an advantage to host range are plasmid stability systems, conjugation functions and anti-restriction systems (del Solar *et al.,* 1996). The host range of a plasmid is often restricted because it must rely on certain host factors for its replication. For instance, replication of plasmid ColE1 is dependent on the DNA polymerase I of *E. coli* (del Solar *et al.,* 1988). In contrast, broad host range plasmids are less restricted by interactions with host factors. Broad host range plasmids may initiate plasmid replication independently of host initiation factors and/or be adaptable to host factors (del Solar *et al.*, 1996). One example of host independent replication is found on plasmid RSF1010, which encodes many of the protein products that are necessary for initiation and elongation of replication (del Solar *et al.*, 1996).

Of the many functions that are encoded by plasmids the most important are those that have to do with plasmid inheritance. This includes both vertical inheritance from mother cell to daughter cell (replication and partitioning functions), and horizontal inheritance from a donor

cell to a recipient cell (conjugation or mobilization functions). Conjugation is considered under "DNA delivery methods" in section 1.3.5.2 and replication will be discussed briefly in this section.

Two main types of replication mechanisms are known for circular plasmids: rolling circle replication and theta replication.

1.3.3.2. Rolling circle plasmid replication

Plasmids that use the rolling circle method of replication are most often small (<10 kb). Rolling circle replicating (RCR) plasmids are commonly found in Gram-positive bacteria, but have also been reported in Gram-negative bacteria (Galli and Le Blanc, 1977; Yasukawa *et al.,* 1991; Holtwick *et al.,* 2001) and Archaea (Zhou *et al.,* 2004a). RCR plasmids are characterized by the generation of single stranded DNA intermediates during replication (te Riele *et al.,* 1986a; 1986b) and detection of ssDNA is diagnostic of this method of replication.

RCR plasmids consist of a number of interchangeable gene modules with most genes transcribed in the direction of replication (Meijer *et al.,* 1998; Projan and Novick, 1988). Two of these gene modules are essential for plasmid replication and are thus always present. The first of these modules contains the *rep* gene encoding the replication initiation protein; and its associated double stranded origin (*dso*) just upstream or within the coding sequence of the *rep* gene (Khan, 1997). The second replication module contains the single stranded origin (*sso*) which serves as the initiation site of lagging strand synthesis. The *sso* is not located adjacent to the *rep* (Meijer *et al.,* 1998) but is normally located just in front of the *dso*.

The Rep proteins of RCR plasmids show conservation at the amino acid level (Guilhabert *et al.,* 2006) and have several motifs in common with mobilization proteins of conjugative plasmids (Ilyina and Koonin, 1992). Three conserved motifs have been identified in RCR Rep proteins. Motif I, the triple C motif, has three conserved cysteine amino acids. Motif II, also known as the two-His motif, consists of the following amino acid residues, HisHydrHisHydrHydrHydr, where His represents histidine and Hydr represents a bulky hydrophobic amino acid residue. The conserved histidine residues may be involved in binding of Mg^{2+} and Mn^{2+} ions which are required for Rep function. This motif occurs in all RCR Rep proteins, and also in mobilization proteins (Ilyina and Koonin, 1992). Motif III contains a conserved tyrosine which is involved in binding of the Rep protein to the nicked

plasmid DNA (Ilyina and Koonin 1992). This tyrosine is indispensable for activity of the replication protein (Noirot-Gros *et al.,* 1994).

The double stranded origins (*dso*'s) of RCR consist of a *bind* site, where the Rep protein initially binds to the plasmid DNA, and a *nick* site, where the Rep protein creates a strand specific cut. The *nick* sites show greater conservation within plasmid families, while the *bind* site is more variable. This is reflected in the conservation of nicking domains of Rep proteins within RCR plasmid families, and the greater divergence in site-specific DNA binding domains of these proteins (Khan, 2005).

The single stranded origins show very little sequence conservation even within plasmid groups. The single stranded origin (*sso*) is usually located just upstream of the *dso*, in a non-coding region of DNA. These regions are characterised by a high degree of secondary structure, which is important for functioning of the *sso* (Khan, 2005; Guilhabert *et al.,* 2006).

The plasmid encoded Rep protein initiates replication of the RCR plasmid by recognising and binding to the *bind* site in the double stranded origin and then creating a single strand nick within the conserved *nick* site. The newly created 5' free DNA end becomes covalently bound to the catalytic tyrosine residue within the active site of the Rep protein monomer. A host-encoded helicase unwinds the plasmid DNA and host-encoded single stranded binding proteins coat the displaced single stranded DNA. The 3' hydroxyl end of the DNA at the *nick* site serves as a primer for leading strand replication by host–encoded DNA polymerase III. Replication continues around the plasmid until the replication fork again reaches the *dso.* This yields one single stranded plasmid circle and one relaxed double stranded plasmid circle. The conversion of the ssDNA circle to dsDNA is dependent on a functional *sso* and host encoded proteins. Host RNA polymerase and DNA polymerase I produce and extend an RNA primer, from which host DNA polymerase III produces the second (lagging) strand of plasmid DNA. Host ligase joins the DNA ends and host DNA gyrase restores supercoiling to both double stranded plasmid circles (Khan, 2005).

1.3.3.3. Theta replication

Theta replicating plasmids are common in Gram-negative bacteria, but also occur in Gram-positive bacteria and Archaea (del Solar *et al.,* 1998). Replication of theta-replicating plasmids produces characteristic θ-shaped replication intermediates as seen by electron

microscopy. Theta replication can be unidirectional or bidirectional and can be initiated at one or more sites. Plasmids using this type of replication mechanism are less interrelated than the rolling circle replicating plasmids, but certain general themes can be identified nonetheless and these are discussed briefly.

Certain DNA sequence features have been identified that are often found within the origins of replication (*oriV*) of theta replicating plasmids. These include specific binding sequences for replication initiation proteins, AT-rich regions, dnaA boxes where host encoded DnaA proteins bind, *dam* methylation sites and binding sequences for host factors like the integration host factor. These sequence features are not universally present in the *oriV* of all theta replicating plasmids. For instance, the binding sites for Rep proteins may or may not be iterons (directly repeated DNA sequences). Some theta-replicating plasmids lack plasmid-encoded Rep proteins and rely on host factors for replication initiation (del Solar *et al.*, 1988).

Replication of theta replicating plasmids is initiated by localized melting of the DNA strands at specific sequences within the plasmid origin of replication (*oriV*). This is mediated either by initiation proteins (plasmid encoded Rep and/or host DnaA) and/or by transcription through the (*oriV*). A primase synthesises a complimentary RNA primer on one of the DNA strands within the melted region and replication of the plasmid DNA proceeds by elongation from the RNA primer. Plasmid replication requires the proper assembly of a replisome complex; which consists of at least the host DnaA protein and the plasmid encoded Rep (if present), and the DNA polymerase III holoenzyme, and contains other host factors as well. Once DNA replication has been initiated, it continues until the replisome complex reaches a replication termination signal (del Solar *et al.,* 1998). DNA synthesis is continuous on the leading strand and discontinuous on the lagging strand.

Strand displacement replication is a variation of the theta method of replication. This method of replication is employed by the IncQ type plasmids. In strand displacement replication, plasmid encoded proteins with helicase, primase and initiator activities are responsible for initiation of plasmid replication. This means that IncQ plasmids are largely independent of host-encoded proteins for the initiation of plasmid replication, which may contribute to the wide host range of these plasmids (del Solar *et al*., 1996).

1.3.4. Important considerations for vector choice

In general, theta replicating plasmids are preferred above RCR plasmids as vectors in molecular biology. Many plasmids replicating via the rolling circle mechanism are less suitable as cloning vectors as they are prone to deletions and / or rearrangements of inserted DNA due to the production of highly recombinogenic single-stranded DNA intermediates during replication (Gruss and Ehrlich, 1989).

Also, many RCR plasmids are not stably inherited by daughter cells of a heterologous host during division (Lee *et al*., 1998; Biet *et al*., 1999). The main cause of such instability is the failure of the *sso* to function in a heterologous host, or the disruption of the *sso* sequence by the insertion of foreign DNA during cloning (Guilhabert *et al.,* 2006). Despite these problems, many successful cloning vectors have been developed from RCR plasmids (Baldini *et al.,* 1999; Biet *et al.,* 1999; Nakashima and Tamura, 2004; Zhou *et al.,* 2004b).

One advantage of using RCR replicons for shuttle vectors is that they often have relatively broad host ranges. For instance, derivatives of the *S. aureus* RCR plasmid pC194 were able to replicate in many Gram-positive bacteria (Fischer *et al*., 1984; Augustin and Götz, 1990; Pulido-Vega *et al*., 1991; Shiroza *et al*., 1997) and even an eukaryote, *Saccharomyces cerevisiae* (Goursot *et al.,* 1982). An RCR plasmid from *Lactobacillus fermentum*, pLF1311 was able to replicate in strains of *Lactobacillus, Lactococcus, Enterococcus,* and *Bacillus* (Aleshin *et al.,* 1999). Plasmid pBM02 replicates in *Lactobacillus* spp., *B. subtilis* and *E. coli* (Sánchez and Mayo, 2003). The wide host range of many RCR plasmids is probably a consequence of the host independent initiation of leading strand replication.

1.3.5. DNA delivery methods

During transformation the genetic material of a cell is altered by introduction of foreign DNA into the cell which may then be expressed to yield a new phenotype. The most challenging element in constructing a genetic system is the introduction of DNA into a novel organism, because multiple variables need to be exactly right at the same time and positive controls are not yet available (Salyers *et al*., 2000).

Biological methods of transformation include natural transformation, conjugation and transduction. Physical methods include electroporation, sonoporation and biolistics.

Chemical/physical methods include PEG- or liposome- mediated protoplast transformation and $LiCl_2$ / $CaCl_2$ transformation. These methods will be discussed in greater detail below in sections 1.3.5.1 to 1.3.5.8.

1.3.5.1. Natural transformation

Natural transformation is the uptake of naked DNA from the extracellular environment by the action of bacterially encoded transformation machinery, which processes the DNA, brings it across the bacterial membrane(s) and cell wall and establishes the incoming DNA in the host genome.

Competence is the highly regulated physiological state during which the natural transformation systems are active. Very few bacteria have been reported to be naturally competent but these species are widely distributed across phylogenetic and ecological divides. It is therefore expected that many more bacterial species can become naturally competent under the right conditions (Dubnau, 2000).

Three types of natural competence systems are known (Fujise *et al.,* 2004), the Gram-positive *Streptococcus-Bacillus* system (Dubnau, 1999), the Gram-negative *Haemophilus-Neisseria* system (Lorenz and Wackernagel, 1994.), and the *Helicobacter pylori* system, which is more similar to type IV secretion systems involved in conjugation (Smeets and Kusters, 2002).

Although some bacteria may take up large DNA fragments or whole circular plasmids, most often the DNA is digested into shorter fragments before uptake by natural competence. This complicates the use of natural transformation as a tool for genetic manipulation, since plasmid vectors must then re-form in the cytoplasm or integrate in the genome (Salyers *et al*., 2000).

Most naturally competent bacteria do not discriminate between DNA fragments for uptake. Bacteria of the genera *Neisseria* and *Pasteurella* are exceptions. They preferentially take up DNA fragments that contain recognition sequences that are common in their genomes, GGCCGTCTGAA for *Neisseria* and AAGTGCGGT or ACAAGCGGT for *Pasteurella* (Dreiseikelman, 1994).

Competence is usually not constitutive, but rather a short lived, highly regulated, physiological state expressed under defined environmental conditions. The conditions

inducing competence vary between species but competence is often induced under adverse conditions (Lorenz and Wackernagel, 1994; Tortosa and Dubnau, 1999).

The competence mechanism of *B. subtilis* will be shortly discussed as a model for natural transformation in Gram-positive bacteria

Regulation of competence development in *B. subtilis* is a affected by a range of stimuli. These stimuli are integrated by regulation of the transcription factor ComK Five transcription factors bind to the *comK* promoter and at least three proteins interact with the ComK protein. ComK is responsible for activation of the rest of the competence regulon. A summary of the main regulation pathways is depicted in Figure 2.1.

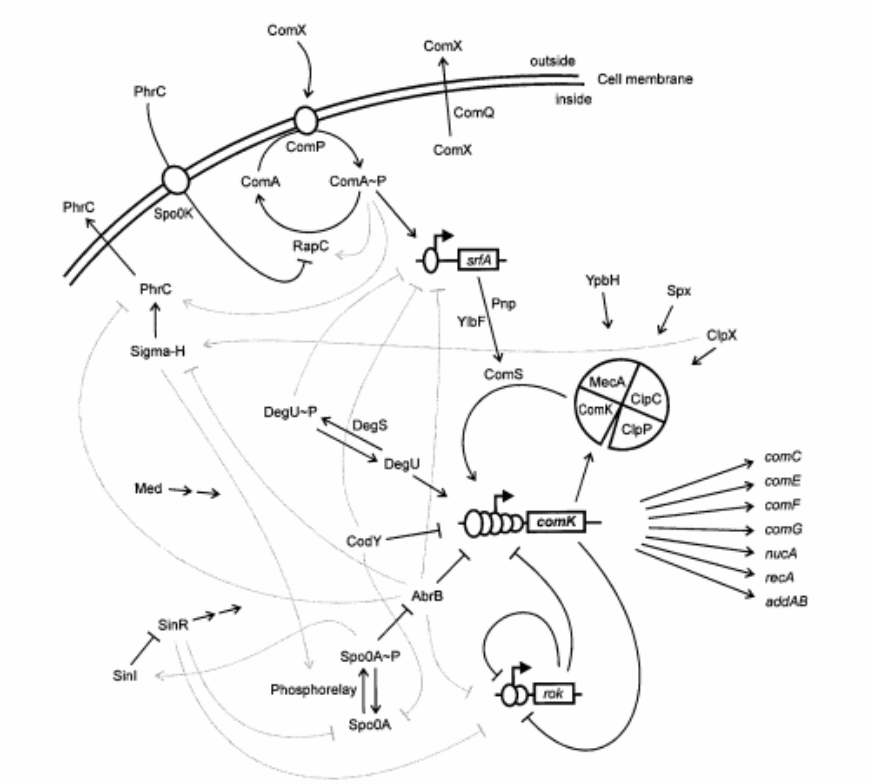

Figure 2.1 Regulation of competence development in *B. subtilis*.

The inputs affecting competence development in *B. subtilis* are depicted schematically. Arrows indicate activation, while closed lines indicate inhibition. Grey lines indicate additional cross-talk between pathways. The main regulatory pathways are explained in the text. This figure is reproduced from Hamoen *et al.,* (2003).

ComK activates its own expression by binding to the *comK* promoter which initiates a positive feedback loop, although binding of ComK to its own promoter is inefficient.

Upon reaching the stationary phase of growth, a percentage of *B. subtilis* cells become competent for DNA uptake, while the rest of the population activates different adaptive mechanisms. The DegS/DegU two component regulatory system provides the basis for one such developmental decision. Unphosphorylated DegU induces competence by increasing the efficiency of ComK binding to the *comK* promoter. When phosphorylated by DegS, DegU activates the production of degradative enzymes instead.

Competence development is initiated only at high cell densities. Two quorum sensing pheromones, ComX and PhrC, are produced, accumulate to threshold levels in the medium and ultimately induce the competence phenotype. ComX induces expression of *comK* via the two component regulatory system comprising of ComA and ComP. Phosphorylated ComA induces the expression of ComS within the *sfrA* locus, which in turn protects ComK from degradation by ClpP. PhrC mediated activation of ComK expression additionally requires the oligopeptide permease Spo0K and functions by inhibiting RapC, a negative regulator of *sfrA* expression.

Several other intracellular and extracellular conditions which affect competence are monitored by a phosphorelay system that culminates in the phosphorylation of the transcription factor Spo0A. These conditions include the metabolic and replication status of the cell and the makeup of the growth medium. SpoA acts through repression of AbrB, another repressor of *comK*.

Once the competence phenotype has been established, the first step of DNA uptake during natural transformation is binding of DNA to the outside of the cell, followed by breaking of DNA into smaller fragments. One strand of DNA is then degraded as the other is internalized. Once inside the cell, DNA is integrated into the genome by homologous recombination.

In *B. subtilis* four classes of proteins are involved in the transport of DNA across the cell envelope. These four classes are class I (ComEA), class II, (ComC, and ComGA to ComGG), class III (ComEC) and class IV (ComFA). ComEA is the primary DNA receptor and is indispensable for binding of double stranded DNA to the cell. The second class of late

competence proteins are necessary for transport of DNA across the cell wall. These proteins are termed PSTC proteins (pilus, secretion, twitching motility and competence proteins). The class III protein, ComEC, forms an aqueous pore in the membrane, while the class IV protein, ComFA, translocates the DNA though the aqueous pore (Dubnau and Provvedi, 2000).

Although some components of the competence machinery remain unknown, a coherent model for the mechanism of natural transformation has been proposed (Chen *et al.,* 2005). Pre-pilin proteins (comGC, comGD, comGE and comGG) are processed by a peptidase (comC) and assemble into a pseudo-pilus which is long enough to traverse the cell envelope. Double stranded DNA binds to the membrane-localised protein ComEA and to the pseudo-pilus via a hypothetical DNA binding protein. The integral membrane nuclease NucA randomly cleaves the dsDNA into smaller fragments. Thereafter, a single strand of the DNA is passed though the water-filled membrane pore created by a ComEC multimer. The other DNA strand is degraded by an unknown nuclease. Transport of the single stranded DNA though the membrane is aided by a helicase/translocase enzyme (comFA), the retraction of the pseudo-pilus which forces the DNA though the membrane pore, and single-stranded DNA binding proteins that act as a "Brownian ratchet mechanism." This means that the single stranded DNA can enter the cell via Brownian motion, but cannot again cross the membrane after binding of the single stranded DNA binding proteins (Dubnau and Provvedi, 2000)

1.3.5.2. Conjugation

Conjugation is the direct replicative transfer of DNA between cells in close contact, mediated by donor-encoded multiprotein complexes. An organism that has been transformed by conjugation is often not regarded as being genetically modified. In fact, conjugation is seen as a food-grade process. Many dairy starter strains that have been transformed by conjugation are used in commercial dairy fermentations (Coffey and Ross, 2002). Importantly, the plasmid(s) used in such a process, must be natural, unmodified plasmids. This may be of importance if strain improvement for biomining processes is attempted because containment of a genetically modified biomining organism would be impossible due to the open, non-sterile nature of the processes.

Conjugative plasmids are self-transmissible, encoding all the elements necessary for their transfer from a donor cell to a recipient cell. Mobilizable plasmids can also be transferred between cells, but require some elements to be supplied by a conjugative plasmid residing in

the same cell. Plasmids can be mobilized by conduction or donation. During conduction, a non-conjugative plasmid is transferred by forming a cointegrate with the conjugative plasmid. During donation, the mobilized plasmid is transferred separately from the conjugative plasmid.

Conjugative transfer of plasmids can occur between organisms that are phylogenetically very distant from each other (Table 2.1, Table 5.1, and Table 5.2). The success of the transfer event is limited by the ability of the introduced plasmid to become established in the new host by replication or integration, rather than by the transfer process.

Table 2.1 Examples of inter-kingdom conjugation

Donor	Recipient	Reference
E. coli	*Saccharomyces cerevisiae*	Heineman and Sprague (1989)
E. coli	Animal cells	Yoshida *et al*., 1997
E. coli	Mammalian mitochondria	Yoon and Koob, 2005
Agrobacterium tumefaciens	Sea urchin embryos	Bulgakov *et al*., 2006
A. tumefaciens	Plant cells	Buchanon-Wollaston *et al*., 1987

The greatest differences between conjugation in Gram-positive and Gram-negative bacteria are found in the initial establishment of cell contact. For most Gram-positive conjugation systems, the mechanism of achieving close cell to cell contact is unknown (Grohmann *et al*., 2003). The processes that follow thereafter share the same broad mechanistic principles in Gram-negative and Gram-positive systems, although the level of DNA homology between different systems may be very small (Zechner *et al.,* 2000). An overview of the main components of conjugation systems is discussed below.

Two functional complexes are required for conjugative plasmid transfer, the mating pair formation system (mpf) and the DNA transfer and replication system (Dtr or relaxosome) (Zechner *et al.,* 2000). The mating pair formation system mediates the contact between the donor and recipient cells while the DNA transfer and replication system is responsible for processing of DNA at the origin of transfer. The two complexes are connected via a coupling protein (Grohmann *et al.,* 2003).

In Gram-negative conjugation systems, the initial cell contact is brought about by an extracellular filament, called a conjugative pilus, encoded by the mpf system of the conjugative plasmid. The pilus of the donor cell makes contact with an unknown receptor on the cell surface of the recipient. The cells are brought together by retraction of the pilus caused by depolymerization of the pilin subunits (Ou and Anderson, 1970). Once the cell surfaces of the mating pair are in contact, the association is stabilised by an unknown mechanism. The exact nature of the channel through which plasmid DNA is transferred remains unknown.

During conjugation, single-stranded plasmid DNA is transferred to the recipient cell. A plasmid encoded relaxase protein introduces a site- and strand-specific nick at the nick site of the origin of transfer (*oriT*) and also recircularises the plasmid DNA after transfer (Yang *et al*., 2007).

1.3.5.2.1. Gram-positive conjugation systems

Four types of conjugative elements have been identified in Gram-positive bacteria: broad host range plasmids, pheromone responding plasmids, conjugative transposons, and plasmids from mycelium forming *Streptomyces* (Zechner *et al.,* 2000). These systems are not as well-understood as the model Gram-negative systems.

1.3.5.2.2. The RP4 system as a model for Gram-negative conjugation systems

RP4 is a wide host range IncP type plasmid. The relaxosome protein complex is assembled at the origin of transfer of the conjugative plasmid. Both plasmid-encoded and host-encoded proteins participate in this complex. Besides the relaxase, TraI, three proteins are required for the formation of a stable relaxosome: TraJ, TraI and TraH. The first step in the assembly of the relaxosome is the binding of TraJ to its palindromic recognition site within the *oriT*. Next, TraI joins the complex by binding both to TraJ and to DNA. TraH stabilizes the relaxosome by binding to TraJ and TraI. TraK binds as a multimer to plasmid DNA in the *oriT* region causing the DNA to be bent around the TraK multimer. The DNA relaxase TraI nicks one DNA strand at the *nic* sequence within the *oriT* and becomes covalently bound to the free DNA end. It has been proposed that the transfer-replication reaction is similar to the replication of rolling circle replicating plasmids(Grohman *et al.,* 2003).

The mating pair formation complex is membrane associated. Proteins of this complex are needed for pilus formation and the establishment of conjugative junctions. TrbC is the pilin subunit; TraF is needed for pilin processing to a mature form. TrbB assists in the assembly of the mating pair formation complex. TrbE is an NTPase and is associated with the inner membrane. It is involved either in the proper assembly of the mating pair formation complex or provides energy for the transfer of DNA. TrbH is located in the outer membrane and anchors the mpf complex in the outer membrane. TrbN breaks down the cell wall locally, to provide a channel for other mpf complex components. TrbK prevents mating between two donor cells. In this system, TraG is the coupling protein that mediates interaction between the relaxosome and the mating pair formation system (Grohman *et al.,* 2003).

1.3.5.3. Transduction

When transduction is used as a transformation method, the life cycle of a bacteriophage is exploited to act as a vehicle for DNA delivery. Phages recognise specific receptors on the cell surface (Dreiseikelmann, 1994) and their host range is therefore restricted to organisms expressing that receptor.

Phages are effective transformation vectors but a suitable phage must first be isolated. It seems unlikely that bacteriophages that infect biomining bacteria are common, since no biomining process has been halted by phage infections in the manner of "stalled" milk fermentations and the hostile environment in which biomining bacteria exist would seem to preclude the existence of bacteriophages. However, a few phages that infect acidophilic and thermophilic organisms have been found (section 1.3.7 "Progress towards genetic systems for biomining bacteria")

1.3.5.4. Electroporation

Electroporation is the creation of transient membrane pores by application of a brief, high intensity electrical pulse, enabling the entry of macromolecules such as DNA or proteins, which would otherwise be excluded.

The process by which membrane pore formation occurs is not well understood and only a brief synopsis of the main mechanism is given here. When the potential difference across the cell membrane exceeds its dielectric strength, the membrane is disrupted and pores are

created. The dielectric strength is the maximum electric field strength that an insulating material, in this case the cell membrane, can withstand without failure of insulation. At the point of failure, electrons are accelerated through the membrane at sufficient velocity to knock more electrons free. This results in a conductive path through an otherwise non-conductive material. The interaction of water dipoles with the electric potential difference at the water/lipid interface contributes to membrane pore formation. This localised current disrupts the cell membrane causing the formation of water-filled transmembrane pores lined with lipid headgroups. Importantly, most membrane pores are quickly resealed by the re-orientation of the lipid molecules of the membrane. If this was not the case, cells could not survive the electroporation procedure. As it is, many cells are killed during a typical electroporation process (Weaver and Chizmadzhev, 1996; Tieleman, 2004).

Organisms that have been transformed by electroporation include all domains of life, from Archaea to Gram-positive and Gram-negative bacteria, yeasts and fungi, insects, vertebrate tissues and plants (Table 2.2).

Table 2.2 Examples of the diversity of organisms that have been transformed by electroporation.

Organism	Description	Reference
Acidithiobacillus ferrooxidans	Gram-negative biomining bacterium. Acidophilic, thermophilic.	Kusano *et al.,* 1992.
Various Gram-positive spp.	Gram-positive rods and cocci from marine-, freshwater- and terrestrial environments, with widely ranging nutritional needs	Table 4.1
Bombyx mori	Silkworm	Thomas, 2003
Ipomea batatas	Sweet potato	Mitchell *et al.,* 1998
Rodents	Post implantation rodent embryos	Osumi and Inoue, 2001
Birds	Chicken embryos	Nakamura and Funahashi, 2001.

Very high transformation frequencies, up to 10^9 *Escherichia coli* transformants per microgram of DNA, are possible using electroporation (Calvin and Hanawalt, 1988; Dower *et al*., 1988), compared to 10^5 to 10^6 transformants per microgram DNA for chemical transformation. Many parameters can affect the electroporation frequency, and these parameters can vary considerably. Knowledge of optimal conditions for one bacterial strain is not necessarily transferable to related strains and some strains remain untransformable by electroporation despite considerable effort. Therefore, optimisation of the procedure requires much trial and error.

The factors affecting electroporation efficiency include growth conditions; the nature and preparation of cells; the vector(s) employed; electroporation pulse parameters; electroporation buffer; recovery medium and selection of transformants after electroporation.

In general, the membranes of cells in the exponential phase are permeablized more efficiently. The size of the vector normally has no effect on electroporation efficiency below 30 kb. Above this limit, electroporation efficiency decreases.

The electrical parameters determined by the electroporation device include the field strength, capacitance, parallel resistance and time constant. The field strength is measured in kilovolts per centimetre ($kVcm^{-1}$). It is determined by the magnitude of the externally applied voltage, divided by the gap between the electrodes. Increasing the field strength increases the number and size of pores in the lipid membrane. Higher field strengths generally increase the transformation frequency, but at the cost of greater cell death. The optimum field strength is dependent on the species and strain being electroporated (Belliveau and Trevors, 1989; Marcus *et al.,* 1990; Gilchrist and Smit, 1991; Table 4.1). Gram-positive bacteria generally require higher voltages than Gram-negative bacteria for successful electroporation (see section "The nature and preparation of cells for electroporation.")

The time constant is a measure of the duration of the electrical pulse and is displayed as milliseconds (ms). It is affected both by the capacitance of the capacitor and the total electrical resistance (Ohms) of the circuit (this includes internal resistance of the machine, the resistance of parallel resistor and resistance of the sample). Capacitance is the ratio of charge to potential across an insulating membrane and is expressed as micro-Farad (μF). The time constant can be varied by changing the composition of the electroporation buffer or the parallel resistor selected on the electroporator. The optimum time constant must be

determined empirically for each type of organism, though in general a shorter time constant results in higher cell survival, but reduced transformation efficiency, while longer time constants can increase the transformation efficiency at the cost of lower survival rate (Gilchrist and Smit, 1991).

Once optimised electroporation protocols have been established, electroporation delivers better reproducibility than most other methods. The size and distribution of electropores can be accurately controlled by pulse parameters, and the method is simple and fast to implement. Recipient cells usually require no special treatments and can in most cases be frozen for future use.

1.3.5.5. Sonoporation

Sonoporation is a relatively new technique that was first applied to eukaryotic cells as an alternative to electroporation (Pepe *et al*., 2004). Ultrasound is used to induce oscillation of encapsulated micro-bubbles, which can make holes in cell membranes though cavitation or sheer stress, and thus enable delivery of macromolecules into cells. This technique has been used to transform *Fusobacterium nucleatum*, a Gram-negative anaerobe (Han *et al*., 2007).

Sonoporation causes less cell death than electroporation, while allowing significant (>20%) delivery of fluorescent macromolecules (Han *et al*., 2007). This may improve the efficiency of transformation, as more of the cells that take up DNA will survive.

1.3.5.6. Biolistic transformation

Biolistic transformation has mainly been used for transformation of eukaryotic cells. However, it has also been applied with success to Gram-positive bacterial cells (Shark *et al*., 1991). During biolistic transformation, DNA-coated micro-projectiles are driven into cells. Although not yet widely used, this method is fast and technically simple in comparison to methods such as protoplast transformation.

1.3.5.7. Production of competent cells by treatment with divalent cations

Induction of competence by treatment of cells with divalent cations (often referred to simply as “chemical transformation” or “heat-shock transformation”) is a standard method of

transforming *E. coli* for routine cloning experiments. A few other Gram-negative bacteria can be transformed using this method, but it is not effective for intact Gram-positive bacteria.

Preparation of cells for transformation by chemically induced competence includes several critical factors. A richer growth medium produces faster growth and also greater transformation efficiency (Tu *et al.,* 2005). Cells must be in the early logarithmic growth phase and must be kept cold. Prolonged incubation, up to 24h, at low temperature in the presence of ions of calcium, magnesium or lithium improves transformation efficiency (Tu *et al.,* 2005). The exact function of these ions has not been unequivocally established, but a number of effects have been proposed. The positively charged ions may neutralize the negative charges on the bacterial cell membrane and the exogenously added DNA. Chloride ions entering the cell are accompanied by water molecules following the concentration gradient. This causes swelling of the cells, which is important for transformation, although the mechanism by which this encourages DNA uptake is unknown (Tu *et al.,* 2005). Addition of DMSO or PEG 8000 before the heat shock step may increase efficiency by up to 300 times (Tu *et al.,* 2005). Cryotransformation is a variation of chemical competence where cells are rapidly frozen in the presence of plasmid DNA and then thawed (Stepanov *et al*., 1990).

Chemical transformation using calcium chloride is simple, fast, yields good transformation frequencies, requires no special equipment, allows storage of competent cells and is repeatable for most *E. coli* strains. Transformation frequency also diminishes with increasing plasmid size. This technique also has limited scope, in that the diversity of organisms that can be transformed using this method is much more limited than for techniques such as electroporation.

1.3.5.8. Protoplast transformation

Protoplasts are produced by the chemical or enzymatic removal of the bacterial cell wall. The production of protoplasts is necessary for polyethylene glycol- and liposome-mediated transformation, and is often combined with electroporation as well.

The use of protoplasts rather than intact cells is an effective strategy for transformation, but is technically challenging and reproducibility is problematic. Recovery of colony forming units after polyethylene glycol (PEG) mediated protoplast transformation is challenging, as the lysozyme treatment, incubation with PEG, washing, and dilution of cells before regeneration

of the cell wall all result in a reduction in the viable cell count (Cue *et al*., 1997). Excessive cell killing during electroporation of protoplasts may sometimes negate the benefits of removing the cell-wall barrier to DNA entry.

1.3.6. Barriers to transformation

There are many barriers to the introduction of DNA into bacteria. These include the physical structures of the cell envelope, secreted or intracellular nucleases, and failure of DNA to integrate, replicate or express in the new host.

1.3.6.1. Cell envelope structures

The bacterial cell envelope can consist of multiple layers, including the cell wall, cell membrane, S-layer and capsule. One or more hydrophobic lipid membranes are always present and are effective at excluding hydrophilic molecules such as DNA. This barrier is overcome by the creation of transient membrane pores (electroporation or sonoporation); by exploiting biological DNA translocation methods (natural transformation, conjugation or phage transduction); or by chemical alterations (polyethylene glycol or divalent cation mediated transformation).

The thick cell walls of Gram-positive bacteria hinder transformation in the case of conjugation and electroporation. The barrier effect of the cell wall can be mitigated by the inclusion of cell-wall weakening agents in the growth media, such as penicillins (Trieu-Cuot *et al.,* 1993), glycine (Holo and Nes, 1989; Cruz-Rods and Gilmore, 1990) or DL-threonine (van der Lelie *et al.,* 1988).

In addition to the cell wall and cell membrane, many Gram-positive and Gram-negative bacteria as well as Archaea possess S-layers. An S-layer is a membrane composed of proteins and glycoproteins that is attached to the outer membrane of Gram-negative bacteria, or to the cell wall of Gram-positive bacteria. An intact S-layer reduced the efficiency of electroporation in *Caulobacter* strains (Gilchrist and Smit, 1991). Strains that did not produce S-layer proteins, or where the S-layer proteins did not attach efficiently to the cell surface, were transformed by electroporation with a maximum efficiency ten times higher than strains that possessed a functional wild type S-layer.

1.3.6.2. Nucleases

Intracellular restriction endonucleases are a strong defence against incoming foreign DNA (Berndt *et al.,* 2003). This is the case for natural transformation (Berndt *et al.,* 2003; Iwai *et al.,* 2004); divalent cation mediated chemical transformation (Reid *et al.,* 1982; Wu *et al.,* 2001), conjugation (Shäfer *et al.,* 1990; Trieu-Cuot *et al.,* 1993; Waldron and Lindsay, 2006) and electroporation (Lefrançois and Sicard, 1997; Vischi and Marchetti, 1997).

Extracellular nucleases also impair transformation by electroporation (Marcus *et al.,* 1990; Kawagishi *et al.,* 1994; Vischi and Marchetti, 1997; Péant and LaPointe, 2004), osmotic shock (Marcus *et al.,* 1990) and $CaCl_2$ mediated chemical transformation (Focareta and Manning, 1991).

Various methods exist for circumventing nuclease barriers to transformation. Strains with low or absent nuclease activity can be selected (Poluektova *et al.,* 2004; Acetto *et al.,* 2005; Bjornsdottir *et al.,* 2005; Waldron and Lindsay, 2006). Nuclease-deficient mutant strains may be constructed (Focareta and Manning, 1991; Berndt *et al.,* 2003). Intracellular nucleases may be temporarily inactivated through heating (Reid *et al.,* 1982; Shäfer *et al.,* 1990; van der Rest *et al*., 1999) and extracellular nucleases may be inactivated by incubation with EDTA or PEG (Vischi and Marchetti, 1997). Nucleases may be removed from the periplasmic space by mild osmotic shock (Kawagishi *et al.,* 1994).

Alternatively, the transforming DNA may be modified by methylation, either by passage through a suitable methylating host strain (Berndt *et al.,* 2003), or by incubation with cell free extracts in the presence of EDTA, which inhibits the endonuclease, but not the methylase (Donahue *et al*., 2000; Kwak *et al.,* 2002; Acetto *et al.,* 2005).

In the case where a methylation-dependent DNA restriction system is present in the recipient bacterium, some protection from restriction can be obtained by isolating the transforming DNA from an appropriately chosen *Escherichia coli* methylation mutant or a starved *E. coli* culture (Ankri *et al.,* 1996).

Salyers *et al.* (2000) proposed that DNA entering a cell through conjugation may escape degradation by restriction endonucleases because such DNA exists in a single stranded form when entering the recipient. This view is supported by experimental evidence: strains of

Streptomyces albus harbouring the *Sal*I enzyme could be transformed by conjugation with plasmid pTO1 which has a *Sal*I recognition site (Voeykova *et al.,* 1998) and transformation efficiency of *E. coli* DH5α harbouring a cloned nuclease from *Vibrio vulnificus* was reduced when electroporation or chemically induced competence were employed, but was unaffected when conjugation was employed (Wu *et al.,* 2001).

However, conjugated plasmid DNA is not protected from nucleases in all cases. For example, conjugation frequencies were still limited by the native restriction enzyme in *Clostridium difficile*, being reduced to undetectable levels when four or more of the recognised restriction sites were present on the incoming DNA (Minton *et al.*, 2004). The *Sau*I type I restriction system in *S. aureus* strains is responsible for low conjugative transfer frequencies, nontransformable phenotype and resistance to bacteriophage attack. The one strain (*S. aureus* RN4220) that was transformable by conjugation with *E. coli* had a mutation in the *sau1hsdR* gene, coding for the *Sau*I restriction enzyme (Waldron and Lindsay, 2006).

1.3.6.3. Delayed growth during plasmid establishment

Within some plasmid-host combinations, the establishment of a plasmid in a host may lead to retardation of growth (Kirby *et al.,* 2002). This slow growth phenotype may be mediated by a mechanism analogous to plasmid stability systems that retard cell division when plasmid copy numbers are low (Ogura and Hiraga, 1983). It is therefore important to allow ample time for development of transformant colonies on selective plates. When transformant colonies eventually develop they can be expected to grow more normally when restreaked on fresh selective media.

Alternatively, the plasmid may be unable to replicate in the host, and colony development follows only in rare cases where a host mutation allows the replication of the introduced plasmid (Kirby *et al.,* 2002).

1.3.6.4. Failure of DNA establishment

As the amount of DNA sequence identity between the donor and recipient decreases, so does the likelihood of integration of donor DNA by homologous recombination (Berndt *et al.,* 2003). Therefore, when homologous recombination is required, the amount of sequence

diversion between the incoming DNA and the resident DNA can determine transformation frequency.

In some cases, the uptake of DNA by natural transformation is regulated by the recognition of a specific nucleotide sequence (Dreiseikelman, 1994). This effectively limits natural transformation to the uptake of DNA from the same or closely related species.

In the case of replicating vectors, failure of any component of the replication system of the vector could preclude successful establishment of the transforming DNA.

1.3.7. Progress towards genetic systems for biomining bacteria

Prerequisites for the development of a genetic system are the ability to cultivate single colonies of the organism on solid media; the availability of suitable vectors and selectable markers; and method(s) for introducing DNA into the organism to be transformed. Few of the bacteria in the biomining environment have a usable genetic system. The available systems or parts thereof will be discussed shortly.

Solid media for the cultivation of most biomining bacteria have been developed (Johnson, 1995); allowing selection of clonal lineages and therefore transformants (Schultheiss and Schüler, 2003). One problem is that many of the autotrophic acidophilic bacteria require an underlay of heterotrophic acidophiles to allow growth on solid media (Johnson, 1995). This complicates selection of transformants, as the bacteria used in the underlay may be sensitive to the selectable marker employed.

The use of antibiotic resistance determinants as selectable markers for biomining bacteria is complicated by the acidic, metal-rich growth medium, which rapidly inactivates most antibiotics. Some progress toward overcoming this barrier has been made by developing media that supports growth at slightly higher pH (Liu *et al*., 2001) or by using resistance to heavy metal ions instead of resistance to antibiotics for selection of transformants (Peng *et al*., 1994b; 1994c; Ghosh *et al.,* 1997). Arsenic resistance determinants, which may be used as selectable markers, have been isolated from *Leptospirillum ferriphilum* (Tuffin *et al*., 2006). A Tn21-like transposon conferring resistance to arsenate and arsenite has also been reported in *Acidithiobacillus caldus* (Tuffin *et al.,* 2005).

Various plasmids that could serve as the basis for replicating DNA vectors have been detected in acidophilic bacteria. Several plasmids have been observed in *Acidithiobacillus acidophilus,* (Davidson and Summers, 1983; Quentmeier and Friedrich, 1994), *Acidithiobacillus ferrooxidans* (Martin *et al.,* 1981; Holmes *et al*., 1984; Rawlings *et al.,* 1984; Domini *et al*., 1997; Rawlings, 2001) and *Acidithiobacillus caldus* (Gardner *et al.,* 2001). Some of these plasmids have been further characterized and / or propagated in *Escherichia coli* (Holmes *et al*., 1984; Rawlings *et al*., 1984; Domini *et al*., 1998; Gardner *et al*., 2001; van Zyl *et al*., 2003).

Two plasmids have been isolated from the *Leptospirillum ferrooxidans* ATCC 49879 (Coram *et al*., 2005).

Five plasmids ranging in size from 6.3 kb to 255 kb were detected in *Acidiphilium cryptum* (Quentmeier and Friedrich, 1994). A plasmid that conferred resistance to Zn^{2+} when transformed into *Acidiphilium multivorum* and *E. coli* was isolated from an *Acidocella* strain (Ghosh *et al.,* 1997). Plasmids originating from *Acidiphilium symbioticum* similarly conferred resistance to Zn^{2+} and Cd^{2+} in *E. coli* (Mahapatra *et al.,* 2002). Plasmids conferring metal resistance phenotypes are especially useful in the biomining setting, firstly because of the suitability of metal resistance determinants for selectable markers in an acidic environment, and because they may be used for the improvement of bacterial biomining strains.

Phage outbreaks, such as those that frequently halt lactic acid fermentations, have not been reported for biomining operations. This implies that phages are not common in biomining environments. Yet phages infecting other extreme thermophiles and acidophiles have been detected (Table 2.3). One reason for the apparent resistance of biomining bacteria to phage attack may be that bacteriophages are inactivated in the low-pH biomining environment (Ward *et al.,*1993), yet some phages isolated from alkaline hot springs survived for 24h at pH 3-13 (Yu *et al*., 2006). Even when phages are rapidly inactivated in the growth medium of their host, they may still be maintained as lysogens, while continuously producing free phage without killing the host. These free phages then have a short time to encounter and infect a new host (Ward *et al.,* 1993). Therefore, although no phage transduction systems have yet been reported for bacteria involved in biomining, it is reasonable to continue searching for such phages.

Table 2.3 Examples of phages infecting acidophilic or thermophilic microbes

Phage name	Host name	Host description	Reference
SNDV	*Sulfolobus* strain STH3/1	Extremely thermophilic, acidophilic archaeon	Arnold *et al.,* 2000
ϕAc1	*Acidiphilium*	Acidophilic, heterotrophic Gram-negative bacterium	Ward *et al.,* 1993
φNS11	*Bacillus acidocaldarius*	Thermophilic, acidophilic, Gram-positive bacterium	Sakaki and Oshima, 1976; Sakaki *et al.,* 1977a; 1977b; 1979.
Myovirus P78-77, Tectivirus P78-76 Inovirus PH75	*Thermus thermophilus*	Extreme thermophile	Yu *et al*., (2006)
φYS40	*Thermus thermophilus*	Extreme thermophile	Sakaki and Oshima, 1975
Acm1	*Acetobacter methanolicus* MB 58/4	Acidophilic facultative methylotroph	Kiesel and Wünsche, 1993

Methods for the introduction of DNA into cells have been developed for a very few biomining bacteria. A single isolate of *At. ferrooxidans,* out of 30 strains tested, was transformed at a very low frequency (200 colonies per milligram DNA) by electroporation (Kusano *et al.,* 1992) and up to 10^4 transformants per µg DNA could be obtained by electroporation of strains of *Acidiphilium* (Glenn *et al.,* 1992; Inagaki *et al.,* 1993).

Conjugative transfer of plasmids to acidophilic iron and sulphur oxidisers has been far more fruitful than electroporation. Several species and strains of *Acidithiobacillus* have been transformed by conjugation (Jin *et al.,* 1992; Peng *et al.,* 1994*;* Liu *et al.,* 2000; 2001; Kotze, 2006).

Less work has been done on other members of biomining consortia. Conjugation between *E. coli* and *Acidiphilium* spp. has been demonstrated (Glenn *et al.,* 1992; Quentmeier and Friedrich, 1994). Matings between *Acidiphilium* strains and *E. coli* S17-1 (pKR415) or *E. coli* S17-1 (pLAFR3) produced 10^{-5} to 10^{-9} transconjugants per recipient, dependent on the

recipient strain (Glenn *et al.,* 1992). Metal resistance plasmids in *Acidocella* (acidophilic heterotroph) strains could be transferred to *Acidiphilium multivorum* and *E. coli* (Ghosh *et al.,* 1997).

With the tools available (selective solid media, integrative or replicating plasmid vectors and transformation methods) a few successes have been achieved. A *recA* mutant of *At. ferrooxidans* has been constructed by marker exchange mutagenesis, using conjugation as the DNA transfer method (Liu *et al.,* 2000). Enteric arsenic resistance genes have been transferred to and expressed in *At. ferrooxidans* (Peng *et al.,* 1994c). Enhanced resistance to arsenite has been conferred to an *At. caldus* strain by introduction of a mobilizable plasmid harbouring a transposon-borne arsenic resistance operon (Kotze *et al*., 2006). The *E. coli* phosphofructokinase was expressed in *At. thiooxidans* (Tian *et al.,* 2003).

Despite the successes mentioned above, transformation of any biomining organism remains a major challenge, as the efficiencies of the existing transformation systems are still very low, and most strains of biomining bacteria remain untransformable. The tools needed to introduce DNA into cells and to create gene knockout mutants are still missing for most biomining organisms (Valenzuela *et al.,* 2006).

There is resistance to using genetically modified organisms for industrial and food applications and genetically modified organisms cannot be contained within biomining operations, since these processes are open and non-sterile (Rawlings, 2002). One way to avoid this problem is to use natural conjugative plasmids that carry desirable traits. This strategy has been used for the improvement of phage resistance in lactococcal dairy starter cultures for the industrial production of cheddar cheese, while avoiding the stigma of the genetic modification label (O'Sullivan *et al.,* 1998).

1.3.8. Developing a genetic system for the genus Sulfobacillus

The aim of this project was to construct a genetic system for the genus *Sulfobacillus*. The availability of such a system would facilitate the study of this interesting and industrially important bacterium.

At the start of this project, the DNA sequence data pertaining to sulfobacilli was limited to a small collection of sequenced integron-associated chromosomal genes (Ghauri *et al.,* 2003)

and the partial or unpublished complete sequences of three small plasmids (Joubert, 2002; Prof Paul Norris, pers. comm.). Recently, the chromosomal arsenic resistance operon of *Sulfobacillus thermosulfidooxidans*, strain DSM 9293^T has been sequenced and characterized (van der Merwe, 2007). Therefore, clear targets for homologous recombination or vector construction were scarce.

The cell envelope structures of sulfobacilli have been investigated (section 1.2.3), giving an indication of the types of physical obstacles that had to be overcome to introduce DNA into the cytoplasm of *Sulfobacillus* cells. Nothing was known of the presence or otherwise of DNA restriction or methylation systems which would present further barriers to DNA establishment.

The strategy for development of a genetic system for the sulfobacilli thus had to start from the very beginning. Strains of *Sulfobacillus* were obtained or isolated and their reaction toward antibiotics was evaluated to determine suitable antibiotics for transformant selection. *Sulfobacillus* strains were also screened for plasmids that could serve as the basis for cloning vectors. Plasmid diversity was assessed by sequence comparisons and candidate cloning vectors were constructed. Finally, two of the most widely used and successful transformation methods, electroporation and conjugation, were used to transform strains of *Sulfobacillus* with plasmid DNA.

Therefore the objectives of this study were as follows:

Assay strains of *Sulfobacillus* for the presence of plasmids.

Analyze and compare the sequences of three plasmids from sulfobacilli and identify the replication mechanisms and accessory genes of these plasmids.

Construct cloning vectors containing an appropriate selectable marker and DNA elements necessary for the replication in, and mobilization from, *E. coli*.

Attempt to introduce plasmid DNA into *Sulfobacillus* cells by electroporation and conjugation.

CHAPTER TWO
PLASMID SURVEY OF SULFOBACILLI

2.1. Aim

The aim of this part of the study was to add local isolates to our collection of *Sulfobacillus* strains and to investigate the plasmid diversity present in these and foreign strains, with the goal of using the plasmids as vectors for molecular biology.

2.2. Introduction

When a cloning vector is needed for a new bacterial species, a good source of replicons exists in the native plasmids of the bacterium in question (Sreenivasan *et al.,* 1991; Salyers *et al*., 2000). It is useful to know whether plasmids are present in a strain since analysis of putative transformants is simplified and plasmid incompatibility may be avoided by using plasmid free strains (Kusano *et al*., 1992). Furthermore, knowledge of the methylation status of plasmids in the native host is helpful when choosing *E. coli* host strains for preparation of shuttle vectors so that restriction barriers to transformation can be avoided.

The presence of large plasmids can be overlooked, even in well characterised bacteria, due to the difficulty in isolating intact forms with standard plasmid isolation protocols (Pedraza and Díaz Ricci, 2002). In-gel (Barton *et al.,* 1995) and in-well (Eckhardt, 1978; Plazinski *et al.,* 1985; Pedraza and Díaz Ricci, 2002) lysis of cells is effective in visualising very large plasmids (Rousseaux *et al.,* 2002).

The characterisation of very large plasmids is made difficult by the complex behaviour of supercoiled forms during electrophoresis. The method of Barton and co-workers (1995) attempted to avoid this problem by the linearization of supercoiled plasmid forms by digestion with S1 nuclease. S1 nuclease has a high degree of specificity towards single stranded DNA, but can introduce breaks in double stranded DNA, RNA and DNA-RNA hybrids at high enzyme or salt concentrations (S1 Nuclease, Certificate of analysis, Fermentas life sciences). The buffer developed by Barton *et al.* (1995) has been optimised to minimise the secondary activity of S1 nuclease. Under these buffer conditions, S1 nuclease acts at sites where plasmid supercoiling has caused DNA strands to pull apart locally, creating a short stretch of single stranded DNA. The result is that a large proportion of plasmid DNA is converted to a linear form, while chromosomal DNA remains mostly intact. Regions of

supercoiling within the chromosome may also be attacked in this way, but the number of chromosomal fragments generated is small relative to the amount of linearised plasmid DNA.

The electrophoretic mobility of nucleic acid species under different electrophoresis conditions must be considered for analysis of plasmid profiles. On standard horizontal agarose gels with agarose concentrations ranging from 0.4% to 0.8% and a variety of running buffers, intact and damaged chromosomal DNA can enter the gel matrix and is observed as a diffuse band, while plasmid bands are more sharply defined. Supercoiled forms of megaplasmids migrate behind the chromosomal DNA, while smaller plasmids migrate ahead of the chromosome (Hansen and Olsen, 1978; Anderson and McKay, 1983; Quentmeier and Friedrich, 1994). The electrophoretic mobility of supercoiled plasmids is the greatest, followed by linear forms and finally open circular forms (Birnboim and Doly, 1979; Pedraza and Díaz-Ricci, 2002).

When pulsed field gel electrophoresis is employed, the relative mobility of nucleic acid species is altered relative to the situation where a constant electric field is applied. During Transverse Alternating Field Gel Electrophoresis (TAFE), intact chromosomal DNA (Schwintner *et al.,* 1998) and relaxed forms of large plasmids (Stillwell *et al.,* 1995) do not enter the gel matrix. However, fragmented chromosomal DNA does migrate into the gel (Schwintner *et al.,* 1998; Cleasson *et al.,* 2006). The relative order of plasmid forms is also altered. In a pulsed field system, linear forms of megaplasmids migrate ahead of damaged chromosomal material (Shimizu *et al.,* 2001) and supercoiled plasmid forms (Stillwell *et al.,* 1995; Schwintner *et al.,* 1998; Cleasson *et al.,* 2006). Open circular (OC) megaplasmids do not enter the gel matrix although the open circular forms of smaller plasmids can do so (Schwintner *et al.,* 1998; Cleasson *et al.,* 2006).

A further factor that may affect the electrophoretic mobility of DNA species is the method of DNA isolation, which may affect the purity of the product. Purified plasmids have a higher migration rate than those from direct lysis in agarose even when the electrophoresis conditions are the same (Schwintner *et al.,* 1998).

In-gel lysis allows visualization of plasmids, but purification of such plasmid bands from gels is not trivial, especially if plasmids are very large, or present in small quantities. Therefore, plasmids need to be purified away from chromosomal DNA and other contaminants in a liquid medium.

Preparative separation of plasmid and chromosomal DNA relies on differences in behaviour between the two DNA forms. These differences include the ability to renature after alkaline denaturation (Birnboim and Doly, 1979; Ish-Horowicz, 1981); variations in buoyant density in the presence of intercalating dyes (Radloff *et al.,* 1967); differences in sedimentation by salt precipitation (Guerry *et al.,* 1973; Hansen and Olsen, 1979) or high-speed centrifugation. Most methods use a combination of factors to achieve a purified plasmid product and are generally effective for small (<50 kb) plasmids. When dealing with megaplasmids, the differences between plasmid and chromosome that are exploited in plasmid isolation procedures become smaller and plasmid purification becomes more challenging. Megaplasmids are vulnerable to sheering, confounding methods which rely on differences in supercoiling and hence buoyant density or ability to renature after alkaline denaturation. Commercial anion exchange columns for plasmid isolation should be used with care, as very large plasmids cannot reliably be eluted from standard columns (Doetkott *et al.,* 1996).

The replication of large plasmids is most often stringently regulated at a low copy number, thus reducing yields. Additionally, plasmids with stringently regulated replication may form a close association with the bacterial membrane (Schukin, 1981) and may become entrapped in the membrane during isolation attempts.

Despite many attempts to optimise plasmid isolation procedures, no one given method can reliably isolate all megaplasmids (above 50 kb) from all bacteria. This is illustrated by the numerous protocols that have been developed for isolation of large plasmids. A selection of the reported experiences with some of the more popular methods are summarised below:

A large plasmid (180 kb) from *Sphingomonas* strain F199 was successfully purified (Stillwell *et al.,* 1995) by the method of Casse *et al.,* (1979) which relies on a highly alkaline lysis solution to remove chromosomal DNA, but methods reliant on selective renaturation after alkaline denaturation (Birnboim and Doly, 1979), alkaline denaturation, salt precipitation and concentration by PEG 6000 (Hansen and Olsen, 1978) and removal of non-supercoiled DNA at high temperatures followed by phenol extraction (Kado and Liu, 1981) were not affective. A second, larger plasmid also present in this strain could not be isolated by any of the above methods.

The method of Kado and Liu (1981) uses a highly alkaline lysis solution and heating steps to eliminate chromosomal DNA. The method of Kado and Liu has been used both as a

screening procedure and as a method for purifying large plasmids, with varying amounts of success. This method has been reported to clearly visualise large plasmids (up to 114 mDa / 172 kb) from *Bacillus anthracis, B. cereus* and *B. thuringiensis* (Battisti *et al.,* 1985). Often this method is not successful in eliminating chromosomal DNA in species other than *Escherichia coli* (Battisti *et al.,* 1985; Quentmeier and Friedrich, 1994) making such DNA preparations unsuitable for further characterization or subcloning of the plasmid. Plasmids ranging in size from 6.3 kb to 225 kb present in *Acidophilum cryptum* and *Pseudomonas putida* were clearly visualised by the method of Kado and Liu, and plasmids ranging in size from 5 kb to 220 kb were visualised in *Thiobacillus acidophilus* by the method of Casse *et al.* (1979), which is very similar to the method of Kado and Liu (1981) but chromosomal contamination was not eliminated (Quentmeier and Friedrich, 1994).

Hansen and Olsen (1979) visualised plasmids of larger than 300 mDa. Their method was based on alkaline lysis of cells, followed by salt precipitation of chromosome-membrane complexes and concentration of plasmid DNA with PEG 6000. They identified a number of factors that were critical to isolation of very large plasmids. Firstly, sheering of plasmid DNA was minimised by avoiding vortexing and pipetting. Instead, only inversion and decanting was employed. Secondly, enzymatic degradation of DNA was minimised by ensuring that lysates were always kept cold, or in the presence of protein denaturing conditions. Thirdly, increasing the concentration of SDS used in the lysis step to 4%, improved the plasmid yield, even though complete cell lysis was observed with lower SDS concentrations. Inclusion of an alkaline denaturation - renaturation step minimised chromosomal contamination, but had a deleterious effect on isolation of large plasmids of the IncP type.

Plasmid isolation from *Acidiphilium* strains was improved by incubation in nitrogen-free non-growing medium, which was hypothesised to cause changes in the membrane makeup, allowing better transport of hydrophilic molecules, including DNA, across the membrane. Two freezing steps in the isolation procedure also increased plasmid yield by weakening the cell envelope (Singh and Banerjee, 2006). They had limited success with the methods of Birnboim and Doly, 1979; Holmes *et al.,* 1986; Quentmeier *et al.,* 1994; Ghosh *et al.,* 1997 and Sujuki *et al.,* 1997.

Cork and Khalil (1995) describe and compare three methods of megaplasmid isolation from *Pseudomonas*. The methods of Kado and Liu (1981) and Casse *et al.* (1979) are similar.

Both use high pH (12.45 for Casse and 12.6 for Kado and Liu), while the Kado and Liu method also incorporates a heating step, and uses phenol: chloroform for extraction instead of phenol alone. The Kado and Liu method was more effective than the method of Casse. The method of Allen (described in this paper but not published elsewhere) is a more gentle method and had greater success than both the Kado and Liu and Casse methods.

Propagation of plasmids in an *E. coli* host is advantageous, as the influence of the host on ease of plasmid isolation is considerable. However, many plasmids are cryptic (do not confer an easily selectable phenotype) and may have a narrow host range that does not include *E. coli*. Systems have been developed for the "capture" of such cryptic plasmids. The EZ::TN<R6Kγori/KAN-2> Insertion Kit (Epicenter, Madison, Wisconsin) can be used to introduce an origin of replication and antibiotic resistance marker that is functional in *E. coli* onto any piece of DNA. If this DNA is circular, it will gain the ability to replicate in any *E. coli* expressing the *pir* gene. The transposon insertion kit uses an *in vitro* transposition system based on the Tn5 system. A transposon cassette has been engineered to contain the conditional R6Kγ origin of replication and a kanamycin resistance gene. A multiple cloning site is included and the whole is flanked by the Tn5 transposase recognition sequence. The purified transposase enzyme included in the kit is capable of transposing any DNA sequence contained within the Tn5 recognition sequences and is functional *in vitro*.

The EZ::TN<R6Kγori/KAN-2> Insertion Kit has been used to capture a 6.2-kb plasmid from *Sulfobacillus* strain DSM 9293^{T} and propagate it in *E. coli* EC100D *pir*$^{+}$ (Honours Study, Joubert 2002, University of Stellenbosch). Two plasmids (28 kb and 29 kb) originating from *Leptospirillum ferrooxidans* ATCC 49879, an iron oxidising acidophile, have been captured in the same manner (Coram *et al.,* 2005). A similar system has been used to capture three plasmids from a 14 year old crude DNA extract of *Mycobacterium avum*, in which only chromosomal DNA could be visualised (Kirby *et al.,* 2002).

The methylation status of a vector must be appropriate for the strain to be transformed. DNA methylation in prokaryotes can be at adenine or cytosine residues and has been implicated in many cellular functions (Løbner-Olesen *et al*., 2005), including DNA replication and repair (Messer *et al*., 1985), and as part of restriction defence systems against foreign DNA (Berndt *et al.,* 2003). Type II restriction modification systems consist of a DNA restriction enzyme that cleaves DNA at a specific recognition site, and an associated DNA modification enzyme

that methylates the DNA of the host organism within the same recognition site to confer immunity to the restriction enzyme. Methylation specific systems also exist, where the restriction endonuclease cleaves only methylated recognition sites (Zotchev *et al*., 1995). Both types of restriction systems have been implicated in the failure to transform bacteria with plasmid DNA (Marrero and Welkos, 1995; Zotchev *et al*., 1995; Acetto *et al.,* 2005).

In addition, modification enzymes exist that are not paired with a specific restriction nuclease. Well-known examples are the *dam* and *dcm* methylases first characterised in *E. coli.* Although not coupled to a DNA restriction system, the methylation patterns produced by these enzymes can affect restriction by other systems. *Dam*- and *dcm*-type methylases are not found in all bacterial lineages. While *dam* methylation is quite widespread in Gram-positive and Gram-negative bacteria, the distribution of *dcm* methylation has been reported to be restricted to genera of the family *Enterobacteriaceae* closely related to *Escherichia* (Gomez-Eichelmann *et al*., 1991).

Characterization of the restriction modification system(s) harboured by potential host bacteria allows the rational design of methods for the preparation of transforming DNA to circumvent the host restriction system (Cue *et al*., 1996). While examining chromosomal DNA can give an indication of the type(s) of methylases that are present in a bacterium, it is important to investigate plasmid DNA as well, since plasmid and chromosomal DNA can have different methylation patterns (Guha and Guschlbauer, 1992).

The availability of DNA (this chapter) and sequence information (Chapter 3) of a plasmid originating from *Sulfobacillus* allowed the investigation of methylation patterns in sulfobacilli.

2.3. Materials and Methods

2.3.1. Growth media

Sulfobacillus cells to be used for DNA extractions were routinely cultivated in FeSYE liquid media, adapted from Johnson *et al.,* 1995 (section 8.1). In contrast to the original FeSo medium, FeSYE medium is buffered, limiting the changes in pH that take place during batch growth of sulfobacilli and thus minimising the formation of iron-sulphur precipitates that form at higher pH.

For the isolation of novel *Sulfobacillus* strains, mixed samples of acidophiles were plated on FeSo solid media for *Sulfobacillus*, adapted from Johnson *et al.,* 1995 (section 8.1). The underlay of *Acidiphilium* SJH used in the original FeSo medium to scavenge the acid hydrolysis products of agarose was not used for this medium.

2.3.2. Isolation of Sulfobacillus *strains*

Samples were obtained from BHP Billiton and Mintek.

Sample dilutions were plated onto modified FeSo plates and incubated at 45˚C for five days. Single colonies showing the typical "fried-egg" morphology were selected. After three rounds of streaking to single colonies; colonies were inoculated into 50 ml FeSo liquid medium and grown at 45˚C.

Sulfobacillus strains DSM 9293^T, ALV, L15, YOO17, G2, GSM, YTF1, TH1, MT13, THWX and GG6-3 (Table 7.2) were gifts from Prof. D.B. Johnson

2.3.3. Preparation of Sulfobacillus *cells for DNA extractions or transformation*

Fresh FeSYE liquid medium was inoculated with 1:20 volumes of actively growing *Sulfobacillus* culture and incubated overnight, with shaking, at 45˚C. Cells were harvested from FeSYE media by centrifugation (9000 x g, 20 min, 15˚C) and resuspended in dH_2O acidified (pH 1.9) with sulphuric acid. The cell suspensions were transferred to Eppendorf tubes and collected by centrifugation at (3500 rpm) in a bench-top centrifuge. The supernatant was removed by aspiration. This yielded a pellet consisting of *Sulfobacillus* cells and precipitate (probably jarosite) with a clear separation between the jarosite- and cell layers. The top, cell-containing, layer was resuspended in acid dH_2O by careful pipetting and transferred to a new tube. The washing process was repeated until no more jarosite could be seen in the cell pellet or by inspection by light microscopy (100 to 400 times magnification). The cell pellet was finally washed once in the starting solution for the specific DNA extraction protocol or transformation procedure.

2.3.4. Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA)

Total DNA was isolated from *Sulfobacillus* strains using the tissue protocol of the Genopure PCR Template Preparation Kit (Roche). Using primers fDD2 and rPP2 (Table 7.3), a 1.5-kb

fragment of the 16S rDNA was amplified. PCR conditions were as follows: an initial denaturing step of 60 s at 94˚C was followed by 25 cycles of denaturing for 30 s at 94˚C, annealing for 30 s at 52˚C (when using Taq polymerase from Bioline) or 55˚C (when using GoTaq from Promega) and elongation for 90 s at 72˚C. A final lengthening step of 120 s was performed at 72˚C and the tube cooled to 4˚C. The reactions were performed in a PCR Sprint Temperature Cycling System (Hybaid).

Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA) was conducted to identify the three isolates. ARDREA has been used to differentiate between different genera of bacteria commonly occurring in acidic, metal rich environments and between groups within the genus *Sulfobacillus* (Schutte, 2004; Johnson *et al.,* 2005). Diagnostic restriction digests of 16S ribosomal DNA PCR products were performed to characterise the isolates, using *Eco*RI, *Eco*RV, *Bam*HI and *Stu*I (Rawlings, 1995; Rawlings *et al.,* 1999; Schutte, 2004) and *Eco*721 and *Sna*BI (Johnson *et al.,* 2005).

2.3.5. Preparation of DNA for pulse field gel electrophoresis

Agarose plugs for Transverse Alternating Field Electrophoresis (TAFE) were prepared by the method used by Barton and co-workers (1995). After the standard cell washing procedure (section 2.3.3), cells were washed twice more in SET buffer (50 mM Tris, 2 mM EDTA, 25% sucrose, pH 8). Cells were finally resuspended in 2 ml SET buffer and the OD at 600 nm adjusted to 2.0 with SET buffer. RNase and lysozyme were added to a final concentration of 20 µg/ml and 1 µg/ml, respectively.

Cells were mixed in a 1:1 ratio with 2% LMP agarose in dH_20 (Agarose Low Melt LM2, SeaPlaque, FMC Bioproducts). The mixture was transferred to a standard mould with a Pasteur pipette and allowed to set at 4˚C for 15 min. The solidified plugs were incubated at 37˚C for 1h in ES buffer (0.5 M EDTA free acid, 1% Na-lauryl sarcosine, pH 8) with the same concentrations of RNase and lysozyme. Plugs were then incubated overnight at 37˚C in ESP buffer (0.5 M EDTA free acid, 1% Na-lauryl sarcosine, 1 µg/ml proteinase K, pH 8). This solution was replaced with fresh ESP buffer and incubated overnight at 50˚C. The ESP solution was replaced with ES buffer and 1-5 mg/ml Pefabloc SC (Roche Molecular Biochemicals) was added. The plugs were incubated in this solution overnight at 4˚C. The

plugs were washed in TE_{50} (30 mM Tris pH 7.6, 50 mM EDTA) overnight on a Bio Dancer rotary shaker (New Brunswick scientific) and stored at 4˚C in TE_{50}.

Digestion of DNA in agarose plugs with S1 nuclease was done as described by Barton *et al.* (1995). Gel plugs were equilibrated by incubation in 10 mM Tris (pH 7.5) for two 15 min intervals. The gel plugs were transferred to 200 µl Barton buffer (50 mM NaCl, 5 mM $ZnSO_4$, 30 mM sodium acetate, pH 4.5) with 1 unit of S1 nuclease and incubated at 37˚C for 45 min. The reaction was stopped by transferring the slices to 100 µl ES buffer on ice.

Lambda concatemers were used as a molecular weight marker for TAFE. To prepare the lambda concatemers, 10 µg of lambda DNA in 250 µl Soln A (2% PEG 8000, 2 mM ATP, 2 mM DTT) was mixed with 3 U of T4 ligase, and an equal volume of 1% LMP agar melted in Soln B (20 mM $MgCl_2$, 100 mM Tris pH 8). The mixture was set a standard mould and allowed to solidify at 4˚C for 15 min. The lambda gel plugs were incubated at room temperature overnight in Soln C (Soln A and Soln B mixed in 1:1 ratio) and stored at 4˚C in 50 mM EDTA.

Sample plugs of lambda marker and cell lysates were applied to the wells of a 1% agarose gel and separated in a TAFE apparatus (Beckman GeneLine™). Electrophoreses conditions were as follows: 250 V, 150 mA, pulse length 15 s, total time 16h, buffer 1x TAFE (0.29 g/l EDTA, 0.5 ml/l glacial acetic acid, 2.42 g/l Tris), recirculated and maintained at 10˚C. The gel was stained with ethidium bromide for 15 min and de-stained for 2x 20 min in 1x TAFE buffer.

The DNA was transferred to a positively charged nylon membrane (Hybond N+) by upward capillary blotting and used in later Southern blotting experiments.

2.3.6. Recovery of DNA from gel bands

Several methods of DNA recovery from gel bands were attempted. Two commercial kits were used, the GFX DNA and gel band purification kit (Amersham Biosciences) and the Cleanmix DNA purification system (Talent, Trieste, Italy)

Electro-elution from gel slices was done as follows: The DNA-containing gel slice was placed in DNA dialysis tubing (Pierce, Rockford, USA) along with 1 ml TAE buffer. The ends of the tube were clipped shut and the dialysis tube was suspended in an electrophoresis tank with

TAE buffer. Electrophoresis was carried out for 4h at 80 V. The contents of the dialysis tubing was removed by aspiration and DNA was recovered by ammonium acetate-ethanol precipitation.

Recovery of DNA from gel slices was also attempted by an adaptation of the "freeze-squeeze" method (Thuring *et al.,* 1975). A gel slice containing the DNA band was cut from the gel and placed in a 1.5 ml Eppendorf tube. The gel slice was crushed using a disposable pipette tip. Five hundred microlitres of buffered phenol (pH 8) were added and the tube vortexed until a milky emulsion formed. The tube was placed at -80˚C for 30 min. The mixture was centrifuged at 13000 rpm for 5 min in a benchtop centrifuge. The top, aqueous phase was removed to a fresh tube and the volume made up to 200 µl with TE buffer. This mixture was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1), then twice with chloroform:isoamyl alcohol (24:1). The aqueous phase was transferred to a fresh tube and DNA recovered by ammonium acetate – ethanol precipitation.

2.3.7. Phenol extraction by the Kado and Liu (1981) method

At first, the method of Kado and Liu was followed exactly as set out in their paper; thereafter steps were changed in an attempt to optimise the protocol for sulfobacilli.

For *E. coli*, 5 ml Luria broth cultures were incubated at 30˚C with shaking until OD_{600} of 0.8 was reached. Three millilitres of this culture were used for plasmid preparation. For *Sulfobacillus* strains, cells were prepared from 500 ml cultures as described earlier and resuspended 1 ml E-buffer (40 mM Tris-acetate, 2 mM sodium EDTA, pH 7.9). Cells were lysed by addition of 2 ml lysing solution (3% sodium dodecyl sulphate, 50 mM Tris, pH 12.6).

The lysate was heated at 50˚C-65˚C for 20 min to reduce the amount of chromosomal DNA and RNA present. To purify the DNA two volumes of phenol-chloroform (1:1) were added and emulsified by shaking briefly. The emulsion was broken by centrifugation for 2 min at 8000 rpm in a benchtop centrifuge.

The upper aqueous phase was transferred to a new tube. The end was cut off the disposable p1000 pipette tip (Greiner bio-one) to avoid shearing DNA.

Optimisations of the method included addition of lysozyme, proteinase K and/or RNase to the E-buffer; increasing the percentage SDS in the lysing solution; vortexing the lysate before the heating step to fragment chromosomal DNA; increasing the temperature and duration of the heating step and rapidly cooling the lysate on ice; using buffered phenol and including extra chloroform extraction steps to remove residual phenol.

2.3.8. Alkaline lysis mini-preparations

Plasmid mini-preparations were performed on *Sulfobacillus* strain DSM 9293^T by the Ish-Horowicz (1981) method, modified as follows. Cells were grown in 500 ml FeSYE and harvested as normal, but not washed further. The cell pellet was resuspended in 2 ml acidified dH_2O (pH 1.9) and transferred to two Eppendorf tubes (1 ml per tube). After pelleting by centrifugation at 3500 x g for 5 min in a bench top centrifuge, one pellet was resuspended in 2 ml Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris, pH 8), while the other pellet was washed further in acid H_2O as described in preparation of cells for DNA extraction, before being resuspended in 2 ml Solution I. Three aliquots of 400 µl each were taken of the washed and unwashed cells, 5 µl H_2O was added to the first, 5 µl of 20 µg/ml lysozyme to the second and 5 µl of 20 µg/ml proteinase K to the third tube. All were incubated at 37˚C for 5 min, before adding 800 µl solution II (0.2 N NaOH, 1% SDS) and incubating at room temperature for 5 min. Finally, 600 µl Solution III (5 M potassium acetate, 2 M glacial acetic acid) was added to each tube and the tubes were incubated on ice for 10 min. The flocculent precipitate was removed by centrifugation at 13000 rpm for 10 min in a bench top centrifuge and the supernatants transferred to new tubes. Isopropanol (0.6 volumes) was added and the solution centrifuged at 13000 rpm for 10 min. The pellet was washed with 70% ethanol, dried, and resuspended in 60 µl TE buffer. Twenty microlitres of each DNA solution was digested with *Sal*I and separated on a 1% w/v agarose gel.

2.3.9. Gentle lysis cesium gradient plasmid isolations

Gentle lysis plasmid preparations were performed essentially as described by Coram *et al.* (2005).

Sulfobacillus cells were grown in 1 litre FeSYE media at 45˚C, harvested and washed as described earlier (section 2.3.3), then washed twice more in SET buffer (50 mM Tris, 2 mM EDTA, 25% sucrose, pH 8.0), and finally resuspended in 2 ml of this buffer.

Cells were diluted to 30 ml in SET buffer and frozen in SET buffer at -20˚C for 1h. After thawing on ice, 200 µl proteinase K (20 mg/ml) was added and cells were incubated at 4˚C for 15 min. Thereafter cells were lysed by incubation with sodium dodecyl sulphate at a concentration of 1% w/v at 4˚C for 10 min, then at 37˚C for 10 min. Lysates were centrifuged at 25000 x g for 60 min to precipitate cellular debris and chromosomal material. Five millilitre fractions of supernatants were transferred to fresh tubes and 1 g/ml cesium chloride and 0.4 mg/ml ethidium bromide were added. This solution was centrifuged at 12000 x g for 10 min to remove remaining cellular debris. Plasmids were purified by cesium chloride gradient centrifugation (Heilig *et al.,* 1998) and resuspended in 100 µl sterile dH_2O.

2.3.10. Plasmid isolations by anion exchange chromatography

The Nucleobond$^{(R)}$ Xtra Midi plasmid purification kit (Macherey-Nagel) was used to purify plasmid DNA via anion exchange column chromatography. Plasmid purification was done according to the low copy number protocol in the kit manual. Five hundred millilitres to one litre of *Sulfobacillus* overnight culture in FeSYE media was used, yielding 50-200 mg wet weight of cells. Cells were washed as normal with an additional washing in 10 mM Tris (pH 8) before resuspending in the first buffer of the kit.

Further optimizations included treatment of cells by three cycles of freezing at -80˚C then thawing at 37˚C in 1 ml SET buffer (50 mM Tris pH 8, 2 mM EDTA, 25% sucrose) before resuspending in the first buffer of the kit. Cells were also treated with lysozyme according to instructions for difficult-to-lyse strains in the Nucleobond$^{(R)}$ Xtra Midi kit manual.

2.3.11. Plasmid capture

The EZ::TN<R6Kγori/KAN-2> transposon rescue kit (Epicenter, Madison, Wisconsin) was used to introduce the R6K plasmid replicon and a kanamycin resistance marker onto plasmids isolated from *Sulfobacillus* strains.

A plasmid solution of 114 ng/µl from YOO17, as measured by absorption at 260 nm, was obtained by pooling the total DNA from two plasmid isolations, dialyzing this against dH_2O, and concentrating the sample by evaporation. Taking the estimated size of YOO17 to be 80 kb, the molar concentration of the plasmid solution was estimated at 2.2×10^{-4} pmol/µl.

Two hundred nanograms (3.85 x 10^{-4} pmol) of plasmid DNA (in 1.75 µl) and 2 x 10^{-3} pmol of transposon DNA (in 0.4 µl) was used in the *in vitro* transposition reaction. Plasmid DNA, transposon DNA and purified transposase were mixed and incubated as directed in the EZ::TN<R6Kγori/KAN-2> Insertion Kit manual.

Thereafter, one microlitre of the transposition mixture was transformed by electroporation (2.5 kV, 200 Ω, 25 µF) into EC100D^{TM} *pir*$^{+}$ electrocompetent *E. coli* (Epicentre, Madison, Wisconsin). Cells were allowed to recover and express kanamycin resistance in SOC buffer (20 g/l tryptone, 5 g/l yeast extract, 8.6 mM NaCl, 1 mM KCl, 20 mM glucose, pH 7.0) for 1h and transformants were selected on plates with kanamycin (50 µg/ml).

As a control for the *in vitro* transposition reaction, control DNA cloned on a pUC19 replicon (supplied with the transposon insertion kit), was used as a target for transposition and transformants were selected on kanamycin. As a control for electroporation efficiency, the same transformation mixture was selected on ampicillin.

Transformants were screened by electrophoresis of alkaline lysis plasmid preparations from single colonies and pools of 48 colonies. Single colonies from kanamycin plates were restreaked onto fresh kanamycin plates and alkaline lysis plasmid isolations were prepared from cells scraped from the plate. Pools of colonies were made by restreaking 48 single colonies on a plate then scraping the cells of 48 streaks from the plate and resuspending in 15 ml Solution I (50 mM glucose, 10 mM EDTA, 25 mM Tris pH 8) and mixing the cells very well. One hundred microlitres of the mixed cells was taken and the standard alkaline lysis protocol was completed.

2.3.12. Southern blotting

DIG labelled probes were prepared from cloned fragments of plasmid DNA of pKara or pYOO17. Hybridisation and detection were done according to standard protocols.

Table 2.1 DIG-labelled probes used to compare plasmids of *Sulfobacillus* strains.

Name	Description	Reference
KaraORF1	550 bp *Nco*I-*Sal*I subclone of internal region ORF1 of pKara	This study
KaraORF2	541 bp PCR product within ORF2 of pKara	This study
KaraORF3	521 bp *Sal*I-*Xba*I subclone of the internal region of ORF3 of pKara	This study
KaraORF4	432 bp PCR product of internal region of ORF4 of pKara	This study
Y-700	700 bp *Sal*I fragment of pYOO17 (gentle lysis prep)	This study
Y-5000	5000 bp *Sal*I fragment of pYOO17 (gentle lysis prep)	This study

2.3.13. Investigation of methylation patterns

Plasmid DNA was isolated from *Sulfobacillus* strain DSM 9293^T and digested with a range of restriction enzymes. The restriction patterns obtained on a 1% agarose gel was compared to patterns predicted from the nucleotide sequence of pKara (Chapter 3).

2.3.14. Ability of pKara to replicate in E. coli

Competent cells of *E. coli* DH5α were prepared by the standard $CaCl_2$ / $MgCl_2$ method (Seidman *et al.,* 1997) and transformed by heat shock (5 min 37˚C) with pSulfTA and pSulfTY (two transposon jumped derivatives of pKara) then selected on Luria agar with kanamycin (50 µg/ml).

2.4. Results

2.4.1. Isolation and typing of Sulfobacillus strains

A diversity of *Sulfobacillus* strains was needed for the dual purposes of screening for novel plasmids and later use in genetic transformation experiments. Of the nine *Sulfobacillus* strains kindly provided by Prof. Johnson, only one (strain MT13) originated from a commercial biomining operation in Africa. To broaden the applicability of this study, local *Sulfobacillus* strains of industrial importance had to be included. Therefore, *Sulfobacillus* strains were isolated from samples originating from BHP Billiton and Mintek inoculums of biooxidation plants in Africa.

A mixed community sample from the first in a series of biooxidation tanks from Zaire (tank 611) grown at 40˚C on a copper concentrate containing chalcocite, covellite and bornite cobalt, was plated on FeSo plates. A variety of colony morphologies was observed. After

several days of incubation, some colonies exhibited the "fried-egg" morphology typical of *Sulfobacillus* colonies. Several rounds of restreaking from single colonies onto fresh FeSo plates produced round and transparent colonies with an uneven margin after two days of incubation. These colonies attained the "fried-egg" colony morphology after several more days of incubation. This isolate was named 611(2005).

Another strain, named Andy, was isolated in the same manner from a sample originating from a BHP Billiton mesophilic inoculum grown at 40˚C on a chalcopyrite concentrate.

Samples received from Mintek were from a mixed semi-batch culture maintained on a pyrite-arsenopyrite concentrate. This sample yielded another *Sulfobacillus* strain, named Mintek45.

Identification to the genus level was done by digestion of a 1500-bp PCR amplicon with *Eco*721, *Eco*RI and *Eco*RV. Johnson *et al.* (2005) predicted 1100-bp, 300-bp and 100-bp fragments for *Sulfobacillus* spp. when this region of the 16S rDNA is digested with *Eco*721, while Schutte (2004) predicted an uncut 1.5-kb band and a doublet at 700 bp for sulfobacilli, when the amplicon is digested with *Eco*RV and *Eco*RI respectively.

Further grouping of the isolated strains was done by digesting the 1500 bp 16S rDNA amplicon with *Sna*BI. Johnson *et al.* (2005) predicted fragments of sizes 550 bp and 950 bp for *Sulfobacillus* group I, and an uncut 1500-bp fragment for *Sulfobacillus* group II.

The same digestion pattern was obtained for all three newly isolated strains and for the control, *S. thermosulfidooxidans* strain DSM 9293^{T}: fragments of 1100 bp, 300 bp and 100 bp after digestion with *Eco*721, an uncut 1500 bp fragment after digestion with *Eco*RV and two fragments of ± 700 bp after digestion with *Eco*RI (Figure 2.1). With this evidence, the newly isolated strains were placed in the genus *Sulfobacillus*.

Likewise, digestion of the 1500 bp 16S rDNA amplicon with *Sna*BI yielded similar digestion patterns for 611(2005), Andy, Mintek45 and DSM 9293^{T}: two fragments of 950 bp and 550 bp respectively (Figure 2.1). Therefore strains 611(2005), Andy and Mintek45 could be placed in the *Sulfobacillus* subgroup I, the *S. thermosulfidooxidans* group.

Partial sequencing of the cloned 16S rDNA gene of Mintek45 confirmed the identification of this strain (Appendix I).

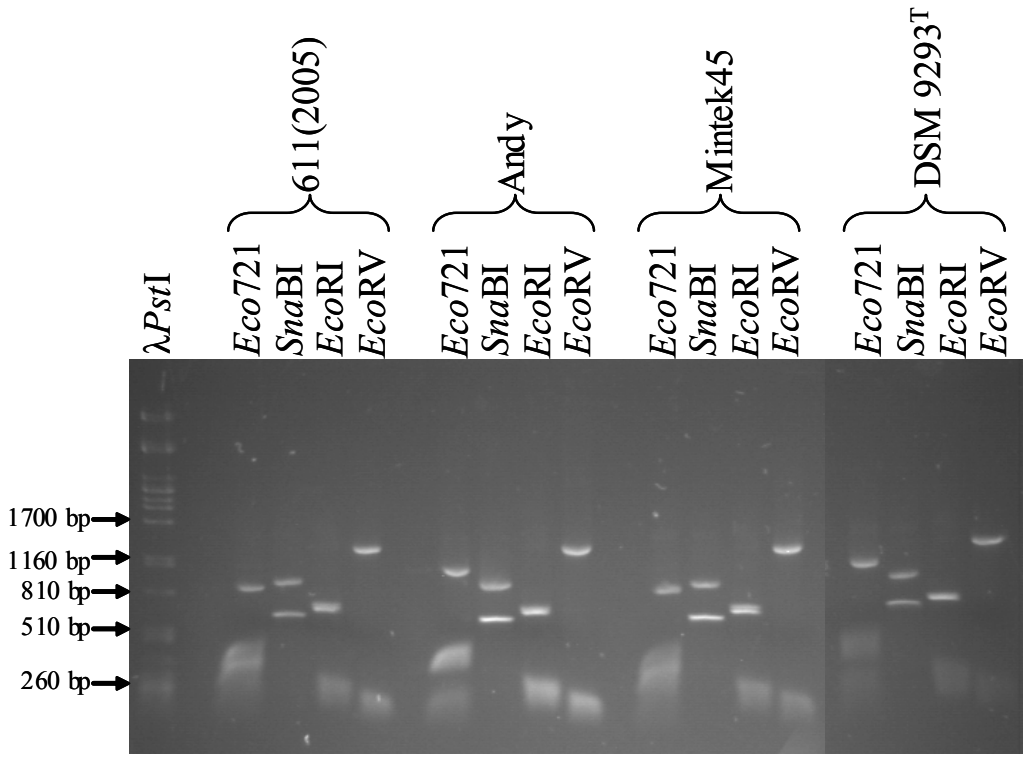

Figure 2.1 ARDREA of *Sulfobacillus* strains 611(2005), Andy, Mintek45 and DSM 9293^T.

An approximately 1500 bp fragment of the 16S ribosomal DNA gene was amplified by PCR and digested with restriction enzymes *Eco*721, *Sna*BI, *Eco*RI and *Eco*RV. *Sulfobacillus* strains 611(2005), Andy, Mintek45 and the *S. thermosulfidooxidans* type strain DSM 9293^T all produced restriction patterns diagnostic of the *S. thermosulfidooxidans* group. λ *Pst*I was used as molecular weight marker.

2.4.2. Plasmid survey by pulsed field gel electrophoresis

Sulfobacillus strains were screened for the presence of plasmids by Transverse Alternating Field gel Electrophoresis (TAFE) of in-gel lysates treated with S1 nuclease. The presence of one or more plasmids was revealed in six of nine *Sulfobacillus* strains tested (Figure 2.2).

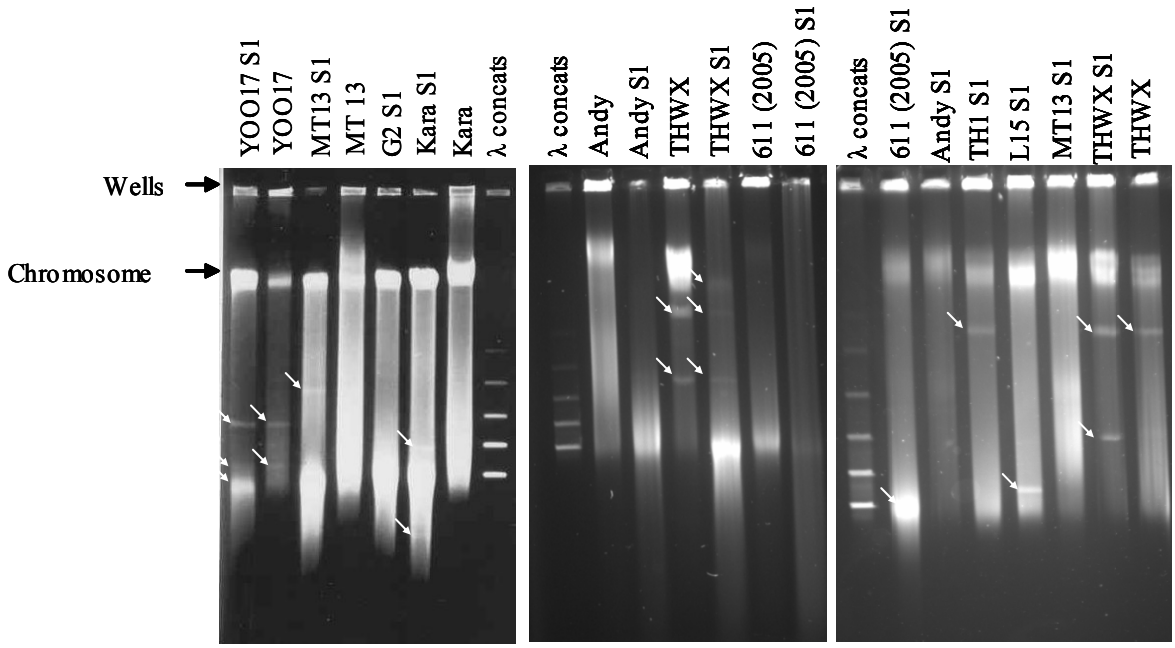

Figure 2.2 Survey of plasmids present in strains of *Sulfobacillus*.

Three gels of in-gel lysates of *Sulfobacillus* strains are shown. DNA species were separated by transverse alternating field gel electrophoresis. Visualization of plasmid bands was improved by S1 nuclease treatment to linearise plasmid DNA. Chromosomal DNA is visible as diffuse bands on the gel photographs, while plasmid bands are sharper. λ concats: molecular size marker composed of concatemers of lambda DNA with sizes of 48.5, 97, 145.5, 194, and 242.5 kb. S1: gel plugs were treated with S1 nuclease. Plasmid bands are indicated with arrows.

Although the presence of plasmid DNA was clearly demonstrated by TAFE, this method did not yield plasmid DNA in a form that was amenable for further manipulation. Plasmid bands were effectively separated from chromosomal DNA and cellular debris, but remained encased within the gel matrix. Further characterization of the observed plasmids required their recovery from the gel matrix, or isolation in a liquid medium.

2.4.3. Recovery of DNA from gel bands

Commercial kits for the recovery of DNA from agarose gels could effectively recover small plasmids, such as the 6.2-kb plasmid of *S. thermosulfidooxidans* strain DSM 9293^T, from agarose gels. The efficacy of these kits did not extend to the larger plasmids seen in other *Sulfobacillus* strains.

The electro-elution and freeze-squeeze methods of isolating plasmid bands from agarose gels did not recover detectable amounts of plasmid DNA.

Since the amounts of DNA of the larger *Sulfobacillus* plasmids that could be isolated from agarose gels was insufficient, other plasmid isolation techniques were attempted.

2.4.4. Plasmid isolations

The ability to screen possible transformants for the correct plasmid or clone is indispensable for a successful genetic system. Therefore, the small scale alkaline lysis plasmid preparation method, which is routinely used as a rapid test for the presence of plasmids, was included in this study.

As the majority of plasmids detected in *Sulfobacillus* strains migrated above the 50 kb molecular size marker (section 2.4.2), plasmid isolation techniques developed for large plasmids were also adopted. The method of Kado and Liu (1981) has been reported to be effective for the isolation of large plasmids from diverse bacterial species. The gentle lysis cesium gradient method was developed specifically for the isolation of large plasmids and has been used in this lab to isolate plasmids of 28 kb and 29 kb from *Leptospirillum,* a Gram-negative, acidophilic iron oxidiser (Coram *et al.,* 2005).

2.4.4.1. Phenol extraction by the Kado and Liu method

The standard plasmid isolation method described by Kado and Liu (1981) resulted in large amounts of intact DNA from sulfobacilli that was resistant to digestion with restriction endonucleases. Inclusion of chloroform extraction steps to remove residual phenol allowed successful restriction of this DNA, which was shown to be mostly chromosomal in nature (Figure 2.3A). No purified plasmid DNA was obtained.

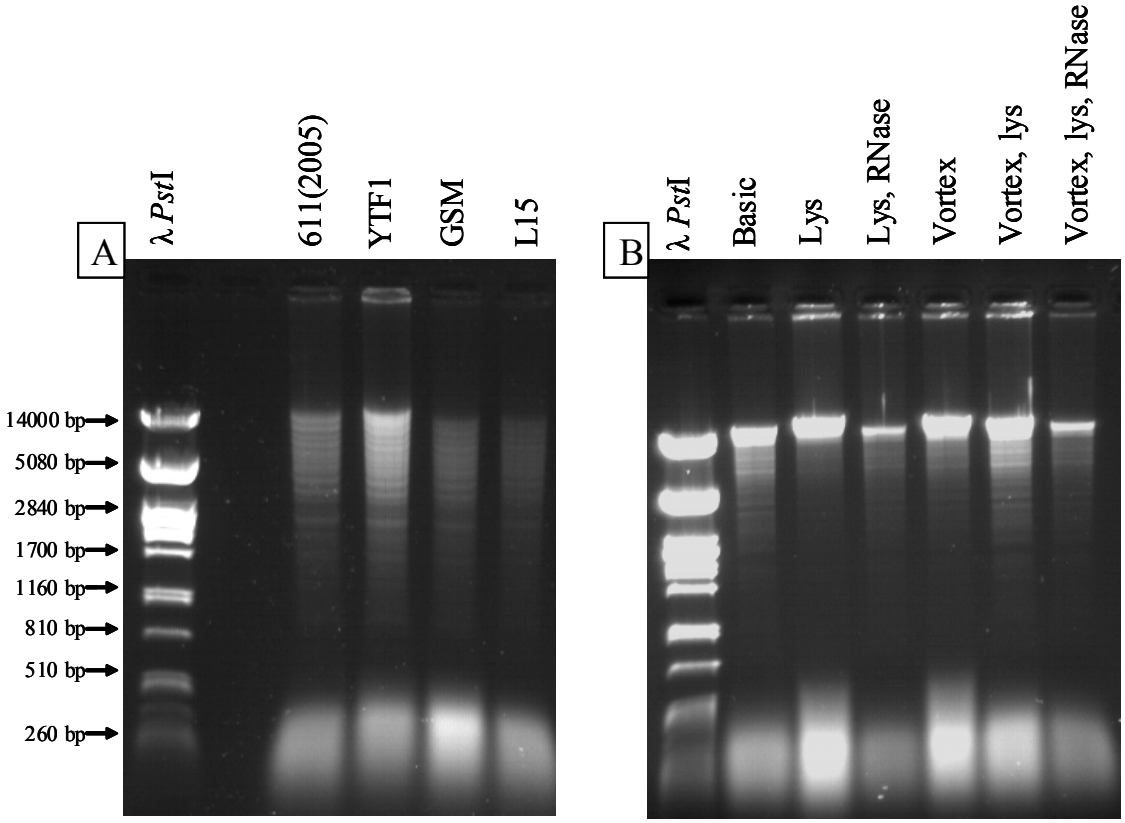

Figure 2.3 Agarose gel of Kado and Liu plasmid preparations from *Sulfobacillus* and *E. coli*

Gel A contains Kado and Liu plasmid preparations from *Sulfobacillus* strains 611(2005), YTF1, GSM and L15, all digested with *Sal*I. The dense, continuous banding pattern is indicative of large amounts of digested chromosomal DNA with little or no enrichment for plasmid DNA.

Gel B contains Kado and Liu plasmid preparations from *E. coli* HB101 harbouring plasmid RP4 cut with *Sal*I. Two brighter DNA bands corresponding to predicted sizes of RP4 fragments on a background of dense, continuous bands are indicative of plasmid DNA enriched in a preparation still contaminated with large amounts of chromosomal DNA. Basic: the standard method as set out in Kado and Liu was followed. Lys: lysozyme was added to the suspension buffer. RNase: RNase was added to the suspension buffer. Vortex: lysates were vortexed to sheer chromosomal DNA. λ *Pst*I was used as molecular weight marker.

The effect of various protocol optimizations on plasmid yield and purity were investigated with a more amenable bacterial host, *E. coli* HB101 harbouring a large plasmid RP4 (51 kb).

In *E. coli* HB101 containing plasmid RP4 (51 kb), addition of lysozyme to the E-buffer improved total DNA yields but did not improve isolation of plasmid DNA. Addition of RNase reduced the amount of RNA in the preparation but also did not improve plasmid isolation. Vortexing the lysate to sheer chromosomal DNA resulted in a reduction of the amount of chromosomal DNA, but did not improve isolation of plasmid RP4 (Figure 2.3B). Addition of proteinase K had no noticeable effect (not shown). Prolonging the heating step resulted only in a slight smearing of the chromosomal DNA band (not shown). Despite the large amounts of chromosomal contamination present in the plasmid preparations of RP4, plasmid bands could still be identified over the background of *Sal*I digested chromosomal DNA

In order to determine whether this degree of plasmid purification was sufficient, the isolation of even larger and smaller plasmids from *E. coli* was attempted. The presence of plasmids Tn11 (66 kb) and pUCBM21 (2.7 kb) could be demonstrated in the host *E. coli* S17-1 *pir*$^{+}$, but the chromosomal DNA band could not be eliminated. Plasmid Tn11 is a plasmid isolated by transposon rescue from *At. caldus* strain MNG (S.M. Dean) an organism that shares the habitat of sulfobacilli. Tn11 is able to replicate in *E. coli* S17-1 *pir*$^{+}$ because of the presence of the EZ::TN<R6Kγori/KAN-2> transposon, which was integrated into the native *At. caldus* plasmid.

No plasmid bands could be visualised in DNA preparations of *Sulfobacillus* strains by using the same method that allowed visualization of a 66-kb plasmid in an *E. coli* host (Figure 2.4).

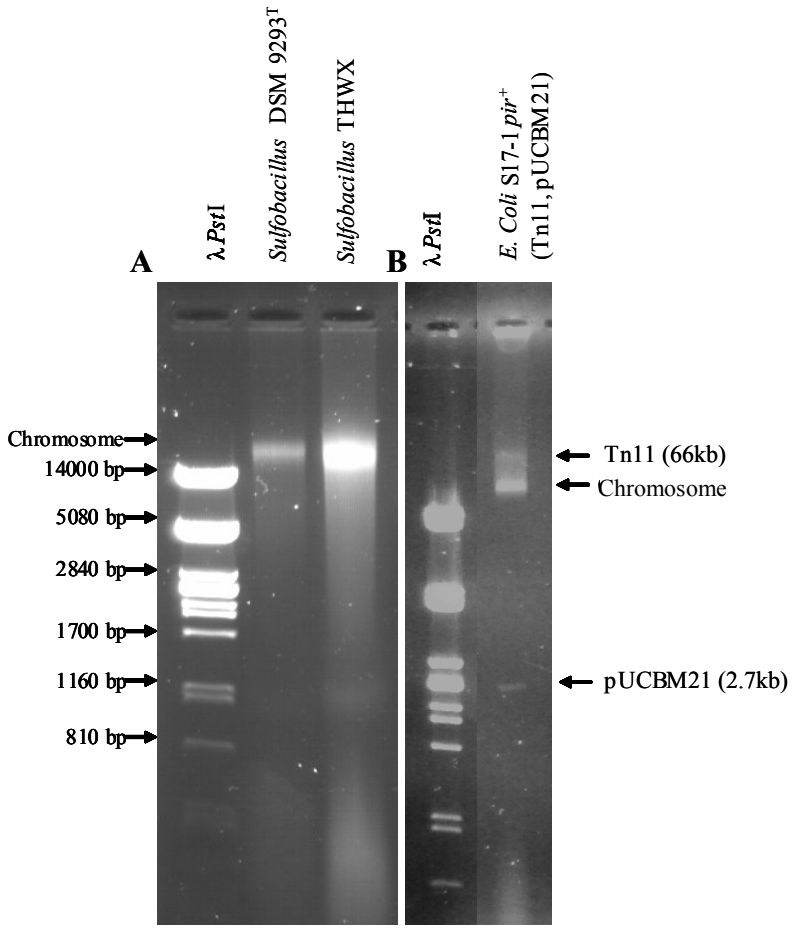

Figure 2.4 Agarose gel of Kado and Liu plasmid preparations from *Sulfobacillus* and *E. coli.*

Two gels are shown, with DNA of *Sulfobacillus* strains DSM 9293^T and THWX on gel A and DNA of *E. coli* S17-1 containing plasmids Tn11 and pUCBM21 on gel B. λ *Pst*I was used as molecular weight marker.

2.4.4.2. Gentle lysis cesium gradient preparations

The gentle lysis method minimises sheering of large plasmids. While chromosomal DNA bands were observed within the cesium chloride density gradients, no plasmid bands were visible below the chromosome. Fractions taken sequentially from the bottom of the tube were analysed and in only two instances yielded tiny amounts of DNA in fractions below the chromosomal DNA band (Figure 2.5).

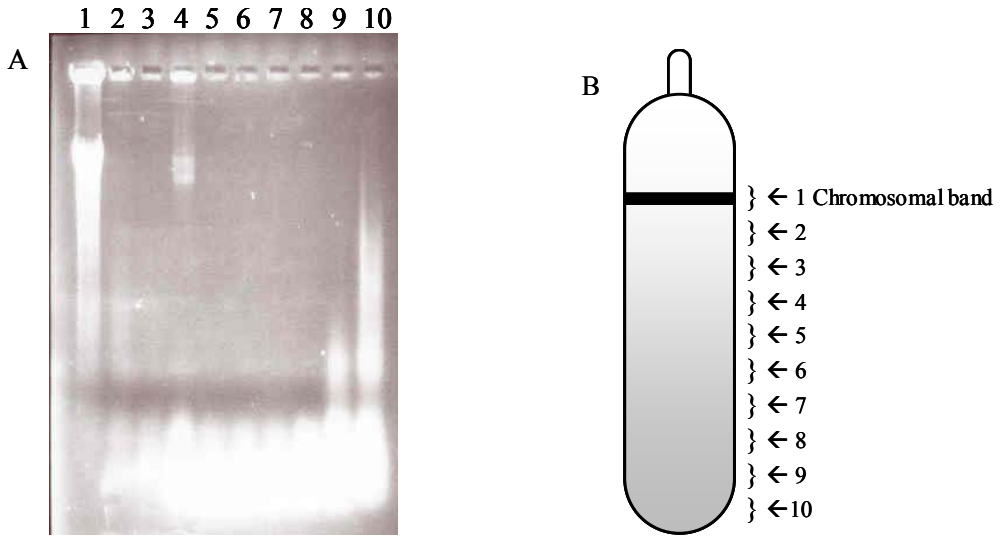

Figure 2.5 Agarose gel of fractions taken from the cesium chloride density gradient of a gentle lysis plasmid preparation from YOO17:

Fractions are numbered from the top of the tube (least dense) to the bottom (most dense). Separation by agarose gel electrophoresis of DNA contained in the gradient fractions showed that fraction 1 contains the chromosomal DNA of YOO17 – a diffuse band with a smear of degraded chromosome below. Fraction 4 showed no visible fluorescence under UV light but contained possible plasmid DNA of YOO17 – a more distinct band. Fractions 9 and 10 contain most of the RNA present in the preparation. A schematic representation of the relative locations of the gradient fractions is shown in B.

When digested with *Sal*I and separated by electrophoresis, the DNA recovered from the cesium chloride gradient fractions differed markedly, with fewer *Sal*I fragments present in the lower fraction than in the higher fraction. The restriction patterns of the lower fraction are consistent with large plasmid species rather than chromosomal DNA (Figure 2.6).

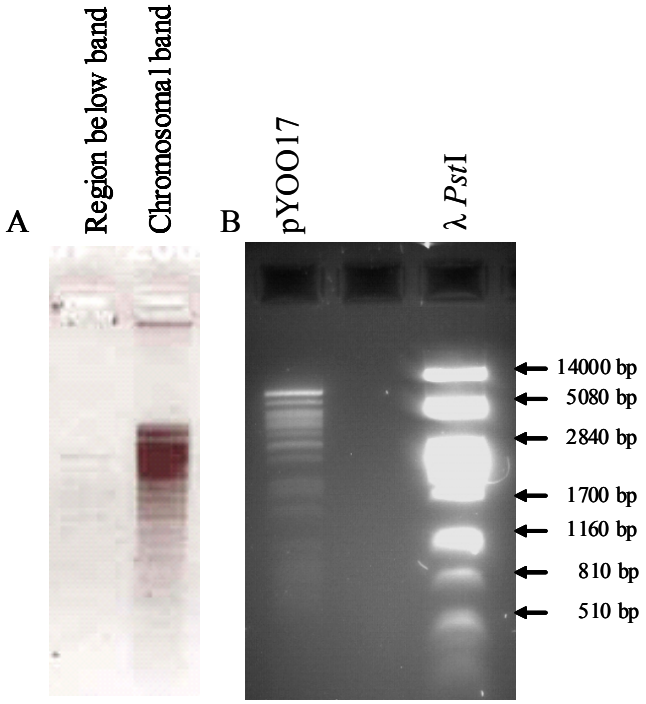

Figure 2.6 Agarose gel of gentle lysis cesium gradient plasmid preparation of YOO17 compared with chromosomal DNA.

A: DNA from the chromosomal band on cesium gradient and the fraction 3 cm below it was cut with *Sal*I and separated on a 0.8% agarose gel. B: The fraction collected below the chromosomal band was digested with *Sal*I and concentrated via the DNA Clean and Concentrator™-5 kit (Zymo Research). λ *Pst*I was used as molecular weight marker.

The *Sal*I fragments from the plasmid fraction were shotgun-cloned into *Sal*I-digested pSK. Twenty-two different-sized inserts were recovered. The sum of the estimated sizes of the cloned fragments was ± 40 kb, which is only half of the estimated size of the smallest plasmid band detected in strain YOO17 and therefore does not represent complete coverage of the plasmid. Two cloned fragments (± 700 bp and ± 5000 bp) were used to make DIG-labelled probes for Southern blotting.

In a plasmid preparation from *Sulfobacillus* strain L15, the cesium chloride gradient was similarly fractionated and plasmid DNA was recovered. In this case, the yield was better than for the YOO17 plasmid, but this preparation was heavily contaminated with chromosomal DNA (Figure 2.7).

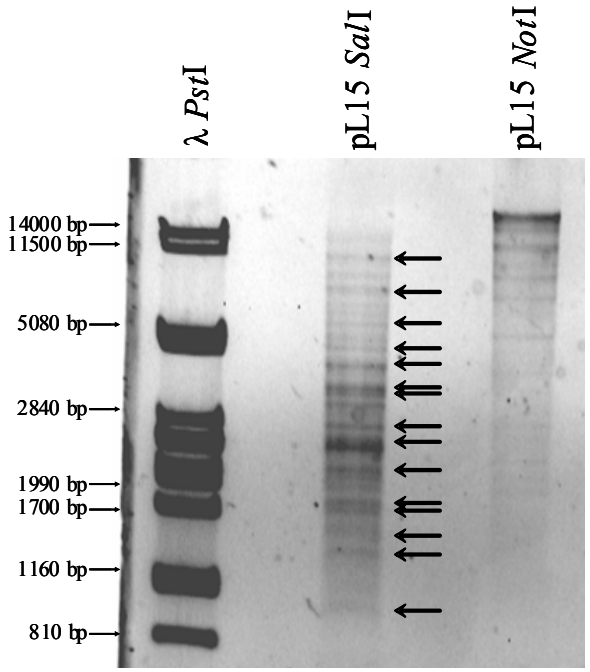

Figure 2.7 Agarose gel of *Sulfobacillus* L15 DNA recovered from a cesium chloride gradient and restricted with *Sal*I and *Not*I.

In this preparation, plasmid DNA is enriched slightly relative to chromosomal DNA. The fragments marked with arrows correspond to plasmid fragments obtained by a different method (Figure 2.10) but the chromosomal contamination is still too severe to allow further unambiguous characterization of the plasmid. λ *Pst*I was used as molecular weight marker.

A more typical result with the gentle lysis cesium gradient method was that mostly chromosomal DNA is recovered (Figure 2.8), but no detectable plasmid DNA.

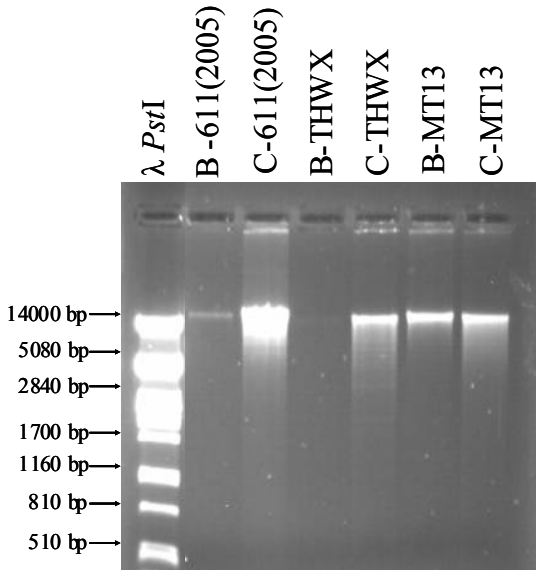

Figure 2.8 Agarose gel of fractions taken from cesium chloride gradients with plasmid preparations from *Sulfobacillus* strains 611, THWX and MT13.

Gentle lysis plasmid preparations from *Sulfobacillus* strains 611, THWX and MT13 were separated in a cesium chloride gradient. Two fractions were taken from the gradient, the chromosomal fraction that was visible under UV light (C), and a fraction just below that (B). λ *Pst*I was used as molecular weight marker.

2.4.4.3. Plasmid isolations by anion exchange chromatography

The way in which anion exchange columns purify plasmids away from other DNA species is very different from the other methods investigated, being based on the strength of binding to a column by total negative charge carried by the DNA molecule. This method could therefore access an aspect of difference that was not investigated with the previous plasmid isolation methods.

When using the standard kit protocol, the Nucleobond$^{(R)}$ Xtra Midi plasmid purification kit (Macherey-Nagel) effectively isolates plasmid pKara (6.2 kb) from DSM 9293^{T}, but very low to undetectable yields are found for the larger plasmids of DSM 9293^{T} (±80 kb), YOO17 (±80 kb and ±120 kb) and MT13 (± 170 kb) (Figure 2.9).

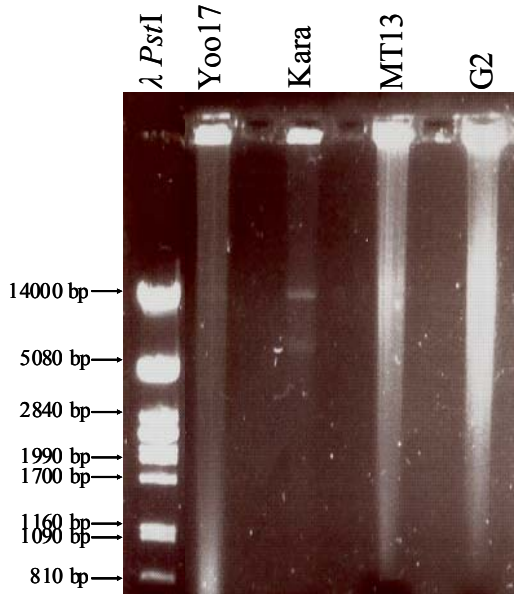

Figure 2.9 Agarose gel of Nucleobond plasmid preparations from *Sulfobacillus* strains YOO17, DSM 9293^{T}, MT13 and G2.

Nucleobond plasmid preparations from *Sulfobacillus* strains YOO17, DSM 9293^{T}, MT13 and G2 revealed plasmid DNA only in DSM 9293^{T}, while smears of genomic DNA were visible in the other preparations. λ *Pst*I was used as molecular weight marker.

When treatment with lysozyme and three cycles of freeze-thawing were incorporated in the method, the yield of plasmid DNA increased markedly (Figure 2.10, Figure 2.11). Digestion of the plasmid preparations with *Sal*I and addition of the fragment sizes resulted in a total of ± 70 kb for the plasmid of L15 and ± 80 kb for the plasmid of YOO17, which is in agreement with the estimated sizes of the linearised plasmids observed after TAFE (Figure 2.2).

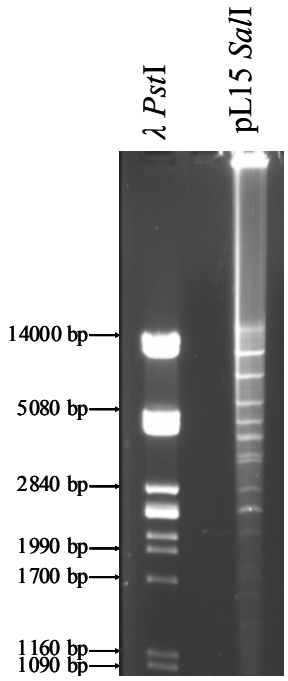

Figure 2.10 Agarose gel of Nucleobond plasmid preparation from *Sulfobacillus* strain L15.

Cells of *Sulfobacillus* strain L15 were subjected to freeze-thaw cycles and lysozyme treatment before using the low copy number protocol of the Nucleobond plasmid isolation kit. Here, the relative enrichment of plasmid versus chromosome is sufficient for further characterization of the plasmid. Plasmid DNA was digested with *Sal*I and separated on a 0.8% agarose gel. λ *Pst*I was used as molecular weight marker.

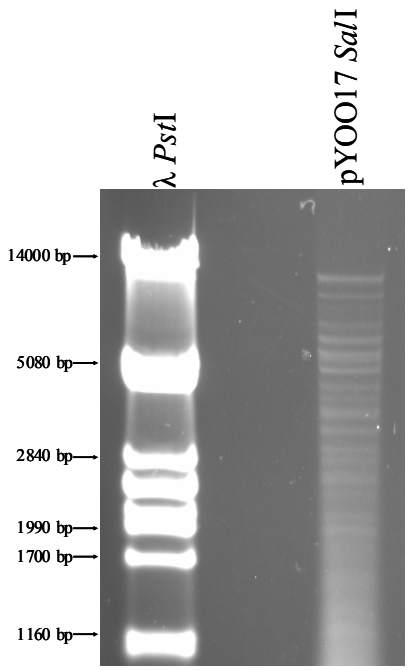

Figure 2.11 Agarose gel of Nucleobond plasmid preparation from *Sulfobacillus* strain Y0017, digested with *Sal*I

One half of the plasmid preparation was digested with *Sal*I, concentrated by evaporation and loaded on this gel. Although the concentration of plasmid DNA was low, plasmid enrichment relative to chromosomal DNA was sufficient to allow visualization of plasmid fragments. λ *Pst*I was used as molecular weight marker.

2.4.4.4. Alkaline lysis minipreparations

Since the yield of large plasmid DNA from *Sulfobacillus* remained nearly undetectable, while large plasmids could be recovered from *E. coli* by the same methods, plasmid isolations from *Sulfobacillus* was optimised by studying the small plasmid of strain DSM 9293^T.

It is possible to isolate DNA of the 6.2-kb plasmid from *Sulfobacillus* strain DSM 9293^T by slight modification of the Ish-Horowicz small scale plasmid preparation method. While lysozyme and proteinase K treatments improved the plasmid yield slightly, thorough washing of cells to remove growth medium constituents had the greatest positive effect on plasmid yield (Figure 2.12).

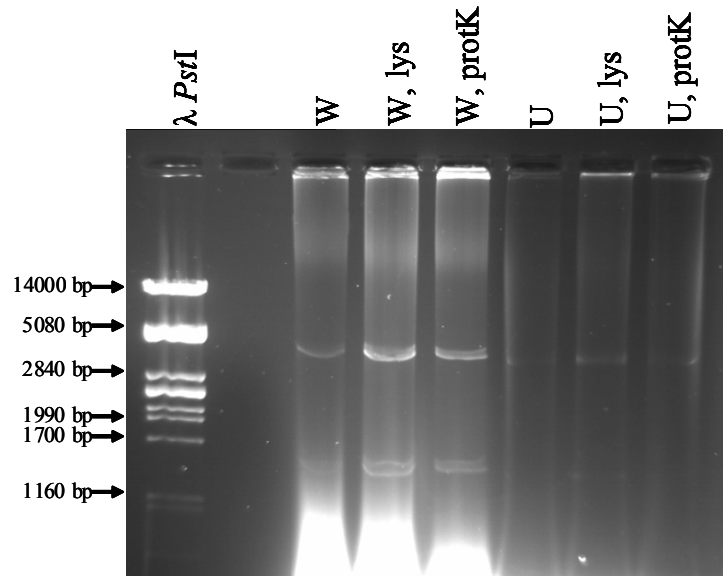

Figure 2.12 Agarose gel of alkaline lysis minipreparations of pKara from *Sulfobacillus* strain DSM 9293^T.

Equal volumes of each plasmid preparation were digested with *Sal*I. The treatment that *Sulfobacillus* cells received before lysis is indicated above each lane. W: washed, U: unwashed, lys: lysozyme treated, protK: proteinase K treated. λ *Pst*I was used as molecular weight marker.

2.4.5. Plasmid capture

Plasmids were clearly visible in pulsed field gels (Figure 2.2), yet the amounts of purified plasmid DNA obtained were too little to allow restriction mapping and cloning of large plasmids of *Sulfobacillus.* Propagation of plasmids from *Sulfobacillus* strains in *E. coli* would facilitate the recovery of larger quantities of plasmid DNA. In a previous study, the 6.2-kb plasmid (pKara) of strain DSM 9293^T was "captured" using the EZ::TN<R6Kγori/KAN-2> Insertion Kit to introduce an *E. coli* origin of replication and kanamycin marker onto the plasmid. The same approach was followed for capturing plasmids of strain YOO17.

Although very little plasmid DNA could be purified from strain YOO17, pooling of two plasmid isolations (Figure 2.5 and Figure 2.11) resulted in a small amount of plasmid-containing solution with a concentration of 114 ng/µl. This DNA was used for the *in vitro* transposon jumping reaction.

The transposon jumping mix was introduced into *E. coli* EC100D *pir*$^{+}$ by electroporation and transformants were selected on kanamycin. Alkaline lysis plasmid preparations from kanamycin resistant colonies resulting from introduction of the *in vitro* transposon jumping reaction mix into *E. coli* EC100D *pir*$^{+}$ were separated on an 0.8% agarose gel.

Of twenty kanamycin resistant single colonies that were screened, all were false positives generated self-integration of the transposon cassette. Six pools of 48 colonies each were made and plasmid DNA prepared from the pools. None of the pools contained DNA species larger than two Kilobases. Thus, of a total of 308 colonies screened, all were false positives. Plasmid bands larger than 2 kb that would indicate successful capture of the *Sulfobacillus* YOO17 plasmid were not observed in any plasmid preparation.

Electroporation efficiency for the pUC19-based control plasmid, expressed as the total number of ampicillin resistant colonies per microgram of electroporated plasmid DNA, was 2.35×10^5. Approximately one in ten ampicillin resistant colonies was also kanamycin resistant, showing that the transposon had been integrated into one tenth of the control plasmids that were successfully transformed into *E. coli*.

2.4.6. Analysis of Sulfobacillus plasmids

2.4.6.1. Comparison of plasmids by Southern blotting.

Subclones of the transposon captured version of the 6.2-kb plasmid of strain DSM 9293^{T} (Honours study, Joubert, 2002, University of Stellenbosch, 2002) were one source of probes. Cloned fragments of DNA isolated from strain YOO17 by the gentle lysis cesium chloride gradient method (section 2.4.4.2, Figure 2.5) was another source.

When DIG-labelled probes derived from pKara were used to probe against DNA from strains YOO17, MT13, G2 and DSM 9293^{T}, plasmid bands were detected in the lanes containing DSM 9293^{T} DNA, but not in lanes containing DNA of Y0017, MT13 or G2 (Figure 2.13). In the S1 nuclease treated sample of DSM 9293^{T} DNA, an additional low molecular weight signal was detected, corresponding to linearised plasmid DNA. The signal above that corresponds to supercoiled plasmid DNA, while the third (top) signal corresponds to open circular (nicked) plasmid DNA. DNA in the wells also hybridised to the DIG-labelled probe.

This is probably plasmid DNA that is still trapped within cellular debris, as the plasmid probe did not bind to the chromosomal DNA that entered the gel matrix.

When a DIG-labelled probe derived from cloned *Sal*I fragment of pYOO17 was used, the probe hybridised with YOO17 DNA but not with DNA of MT13, G2, or DSM 9293^T. The clear, sharp plasmid bands visible on the gel photograph are not detected by Southern blotting. Rather, DNA in the wells and chromosomal region are detected, and an additional band that appears after S1 treatment of the gel plug. Chromosomal DNA of other *Sulfobacillus* strains was not detected. This implies either that the probe is derived from a plasmid that is not visible on the gel photograph, or that the probe is a chromosomal fragment that is unique to the YOO17 strain.

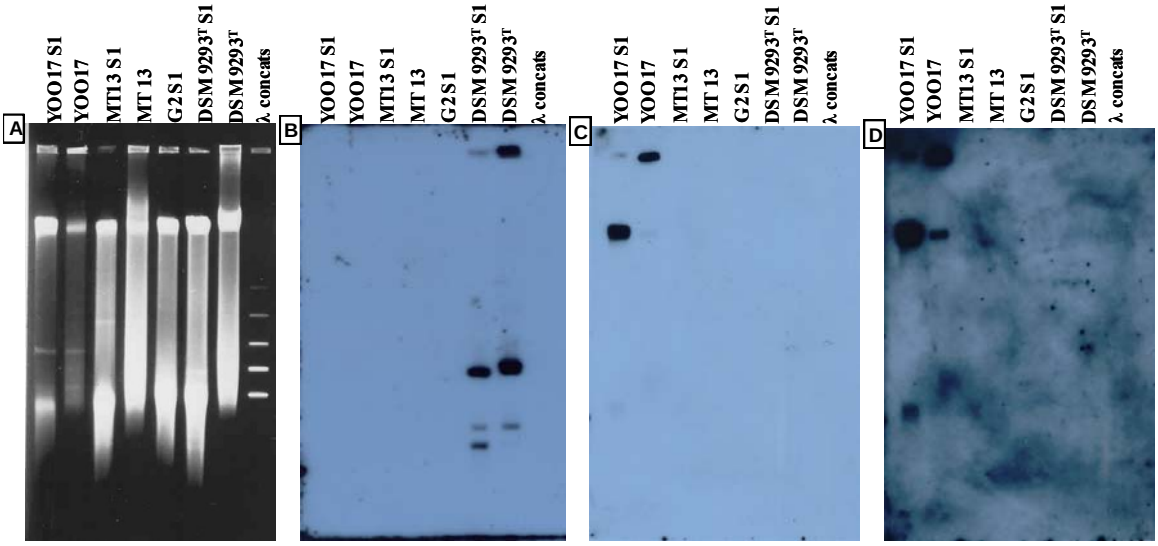

Figure 2.13 Comparison of *Sulfobacillus* plasmids by Southern blotting.

[A] TAFE gel showing DNA of strains Y0017, MT13, G2 and DSM 9293^T. [B] Southern blot of TAFE gel probed with pKara fragment. [C] Southern blot of TAFE gel, probed with YOO17 fragment. [D]: longer exposure of C.

λ concats: concatemers of lambda DNA. S1: DNA was treated with S1 nuclease.

2.4.6.2. Methylation patterns

To determine the appropriate methylation status of vectors used for transformation of *Sulfobacillus* in order to minimise degradation by endogenous nucleases, the methylation status of native *Sulfobacillus* plasmids was investigated.

Plasmid DNA was isolated directly from *Sulfobacillus* strain DSM 9293^T by using a Nucleobond plasmid isolation kit and digested with a selection of restriction endonucleases that are all affected by some type of methylation at the recognition site. The digested plasmid fragments were separated on an agarose gel and compared to the fragment sizes predicted from sequence analysis. Restriction enzymes *Apa*I, *Bgl*II, *Nco*I, *Sac*I, *Sal*I, *Sma*I, *Spe*I and *Xba*I all cut plasmid pKara at their predicted unique recognition sites. *Cla*I cut DNA of pKara at only one of three recognition sites, and *Stu*I did not digest pKara at its unique recognition site. The activity of the *Stu*I enzyme was verified by digestion of standard cloning vectors.

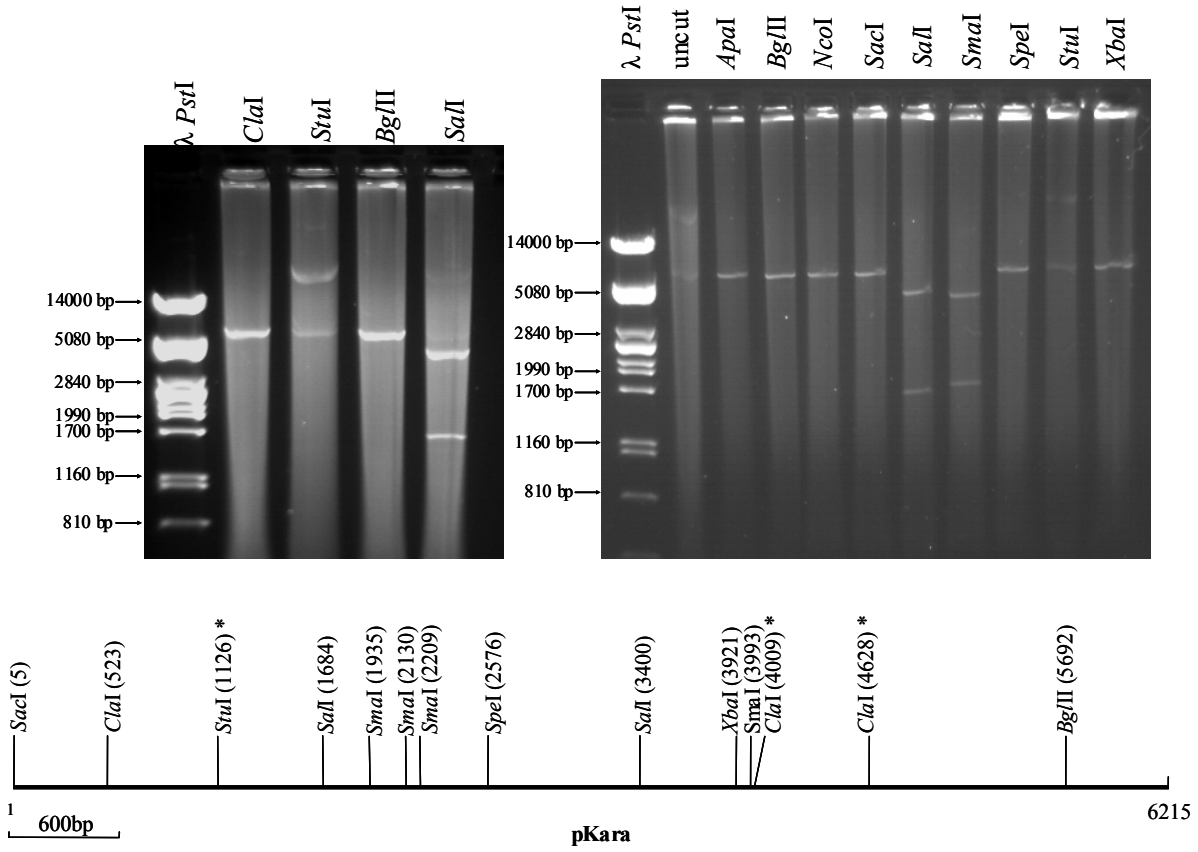

Figure 2.14 pKara methylation test.

Plasmid DNA was isolated from *Sulfobacillus* strain DSM 9293^T by using a Nucleobond plasmid isolation kit, digested with restriction endonucleases and separated on an agarose gel. A plasmid map of pKara, with relevant restriction sites predicted from sequence (Chapter 3), is shown. Restriction sites with overlapping *dam* or *dcm* methylation sites are indicated with asterisks. λ *Pst*I was used as molecular weight marker.

2.5. Discussion

2.5.1. Isolation and grouping of Sulfobacillus strains

Three strains of *Sulfobacillus*, originating from commercial biomining operations in Africa, were purified and added to our culture collection. The isolation of pure cultures of *Sulfobacillus* strains from mixed cultures is relatively simple. The sugars released by acid hydrolysis of agarose inhibit many Gram-negative acidophiles that share the same habitat as sulfobacilli, while all strains of *Sulfobacillus* that were tested could grow on simple FeSo solid media, without the underlay of acidophilic heterotrophs that is needed for growth of more fastidious iron oxidising acidophiles such as *Acidithiobacillus caldus* (Johnson, 1995). This is in contrast to Bridge and Johnson (1998) who found that *Sulfobacillus* strains THWX and YTF1 did not grow on plates without the underlay. Omitting the underlay thus eliminated many of the acidophilic bacteria that share the same ecological niche, but which are sensitive to organic sugars. Similar results were found by Atkinson *et al.* (2000) where *Sulfobacillus* and *Alicyclobacillus* strains formed colonies on phytagel-solidified plates without an underlay, while *Acidimicrobium* strains could only be recovered on plates with an underlay. The characteristic "fried-egg" colony morphology of the sulfobacilli, coupled with their fast growth on solid media allowed a rapid crude screening before final identification using Amplified Ribosomal DNA Restriction Enzyme Analysis (ARDREA) (Rawlings, 1995; Rawlings *et al.,* 1999; Schutte, 2004; Johnson *et al.,* 2005).

ARDREA analysis placed the newly isolated strains, 611(2005), Andy and Mintek45 in *Sulfobacillus* subgroup I (the S. *thermosulfidooxidans* group) (Johnson *et al.,* 2005). This is not surprising, as it has been reported that conditions found in bioreactor-type environments favour sulfobacilli of the *S. thermosulfidooxidans* group over other species within this genus (Robertson *et al.,* 2002; Cleaver *et al*., 2007).

2.5.2. Plasmid survey

At the start of this project, three *Sulfobacillus* plasmids were known, pKara (Honours study, University of Stellenbosch, 2002), pSulfBC1 and pTHWX (personal communication, Paul Norris). In this study, plasmid bands were observed in six of nine strains tested.

A simple small scale alkaline lysis plasmid preparation procedure was sufficient to visualise the 6.2-kb plasmid in *Sulfobacillus* strain DSM 9293^T (Figure 2.12) but no other plasmids could be isolated with this method. Simple alkaline lysis procedures are effective in isolating plasmids smaller than 40 mDa, but gentler techniques are required to visualise larger plasmids.

To detect larger plasmids, which might be missed with standard isolation techniques, the in-gel lysis method of Barton and co-workers (1995) was adopted. This method revealed the presence of plasmids in almost all strains of *Sulfobacillus* that were tested (Table 2.2). The plasmid profiles differed between strains of sulfobacilli (Figure 2.2), indicating a substantial diversity of plasmids present in strains of this genus. With the exception of the 6.2-kb plasmid of strain DSM 9293^T, the plasmid bands visualised by the method of Barton and co-workers all migrated above the 50 kb linear size marker (Figure 2.2).

Table 2.2 Summary of plasmid survey results

Strain	Method	Plasmid(s) detected
Andy	Barton *et al.,*1995	No plasmids
611	Barton *et al.,*1995	One plasmid ± 50 kb
DSM 9293^T	Barton *et al.,*1995 Ish-Horowicz plasmid minipreparations Nucleobond plasmid preparation kit	Two plasmids 6.2 kb and ± 80 kb One plasmid, 6.2 kb One plasmid, 6.2 kb
L15	Barton *et al.,* 1995 Nucleobond plasmid preparation kit, with freeze-thaw cycles	One plasmid ±70 kb One plasmid ±70 kb
Y0017	Barton *et al.,*1995 Nucleobond plasmid preparation kit, with freeze-thaw cycles	Two plasmids ± 80 kb and ± 120 kb One plasmid ± 80 kb
MT13	Barton *et al.,*1995	One plasmid ± 170 kb
G2	Barton *et al.,*1995	No plasmids
THWX	Barton *et al.,*1995	One plasmid ± 260 kb
TH1	Barton *et al.,*1995	One plasmid ± 260 kb

Although techniques utilizing in-gel lysis and pulsed field gel electrophoresis are effective and widely used, variability across species and research groups is considerable (Stillwell *et al.,* 1995). Even within the same method and strain, reproducibility can be problematic. Compare the plasmid profiles obtained for plasmid preparations of *Sulfobacillus* strains MT13 and THWX done on different days (Figure 2.2). A disadvantage of this method is that chromosomal DNA is not eliminated and can enter the gel matrix (Claesson *et al.,* 2006). This may mask the presence of plasmids that co-migrate with the chromosomal DNA band (Anderson and McKay, 1983).

2.5.3. Plasmid isolations

Although plasmids ranging from 6.2 kb to 260 kb were easily visualised in *Sulfobacillus* strains by pulsed field gel electrophoresis, isolation of workable amounts of the larger plasmids was problematic. Purifying large plasmids from gel band is not trivial, especially when working with very small quantities. Plasmid bands visualised on TAFE gels could not be recovered from the gel matrix. Commercial kits available for recovery of DNA from gel bands did not cater for DNA fragments above 48 kb (GFX PCR DNA and gel band purification kit, Amersham).

The "freeze-squeeze" method, where the gel matrix is destroyed by phenol treatment and centrifugation also did not yield workable amounts of plasmid DNA. No plasmid DNA was recovered by electro-elution from gel slices.

Attempts to purify *Sulfobacillus* plasmids from liquid cultures met with varying amounts of success. The small 6.2-kb plasmid from *Sulfobacillus* strain DSM 9293^T could be easily isolated via simple alkaline lysis procedures or by the standard protocol of the Nucleobond plasmid isolation kit. The most important factor in the isolation of this smaller plasmid was thorough washing of cells to remove as much iron as possible. Ferrous iron has been shown to induce single stranded breaks in plasmid DNA, converting supercoiled forms to open circular forms (Chen *et al.,* 2007). Open circular plasmid DNA is not recovered by most plasmid isolation procedures, therefore, a marked decrease in plasmid recovery results when plasmid DNA is damaged in this way.

The method of Kado and Liu could neither purify nor visualise plasmids from any strain of *Sulfobacillus,* even though large and small plasmids could be demonstrated in *E. coli* with the

same technique. The gentle lysis cesium gradient method could isolate minute amounts of plasmid DNA from a two plasmid-containing *Sulfobacillus* strains. Finally, a modification of the Nucleobond kit protocol, incorporating freeze-thaw cycles to weaken the cell envelope, allowed the purification of small amounts of plasmid DNA.

2.5.4. Plasmid capture

Attempts to use the transposon capture method to propagate plasmids in the range of ± 80 kb from *Sulfobacillus* strain YOO17 in *E. coli* have failed.

It has proven to be very difficult to obtain plasmid preparations of high quality from sulfobacilli. With the exception of preparations of plasmids pKara from *Sulfobacillus* strain DSM 9293^T and pL15 from strain L15 (which was isolated after the attempt to capture plasmids from YOO17); all plasmid preparations from sulfobacilli had low concentrations of plasmid DNA and were contaminated with chromosomal fragments. Most plasmids detected in strains of *Sulfobacillus* were larger than 50 kb. These conditions are not ideal for transposon rescue of plasmids.

In spite of these limitations, the *in vitro* transposition system seemed to be a viable option for capturing plasmids from *Sulfobacillus* plasmid preparations. A similar system has been used to capture three plasmids from a 14-year-old crude DNA extract of *Mycobacterium avum*, in which only chromosomal DNA could be visualised (Kirby *et al.,* 2002). The plasmid preparations from strain YOO17 were at least highly enriched for plasmid DNA and should have been acceptable targets for *in vitro* transposition.

According to the EZ::TN<R6Kγori/KAN-2> Insertion Kit manual, the *in vitro* transposition reaction requires 200 ng of target DNA in less than 8 µl. This amount of YOO17 plasmid DNA could be obtained by pooling and concentrating available plasmid samples.

After transposition and transformation, many kanamycin resistant colonies were obtained, but all were false positives. The large number of false positives generated are the result of a transposon being inserted into itself in regions that are not essential for replication or kanamycin resistance, and thus generating a circular molecule capable of autonomous replication (Kirby *et al.,* 2002). For the *in vitro* transposition reaction, transposon and plasmid DNA should be mixed in a 1:1 molar ratio to ensure that, on average, one transposon

insertion per plasmid molecule occurs. The actual molar ratio of transposon to plasmid was difficult to establish with accuracy in this case, since the inferior quality of the plasmid preparation allowed only rough estimates of plasmid size and concentration. The large number of false positives suggested that the plasmid concentration, and thus the number of plasmid targets for transposition, was lower than estimated.

Two strategies now exist for improving the plasmid capture procedure. Firstly, a much lower concentration of transposon DNA should be used in the *in vitro* transposition reaction. Unfortunately, most of the plasmid DNA obtained from *Sulfobacillus* strain YOO17 was used in the first attempt, therefore this strategy must wait on isolation of more plasmid DNA.

A second strategy would be to prepare a DIG-labelled probe from YOO17 plasmid DNA and re-screen the transposon bank by colony hybridization. The YOO17 plasmid preparation is contaminated with chromosomal DNA, so there is risk of non-specific hybridization with chromosomal DNA of *E. coli*. However, there is a considerable phylogenetic distance between the sulfobacilli and *E. coli,* therefore background hybridization between chromosomal fragments should be resolved by increasing the stringency of the washing steps.

Despite the initial failure of the transposon-capture method, it remains an attractive option and is worth pursuing.

2.5.5. Analysis of Sulfobacillus plasmids

2.5.5.1. Comparison of plasmids by Southern blotting

When the plasmids of strains Y0017, MT13, G2 and DSM 9293^T were compared by Southern blotting, three probes derived from different cloned regions of plasmid pKara hybridized to DSM 9293^T DNA only at a position corresponding to the 6.2-kb plasmid (Figure 2.13B), indicating that there is not a high degree of nucleotide similarity between pKara and DNA of *Sulfobacillus* strains Y0017, MT13 and G2. In the S1 nuclease treated DSM 9293^T sample, the hybridization signal at the position of the wells diminished in comparison to the untreated sample and an additional faster migrating plasmid band appeared. It is possible that the signal at the position of the wells represents plasmid DNA that was trapped within cell debris or chromosomal DNA that remained in the well and that was linearised and thus released by the S1 treatment. If the conventional migration order of DNA species in a pulsed field system is

preserved, the second and third lowest plasmid bands should correspond to supercoiled and open circular plasmid forms. However, one would expect the supercoiled DNA signal to diminish in the S1 treated sample and yet the relative intensity of this band stayed the same in the treated and untreated samples. It is possible that the torsional stress due to supercoiling is less in a smaller plasmid molecule than in larger plasmids, and therefore was not sufficient to pull apart the DNA strands. The S1 nuclease treatment would then only act on open circular forms of the plasmid. The third fastest migrating DNA species behaved like open circular plasmid DNA as the intensity of this band diminished in the S1 treated sample.

Probes derived from YOO17 plasmid DNA isolated by the gentle lysis method (Figure 2.6) hybridised only to DNA of YOO17 (Figure 2.13C and D), indicating that plasmid pYOO17 also contains unique DNA sequences. The YOO17 probe did not hybridise to the visible plasmid bands migrating at 80 kb and 120 kb but rather to positions corresponding to the wells and diffuse chromosomal DNA as well as a diffuse band appearing below the 48-kb linear marker in the S1 treated sample. Once again, S1 treatment released DNA from the wells.

Two possible explanations exist for the hybridisation pattern seen with the YOO17 probe. The first possibility is that more than one plasmid is present in strain YOO17 and that the cloned *Sal*I fragment is derived from a plasmid that co-migrates with the diffuse chromosomal band on the TAFE gel and was therefore undetected before Southern blotting.

The second possibility is that the cloned *Sal*I fragments used to make the DIG-labelled probes were in fact chromosomal fragments that are present in the YOO17 strain but not in MT13, G2 or DSM 9293^{T}. The possibility of cloning chromosomal fragments of YOO17 was introduced because of the nature of the source of DNA used for making the DIG-labelled probe. Unlike pKara, plasmids from YOO17 have not been cloned or propagated in *E. coli*. Therefore plasmid DNA was isolated directly from *Sulfobacillus* strain YOO17. A fraction drawn from a cesium chloride density gradient, three centimetres below the visible chromosomal band, contained small amounts of DNA (Figure 2.5). This DNA was digested with *Sal*I and cloned and propagated in *E. coli* before making the probe. It is possible that chromosomal DNA still contaminated the plasmid preparation and was cloned instead of plasmid fragments.

2.5.5.2. Methylation patterns

DNA of plasmid pKara could be easily isolated and the DNA sequence of pKara was known (Honours study, University of Stellenbosch, 2002 and Chapter 3). Therefore restriction patterns could be predicted and compared to patterns obtained by digestion of plasmid DNA.

Both *dam*- and *dcm*-type methylases were active in *Sulfobacillus* strain DSM 9293^T. This was concluded from the observation that plasmid DNA isolated from *Sulfobacillus* strain DSM 9293^T was not digested at restriction endonuclease recognition sites that have overlapping *dam* or *dcm* methylation sites. Three recognition sites for *Cla*I were predicted from sequence analysis, with two sites having an overlapping *dam* methylation site, but digestion of pKara yielded one fragment only. Only one *Stu*I recognition site is predicted on pKara and it has an overlapping *dcm* methylation site. Plasmid pKara remained uncut when digested with *Stu*I. *E. coli* vector DNA was restricted by *Stu*I (not shown) and other enzymes were able to digest DNA pKara from the same preparation, therefore lack of restriction of pKara by *Stu*I was caused by modification of the plasmid DNA at the recognition site of *Stu*I, rather than a defective enzyme or impure plasmid DNA. Other enzymes which are susceptible to a variety of overlapping methylation patterns were not inhibited, indicating that a general cytosine or adenine methylase was not present.

The role of the *dam* and *dcm* methylases in *Sulfobacillus* DSM 9293^T may be analogous to those of *dam* and *dcm* methylases in *E. coli*. Alternatively, they may form part of restriction modification systems. This finding must be kept in mind when preparing DNA that is to be transformed into *Sulfobacillus* strains. It is important that the *E. coli* host from which such DNA is to be prepared must be both *dam* and *dcm* positive, to decrease the chances of an endogenous *Sulfobacillus* endonuclease destroying incoming DNA

2.5.6. Conclusions

The reported prevalence of *S. thermosulfidooxidans* strains in bioreactor-type environments (Robertson *et al.,* 2002) is supported by isolation of three *S. thermosulfidooxidans*-like strains from African biomining operations.

Plasmids are common in species of *Sulfobacillus*. The diversity of the observed plasmids is considerable, with the estimated sizes ranging from 6.2 kb to 260 kb. Isolation of plasmid

DNA from *Sulfobacillus* is possible, although further optimization of methods is needed to improve yields of larger plasmids.

Plasmid DNA in *S. thermosulfidooxidans* DSM 9293^{T} is modified at both *dam*- and *dcm*-recognition sites, therefore plasmids that are to be used during attempts to transform sulfobacilli should be prepared from *dam* and *dcm* positive hosts.

CHAPTER THREE
PLASMID SEQUENCING AND ANNOTATION

3.1. Aim

The aim of this part of the study was to investigate the sequence diversity of *Sulfobacillus* plasmids and to identify the replication mechanism(s) and accessory genes of three sequenced plasmids.

3.2. Introduction

Plasmids are circular or linear DNA species that replicate separately from the host chromosome(s). Besides the genetic determinants that are essential for the persistence (replication and stability) and dissemination (conjugation or mobilization) of the plasmid, other nonessential genes may also be encoded on plasmids.

Knowledge of the DNA sequences of native plasmids of a bacterial species is useful in three areas. Firstly, in the absence of whole genome sequences, it provides a glimpse into the mobile gene pool that is accessible and useful to that bacterium. Native plasmids may bear genes that confer an adaptive advantage to a bacterium under certain conditions (e.g. antibiotic or metal resistance). It is also of interest to discover whether the mobile gene pool of biomining bacteria such as *Sulfobacillus* is separate from that of bacteria in other environments, or whether plasmids of these bacteria can acquire genes from outside the biomining environment.

Secondly, native plasmids have often been used as the backbone for the construction of cloning vectors. For this purpose, it is useful to identify the regions of the plasmid that are required for replication and maintenance and which areas of the plasmid can be cut away to construct a more compact vector. Identifying the replication mechanism of a plasmid is important, as this can affect the usefulness of the plasmid for specific applications. For instance, a plasmid that replicates via the rolling circle mechanism may be expected to be incapable of supporting large DNA inserts without DNA deletions or rearrangements, but may be suitable for cloning of smaller inserts. Resistance genes encoded on indigenous plasmids can sometimes be used as selectable markers in the construction of a cloning vector.

Thirdly, a detailed knowledge of the replication regions of native plasmids may lead to the identification of existing cloning vectors with similar replicons that may also be supported in the bacterial host, and/or may assist in avoiding problems of plasmid incompatibility when introducing highly similar plasmids into the same cell.

The availability of the DNA sequences of plasmids pSulfBC1, pTHWX and pKara, isolated from *Sulfobacillus* strains, BC1, THWX and DSM 9293^{T} respectively, presented an opportunity to identify what types of replicons are maintained in sulfobacilli, which accessory genes are selected for in their environment and to what extent these differ from plasmids of other species or habitats.

Gene annotation is assisted by the availability of large publicly accessible databases of annotated sequence data to which novel sequences can be compared. In addition, software suites further streamline this process. However, the nature of the DNA sequence under investigation and the theoretical basis of the algorithms used by the software must be taken into account when evaluating the quality of data obtained from these aids.

The Glimmer2 software was designed to find genes in the genomes of bacteria, archaea and viruses. It has been used with success by The Institute for Genomic Research (TIGR) to annotate complete genomes of bacterial species, where the accuracy of gene prediction approaches 99%.

Glimmer2 uses interpolated Markov models to formulate sets of gene predictions. The basis for this method lies in the ability of interpolated Markov models to define patterns in datasets. Therefore, when supplied with a set of known bacterial genes, the Glimmer software can extract a characteristic gene pattern from the dataset, which it then uses to find uncharacterized genes within the same genome. The quality of the gene predictions generated by the software is dependent on the quality of the dataset provided. An ideal dataset would consist of genes that have been identified though experimental methods (e.g. RNA was detected) or though significant similarity to other characterized genes. In the absence of this knowledge, a dataset can be generated by collecting sequences of large open reading frames that are statistically unlikely to occur randomly (Delcher *et al*., 1999). When using Glimmer to find genes on plasmids, one must remember that the dataset used to create a predictive Markov model is considerably smaller and therefore the sensitivity and accuracy of predictions may be eroded.

3.3. Materials and Methods

3.3.1. Plasmid sequencing

Two unannotated plasmid sequences (pSulfBC1 and pTHWX) were provided by Prof. Norris and were further annotated during this study. Plasmid pSulfBC1 was isolated from *Sulfobacillus* strain BC1 which originated from a coal spoil in the United Kingdom (Norris *et al.,* 1996). Plasmid pTHWX was isolated from *Sulfobacillus* strain THWX which originated from a self heating coal spoil in Wrexham, North Wales (Ghauri and Johnson, 1991). The sequences of pKara, pSulfBC1 and pTHWX were compared to each other.

Plasmid pKara had been isolated, restriction mapped and partially sequenced in this laboratory. The EZ::TN<R6Kγori/KAN-2> transposon rescue kit (Epicenter, Madison, Wisconsin) was used to introduce an *E. coli* origin of replication and selectable marker onto the native 6.2-kb plasmid of *Sulfobacillus* strain DSM 9293^{T}. The library of random transposon insertions made during my honours study (Joubert, University of Stellenbosch, 2002) allowed partial sequencing of pKara. Sequencing and annotation of pKara were completed during this study.

In order to complete sequencing of pKara, subclones were made of pSulfTA, one of the transposon-rescued derivatives of pKara (Figure 3.1). DNA fragments to be used for cloning were excised from agarose gels and extracted using the GFX™ kit (Amersham® Biosciences Corporation).

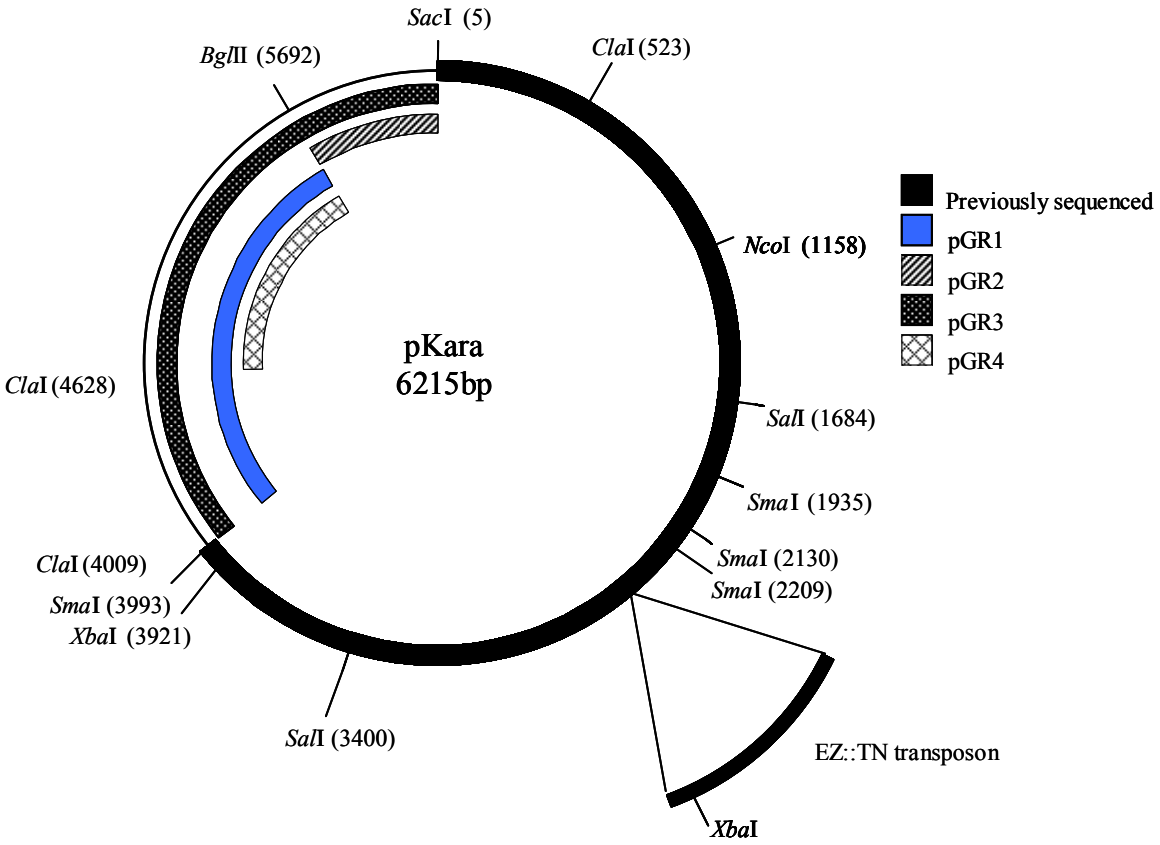

Figure 3.1 Restriction map of pKara, with the subclones used for sequencing indicated.

Only restriction sites that were relevant to the subcloning strategies are shown. pGR3 is the 2222 bp *Sma*I to *Sac*I fragment cloned in pUCBM21. pGR2 is the 523 bp *Sac*I-*Bgl*II fragment cloned in pUCBM21. pGR1 is the 1683 bp *Bgl*II-*Cla*I(4009) fragment cloned into pUCBM21. pGR4 is the 1064 bp *Bgl*II-*Cla*I(4628) fragment cloned in pUCBM21. The *Cla*I site at bp 4628 has an overlapping *dam* methylation site.

Plasmid pSulfTA was digested with *Sac*I and *Xba*I, to produce three fragments of 1610, 2299 and 4316 bp respectively. The 2299 bp *Sac*I-*Xba*I fragment was gel-excised and digested with *Sma*I. The 2227 bp *Sac*I-*Sma*I fragment was ligated to *Sac*I-*Sma*I digested pUCBM21, producing construct pGR3, and transformed into *E. coli* DH5α. This fragment was further subcloned in two pieces. A *Sac*I-*Bgl*II fragment was ligated into *Sac*I-*Bam*HI digested pUCBM21 to yield construct pGR2. The *Sma*I-*Bgl*II fragment was recovered by digesting pGR3 with *Bgl*II and *Eco*RI (cuts within the multiple cloning site of pUCBM21). This fragment was ligated to *Bam*HI-*Eco*RI digested pUCBM21 and transformed into DH5α to

yield construct pGR1. The *Sma*I-*Bgl*II and *Sac*I-*Bgl*II fragments of pKara were sequenced using the M13 sequencing primers.

To obtain the last part of the pKara sequence, construct pGR4 was created by cloning the 1064 bp fragment stretching from the *Bgl*II site to the adjacent *dam*-methylated *Cla*I site (at position 4628) of pKara. First, the *Sma*I-*Bgl*II clone (pGR1) was transferred to the *dam*$^-$ *E. coli* GM41. Unmethylated plasmid DNA isolated from this strain was digested with *Sal*I and *Cla*I. The *Sal*I recognition site fell within the pUCBM21 multiple cloning site. The 1105 bp *Sal*I-*Cla*I fragment was ligated into *Sal*I-*Cla*I digested pBluescript KS$^+$ vector. This construct was named pGR4 and sequenced using the M13 sequencing primers.

The complete sequence of the plasmid was assembled using the DNAMAN software package (Lynnon Bio-Soft), and the integrity of the assembled sequence was checked by comparing restriction digest patterns with patterns predicted from sequence data.

3.3.2. Bioinformatic analysis of plasmid sequences.

The DNA sequences of the three plasmids were analysed with the following software and web-resources: Glimmer2, RBS finder and Transterm (Salzberg *et al.,* 1988; Delcher *et al.,* 1999; www.tigr.org/softlab); BLAST (Altschul *et al.,* 1997; http://www.ncbi.nlm.nih.gov); Proteinfinder (http://nbc11.biologie.uni-kl.de/framed/left/menu/auto/right/glimmer2.02/); DNAMAN (Lynnon Bio-Soft); Artemis (Rutherford *et al.,* 2000); TMHMM (http://www.cbs.dtu.dk/services/TMHMM/); ClustalW (Wilbur and Lipman, 1983; http://www.ebi.ac.uk/Tools/clustalw/index.html#) and InterProScan (Zdobnov and Apweiler, 2001; http://www.ebi.ac.uk/InterProScan/)

3.3.3. Prediction of the most likely protein coding sequences

To find regions of the plasmids that could encode proteins with similarity to known proteins in the database, 1000 bp fragments of the plasmid sequences, each overlapping the next by 500 bp were used as input for the *tblastx* program. The Glimmer2 predicted protein coding sequences, and other ORFS longer than 150 bp, were translated and used for *blastp* searches.

To find protein coding sequences that were not similar to known sequences in public databases and might be missed by similarity searches, the *Glimmer2* program was employed.

Two methods were used to generate the interpolated Markov model used for gene predictions. The first training set for the construction of the interpolated Markov model was a collection of the longest open reading frames which were extracted from the plasmid sequences. The second training set was composed of published open reading frames from *Sulfobacillus* chromosomal sequences.

To find regions of plasmid DNA with similarity to non-coding DNA sequences in public databases, 1000 bp fragments, overlapping by 500 bp, of the plasmid sequences were used as input for the *blastn* algorithm. Possible ribosome binding sites were detected using the *RBS finder* software and visual inspection of sequences upstream of putative start codons. Direct and inverted repeat sequences were located using the DNAMAN software package. Sequence annotations were compiled using the *Artemis* software package.

3.4. Results and Discussion

The complete nucleotide sequence of plasmid pKara from *Sulfobacillus* DSM 9293^T was determined in this study. The unannotated sequences of pSulfBC1 and pTHWX were kindly provided by Prof Norris.

3.4.1. pKara

Plasmid pKara was completely sequenced (Appendix 3, section 9.1.2) in both directions by using a combination of subcloning and sequencing from primers located within the randomly inserted transposon sequences. Restriction digest patterns of pKara (Chapter 2, Figure 2.14) agreed with patterns predicted from sequence analysis, confirming the accuracy of sequence assembly.

3.4.1.1. General features of pKara

Plasmid pKara is 6215 bp in length and has an overall GC ratio of 54.89%. This plasmid had very little homology to known sequences, both at the nucleic acid level and the level of amino acid sequences predicted from the DNA sequence. pKara was predicted to encode five proteins. In no case did a high percentage of sequence similarity to proteins in publicly accessible databases extend over more than half the predicted protein. The only indication of possible protein functions came from short conserved domains and secondary structure

predictions found within the predicted proteins. The gene predictions for plasmid pKara are summarised in Table 3.1.

The web-based Glimmer2 program predicted the same five possible protein coding open reading frames irrespective of where the start of the sequence was defined. All five genes start with a methionine residue and are preceded by a ribosome binding site. All the predicted open reading frames are in the same orientation.

3.4.1.2. Replication region of pKara

Certain common characteristics of plasmid origins of replication can be used to assist prediction of novel plasmid replicons. Plasmid origins of replication are often located in regions of DNA with intrinsic curvature or with high potential of forming secondary structures such as base-paired stem-loops. Other common features found in replication origins are short DNA repeats (iterons), AT-rich sequences and consensus motifs for binding of host initiation factors (e.g. *dna*A boxes) or plasmid-encoded replication proteins (e.g. nick sites of rolling circle replication initiation proteins) (del Solar *et al*., 1998).

A region of DNA (from bp 4142 to bp 6167) was identified within the sequence of pKara that has the potential to form extensive secondary structures and that contains four 21 or 22 bp imperfectly repeated iterons (bp 5191 to bp 5274). Inverted repeats with the potential to form stem-loop structures occur on both sides of the iterons. The first pair of iterons, with the sequence: 5'-TAGTGTCAGAGAGACACTACC-3', are identical to each other. The sequence of the second pair of iterons: 5'-CCTAGTGTCAGAGGGACACC-3', differs slightly from that of the first pair. An 11 bp core sequence (TAGTGTCAGAG) is conserved within all four iterons. The spacing of the iteron pairs is such that they fall on the same face of the DNA helix (that is, close to multiples of 21 bp and 22 bp respectively). This arrangement is often found for the iterons of circular plasmids (del Solar *et al.,* 1998).

Although no AT-rich regions or consensus binding motifs for host- or plasmid-encoded replication associated proteins could be identified, the presence of four correctly spaced iterons and extensive secondary structures was sufficient to allow the designation of a putative replication initiation region for pKara between bp 4142 and 6167.

Table 3.1 Gene predictions for pKara

ORF or feature [Position including stop]	No. of amino acids	Size (kDa)	Amino acid identity to informative database match	RBS position and sequence	Additional information
ORF1 [526-714]	62	7.12	No significant database match	513-519 GGGAGGA	Glimmer2 score: 93 50.26%GC
ORF2 [931-2178]	415	43.33	Autolysin (N-acetylmuramoyl-L-alanine amidase of *Staphylococcus epidermidis* ATCC 12228 (324 aa). Identity/similarity: 44/62% over 111 aa	917-921 AGGGG	Glimmer2 score: 99 56.81%GC LysM domain; Cleavable N-terminal signal sequence and transmembrane region Membrane targeted protein
ORF3 [2213-2851]	212	22.58	Membrane protein of *Silicibacter pomeroyi* DSS-3 (355 aa) Identity/similarity: 33/41% over 106 aa	2119-2203 AGGAGA	Glimmer2 score: 99 59.62%GC Cleavable N-terminal signal sequence; Four transmembrane regions; Membrane targeted protein
ORF4 [2905-4254]	449	47.10	Glycoside hydrolase of *Bacteroides vulgatus* ATCC 8482 (474 aa) Identity/similarity: 45/54% over 75 aa	2889-2893 AGGAG	Glimmer2 score: 99 56.74%GC Six parallel beta helix repeats
ORF5 [4817-5971]	384	43.30	Hypothetical protein GlovDRAFT_1775 of *Geobacter lovleyi* SZ (328 aa) Identity/similarity: 29/51% over 149 aa	4802-4808 AGGAGGA	Glimmer2 score: 99 53.59%GC Winged helix DNA binding domain

Many, but not all plasmids, encode proteins involved in initiation of plasmid replication, while some plasmids are dependent on host proteins for initiation of replication, encoding only proteins involved in the regulation of plasmid replication (del Solar *et al.,* 1998).

Within the set of predicted proteins of pKara, only ORF5 had any similarity to proteins known to be involved in initiation or regulation of plasmid replication. The amino acid sequence of ORF5 contains a "winged helix" DNA-binding domain similar to those found in various DNA binding proteins including replication proteins and transcriptional regulators. The rest of the amino acid sequence of ORF5 did not align well to any characterized protein. The absence of amino acid motifs known to have DNA-breaking or -synthesis functions lead to the conclusion that ORF5 is not a replication initiation protein, but it may be involved in regulation of plasmid replication.

Despite identification of a putative origin of replication the replication mechanism of this plasmid remains unknown. Iterons have been reported in the origins of replication of theta-strand displacement and rolling circle replicating plasmids (del Solar *et al.,* 1998) and therefore are not informative in the identification of the replicon type.

3.4.1.3. Accessory region of pKara

The proteins encoded by ORF2, ORF3 and ORF4 have features suggesting an association with the cell envelope. The products of both ORF2 and ORF3 have predicted transmembrane helixes and cleavable signal peptides, both of which are normally found in membrane targeted proteins and the ORF2 product contains a conserved LysM domain which has been reported to have a general cell wall (peptidoglycan) binding function (Bateman and Bycroft, 2000).

ORF4 contains a conserved pectate lyase fold (polygalacturonase domain) at the amino terminal end of the predicted protein and six parallel beta helix repeats. Proteins containing parallel beta helix repeats are most often enzymes with polysaccharide substrates (Jenkins *et al*., 1998). ORF4 (449 aa) is 45% identical over 75 amino acids to a glycoside hydrolase protein of *Bacteroides vulgatus* ATCC 8482 (474 aa). The similarity of these two proteins is restricted to the polygalacturonase domain which has been reported to occur in proteins with cell envelope biogenesis functions (Marchler-Bauer *et al.,* 2005; 2007).

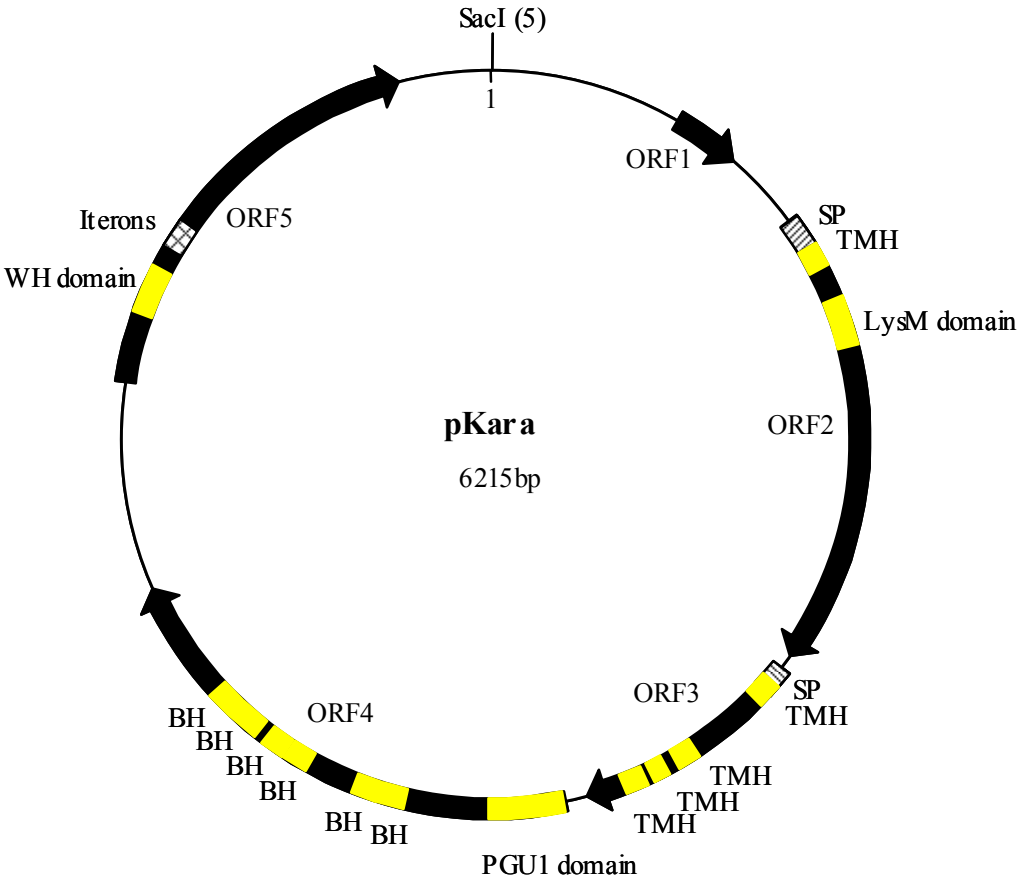

Figure 3.2 Plasmid map of pKara depicting predicted ORFs.

Open reading frames and their direction of translation are indicated with arrows. ORFs are numbered clockwise from the unique *Sac*I site. Shaded boxes are conserved domains or regions of predicted secondary structure. TMH: Transmembrane helix. SP: Signal peptide. BH: Beta helix. WH domain: Winged helix DNA binding domain.

3.4.2. pSulfBC1

3.4.2.1. General features of pSulfBC1

Plasmid pSulfBC1 is 2600 bp in length and has an overall GC ratio of 45.35%. Plasmid pSulfBC1 has many typical features of rolling-circle replicating plasmids of the pC194 group. It was predicted to encode only two proteins, both in the same orientation (Figure 3.3). The protein predictions for pSulfBC1 are summarised in Table 3.2.

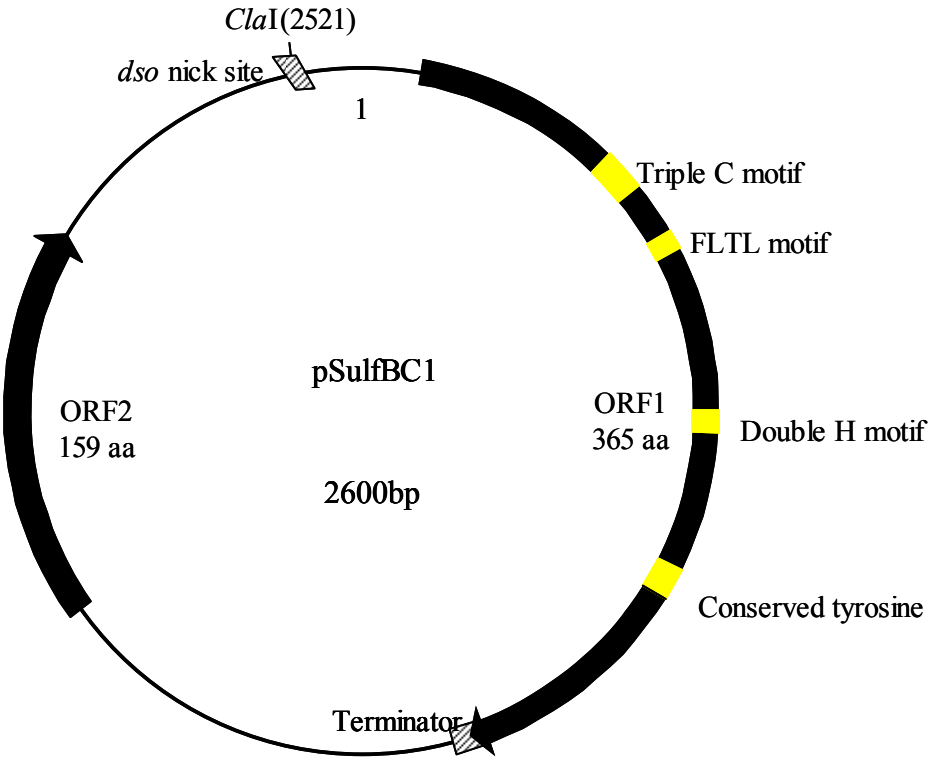

Figure 3.3 Plasmid map of pSulfBC1 depicting predicted ORFs.

Predicted protein coding sequences and their direction of translation are indicated with arrows. ORF1: RCR-type replication initiation protein, ORF2*:* Chaperone-like protein. Conserved motifs of RCR replication proteins are indicated within ORF1 as shaded blocks. The conserved double stranded origin of replication and rho independent terminator are indicated as striped blocks.

Table 3.2 Gene predictions for pSulfBC1

ORF or feature [Position incl stop]	No. of aa	Size (kDa)	Amino acid identity to informative database match	RBS position and sequence	Additional information
ORF1 [72-1169]	365	42.53	Rep protein of plasmid pBP614 from *Paenibacillus popilliae* (371 aa) 32/52% Identity/similarity over 347 aa	56-60 AGGAG	Glimmer score 99 45.17%GC
Terminator [1155-1190]	-	-	Rho independent terminator	-	
ORF2 [1696-2175]	159	18.66	clpB of *Mycobacterium bovis* (877 aa) 56/75% Identity/similarity over 41 aa	1679-1684 GAGAAA	Glimmer score 99 45.75%GC
dso nick site [2510-2516]			Nick sites of pC194 family of RCR plasmids		CTTGATA

3.4.2.2. Replication region of pSulfBC1

The putative replication region of pSulfBC1 was located in the region stretching from bp 2383 to 2521. This region is similar to the double stranded origins of replication of plasmids of the pC194 family of rolling circle replicating (RCR) proteins at structural and sequence levels (Figure 3.4).

At the sequence level, a well conserved double stranded origin of replication (*dso*) *nick* site was identified and aligned to *nick* sites of characterised RCR plasmids (Figure 3.4). Invert and direct sequence repeats are located in front of the nick site, constituting the plasmid specific *bind* site, where the replication initiation proteins bind during rolling circle replication, before initiating replication by creating a single stranded nick at the *nick* site.

As is the case for most RCR plasmids, the double stranded origin of replication of pSulfBC1 is located upstream of the replication initiation protein (Figure 3.3).

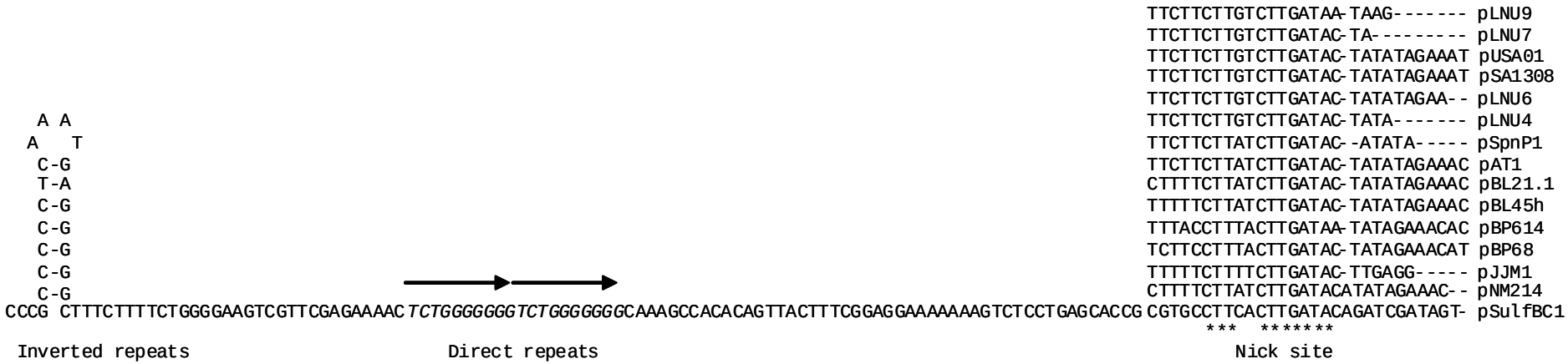

Figure 3.4 Schematic representation of the predicted structure of the pSulfBC1 double stranded origin of replication

Inverted repeats capable of forming a stem-loop structure and two direct repeats are present in front of the consensus *dso* nick site (CTTnnCTTGATAC). A ClustalW alignment of double stranded origins of other RCR plasmids is included. Asterisks indicate conserved nucleotides at the nick site.

The amino acid sequence of ORF1 (365 aa) shares 32% identity over 183 aa with the replication initiation protein of plasmid pBP68 (371 aa) and 31% identity over 354 aa with the replication initiation protein of plasmid pBP614, both from *Paenibacillus popilliae.* The family *Paenibacillaceae*, along with the family *Alicyclobacillaceae* of which *Sulfobacillus* is a member, falls within the order *Bacillales* indicating a moderately close evolutionary relatedness. Furthermore, ORF1 possesses the four conserved domains commonly found in replication initiation proteins of RCR plasmids – the triple C motif, FLTL motif, double H motif and conserved active site tyrosine (Ilyina and Koonin, 1992; Figure 3.5).

```
pSA1308         ----------------------MQYNTTRSITENQDNKTLKDMTKSGKQRPWREKKIDN
pUSA01          ----------------------MQYNTTRSITENQDNKTLKDMTKSGKQRPWREKKIDN
pKH8            ----------------------MQYNTTRCIDENQDNETLKDMTKSGKQRPWREKKIDN
pSK108          ----------------------MQYNTTRCIDENQDNETLKDMTKSGKQRPWREKKIDN
pLNU4           ----------------------MQYNTTKYIDENQDNETLKDMTKSGKQRPWREKKIDN
pLNU6           ----------------------MQYNTTKYIDENQDNETLKDMTKSGKQRPWREKKIDN
pST827          ----------------------MQFNTTRYIDENQDNETLKDMTRSGKQRPWREKKIDN
pGT232          ---------------------------------MYKKEILKDTSSNGKVRPWKQKKLAN
pBP68           MSIVTDKKNIDNDFDWDSHYKYFDFLGAPQPMEEREVGEQLVDLSSTGKIKPWIGHKSAT
pBP614          MSIVTKNSLVNNDFDWEKHYKSLDFLGAPQSVPFREVKEELIDLSATGKIKPWKSHKGAT
pSulfBC         ------------------------MDTIIYDFYEKIQVALDDRNKAGKERPWRKWKLAA
                                                 * *    ** .**   *

                                                          Triple C Motif
pSA1308         VSYADILEIL-----KIKKAFNVKQCGNILEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pUSA01          VSYADILEIL-----KIKKAFNVKQCGNILEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pKH8            VSYADILEIL-----KIKKAFNVKQCGNVLEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pSK108          VSYADILEIL-----KIKKAFNVKQCGNVLEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pLNU4           VSYADILEIL-----KIKKAFNVKQCGNVLEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pLNU6           VSYADILEIL-----KIKKAFNVKQCGNVLEFKPTDEGY-LKLHKTWFCKSKLCPVCNWR
pST827          VSYADILEIL-----KIKKAFNVKQCGNVLEFKPTDEGY-LKLYKTWNCKSKLCPVCNWR
pGT232          LTYSQYLEVL-----NFKKAHNVSKCGEVLQFAKTDNG--LKLYQTWFCHSRLCPLCSWR
pBP68           MLLSESFYRLG----KLNKAESVLYCGTRLKFACCPEGHYKRLKWASFCRVRLCPMCGWR
pBP614          MLLSESFYRLG----KLNKAESVLYCGSRLKFAWFPEGHYKRLKWASFCRVRLCPMCGWR
pSulfBC         QRASKALQSWDSEKAMQRQGQRMMVCGSWLQFGTCPDGDGKWLRKANFCNVKTCPTCMWR
                   .              .   .  **  * *     *    *  .  *  . ** * **

                                      FLTL motif
pSA1308         RAMKNSYQAQKVIEEVIKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTKA
pUSA01          RAMKNSYQAQKVIEKVIKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTKA
pKH8            RAMKNSYQAQKVIEEVVKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTKA
pSK108          RAMKNSYQAQKVIEEVVKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTKA
pLNU4           RAMKNSYQAQRVIEEVVKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTES
pLNU6           RAMKNSYQAQKVIEEVVKEKPKARWLFLTLSTRNAIDGD---------TLERSLKHLTES
pST827          RSMKNSYQAQKVIEAVVKEKPKARWLFLTLSTKNAIDGD---------TLEQSLKHLTES
pGT232          RSMKNSYELRQILDTAYKKDPSAIFLFLTLTEE-SSKLG---------ELRKNLTSMNRS
pBP68           RSLKVAHQVKRVAHTAMESAN-LRWVFLTLTVR-NVSGD---------KLSDEMDHLTKS
pBP614          RSLKVAHQVKRVAHTAMESTN-LRWVFLTLTVK-NVSGD---------KLSDEMDHLTKS
pSulfBC         RSLRQGQQLQLVTQALAAERK-VRWLFITLTAKNFVPDPNDSEAETARKLSEYIKHLLQS
                *...   . . .             .*.**.                  *   .  .  .

                                                                 Active site
                                                 Double H motif     Tyrosine
pSA1308         FDRLSRYKKVKQN--LVGFMRSTEVTVNKN-----DGSYNQHMHVLLCVENAYFRKKENY
pUSA01          FDRLSRYKKVKQN--LVGFMRSTEVTVNKN-----DGSYNQHMHVLLCVENAYFRKKENY
pKH8            FDRLSRYKKVKQN--LVGFLRSTEVTVNKN-----DGSYNQHMHVLLCVENSYFKNKANY
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pSK108          FDRLSRYKKVKQN--LVGFLRSTEVTVNKN-----DGSYNQHMHVLLCVENSYFKNKANY
pLNU4           FRRLFKYKKVSKN--LIGFMRSTEVTVNKN-----DGSYNQHMHVLLCVENSYFKNKANY
pLNU6           FRRLFKYKKVSKN--LIGFMRSTEVTVNKN-----DGSYNQHMHVLLCVENSYFKNKANY
pST827          FRRLFKYKKVSKN--LIGFMRSTEVTVNKN-----DGSYNQHMHVLLCVENSYFKNKSNY
pGT232          IYRLFQYKDVKKD--LLGYVRSTEITVNRD-----SLTFHQHVHILLMVKSSYFS-KGHY
pBP68           FRRFFQRKRLKKI--VLGFFRGLEVTYNKD-----KDTYHPHYHVLLAVTPSYFDGKS-Y
pBP614          FRRLFQRKRFKKT--VLGFFRGLEVTYNKD-----KDTYHPHYHVLLAVTPDYFTRN--Y
pSulfBC         FRRLTMQRFWSNRDVILGFYRTLEVTRNLNRHNHSYGSYHPHLHILVAVAPSYFSGKK-Y
                  *    .       ..*. *  *.* *         ... * *.*. *   **     *

pSA1308         ITQEEWVNLWQRALQVDYRPVANIKAIKPNKKG----------DKDIESAIKETSKYSVK
pUSA01          ITQEEWVNLWQRALQVDYRPVANVKAIKPNRKG----------DKDIESAIKETSKYSVK
pKH8            ITQEEWVNLWQKALQVNYRPVANIKSIKPNQKG----------DKDIQAAIKETSKYSVK
pSK108          ITQEEWVNLWQKALQVNYRPVANIKAIKPNQKG----------DKDIQAAIKETSKYSVK
pLNU4           ITQEEWVSLWQKALQVDYRPVANIKAIKPNQKG----------DKDIQAAIKETSKYSVK
pLNU6           ITQEEWVNLWQKALQVNYRPVANIKAIKPNQKG----------DKDIQAAIKETSKYSVK
pST827          ITQEEWINLWQKALQVDYKPVANIKAIKPNKKG----------DKDIQAAIKETSKYSVK
pGT232          LTQENWSQLWKRARKLDYKPIVNIKKIRASKK-----------DNSLIASAKEVSKYQVK
pBP68           IKKSEWSELWRDAARLDYDPIVDVRVVKDGRRLEKEQGILVEKGYIDPGIISEMSKYTVK
pBP614          IKKSEWSELWRDAARLDYDPVVDVRVVKDGRRVGKEQEILVDKGYIDPGIVSEMSKYTVK
pSulfBC         VKQDEWVARWREALKADYNPIVDIRIPRAKAEADE--------NSVPISALLEASKYATK
                . .  *   *. * .  * *.  ..  .                          * ***  *

pSA1308         SSDFLTDDD-EKNQEIVSDLEKGLYRKRMLSYGGLLKQKHKILNLDDAEDGNLINTSDED
pUSA01          SSDFLTDDD-EKNQEIVSDLEKGLYRKRMLSYGGLLKQKHKILNLDDVEDGNLINASDED
pKH8            SSDFLTDDD-ERNQEIVNDLEKGLYRKRMLSYGGLLKQKHKILNLDDAEDGNLINTSDED
pSK108          SSDFLTDDD-ERNQEIVNDLEKGLYRKRMLSYGGLLKQKHKILNLDDAEDGNLINTSDED
pLNU4           SSDFLTDDD-ERNQEIVNDLEKGLYRKRMLSYGGLLKQKHKILNLDDAEDGNLINTSDED
pLNU6           SSDFLTDDD-ERNQEIVNDLEKGLYRKRMLSYGGLLKQKHKILNLDDAEDGNLINTSDED
pST827          SSDYLTGNH-EKDAEIVQDLEQGLYRKRMLSYGGLLKQKHKLLNLDDAEEGNLIQTSDEE
pGT232          DYDYITDDE-KGDLVVVDELEHALAGTRQLSFAGLLKEIHHELLLDEKEDD-LINVDDE-
pBP68           AADYLVEDDVELTDKVVSTLERALLGRRLFAFGGMLKDVYEWLKNQDAMDDAESDKACLV
pBP614          SSQYLFKDDERLTDKVVLTFERALSGRRLFAFGGLLKEVYNCLKNQVALDDAESDKADLL
pSulfBC         PSFVLPQASEKEAIETTRIIAKSLHMKKMTAFGGELLRIHKELNLQDVESENVNLAEDEK
                    .               . *   .  .. * *      *

pSA1308         KXTDEEEKAHSITAIWNFEKQNYYLR--------------H
pUSA01          KTTDEEEKAHSITAIWNFEKQNYYLRH--------------
pKH8            KTTDEEEKAHSITAIWNFEKQNYYLKDLK-R----------
pSK108          KTTDEEEKAHSITAIWNFEKQNYYLKDLKR-----------
pLNU4           KTTDEEEKAHSITAIWNFEKQNYYLKNLK-R----------
pLNU6           KTTDEEEKAHSITAIWNFEKQNYYLKNLKR-----------
pST827          KTTEEEQKAHSITAIWNFEKQNYFLKKL-------------
pGT232          KEKDD--VVDTVIYRWNSKVSNYVRWE--------------
pBP68           KVDDDLSTCTCPTCNSTLLEEMYRWLPDRGAYH-R------
pBP614          RVDDDLSNCTCPTCNSTLLEEMYRWLPDRGAYHR-------
pSulfBC         LLTTMIDKHSCPICGSELIDQLFRWQNQNYFLYVPESDHV-
                          .           .
```

Figure 3.5 ClustalW alignment of amino acid sequences of rolling circle replication initiation proteins, with consensus motifs indicated above the alignment.

3.4.2.3. Accessory genes of pSulfBC1

Besides the putative replication initiation protein, pSulfBC1 has only one other putative protein-coding open reading frame, ORF2. The product of ORF2 (159 aa) shares 56% identity over 41 aa with a the much larger (877 aa) putative chaperone protein, clpB, of *Mycobacterium bovis.*

This gene arrangement is similar to that of a number of RCR plasmids found in *Streptococcus thermophilus*. Plasmids encoding small chaperones involved in heat shock response are common in strains of *S. thermophilus* (Petrova *et al.,* 2003; Petrova and Gouliamova, 2006). Like pSulfBC1, many of these plasmids encode only two proteins, a replication protein and a heat shock protein. These *S. thermophilus* plasmids all belong to the pC194 family of small rolling circle plasmids (Somkuti *et al.,* 1998; Petrova and Gouliamova, 2006). In *S. thermophilus*, the plasmid-encoded small heat shock proteins serve a protective function during heat shock without pre-adaptation, as plasmid-containing strains showed a higher percentage of survival than plasmid-cured strains after heat shock (Petrova and Gouliamova, 2006, O'Sullivan *et al.,* 1999). However, despite the conserved functional theme, the chaperone-like protein encoded by pSulfBC1 shows little sequence similarity to those encoded by the *S. thermophilus* plasmids.

3.4.3. pTHWX

3.4.3.1. General features of pTHWX

Plasmid pTHWX is 19861 bp in length, with an overall GC ratio of 53.84%. It has a more complex structure than pKara or pSulfBC1. The gene predictions for pTHWX are summarized in Table 3.3. For the purposes of this discussion, the plasmid has been divided into three regions based on what appear to be common origins or functions (Figure 3.6).

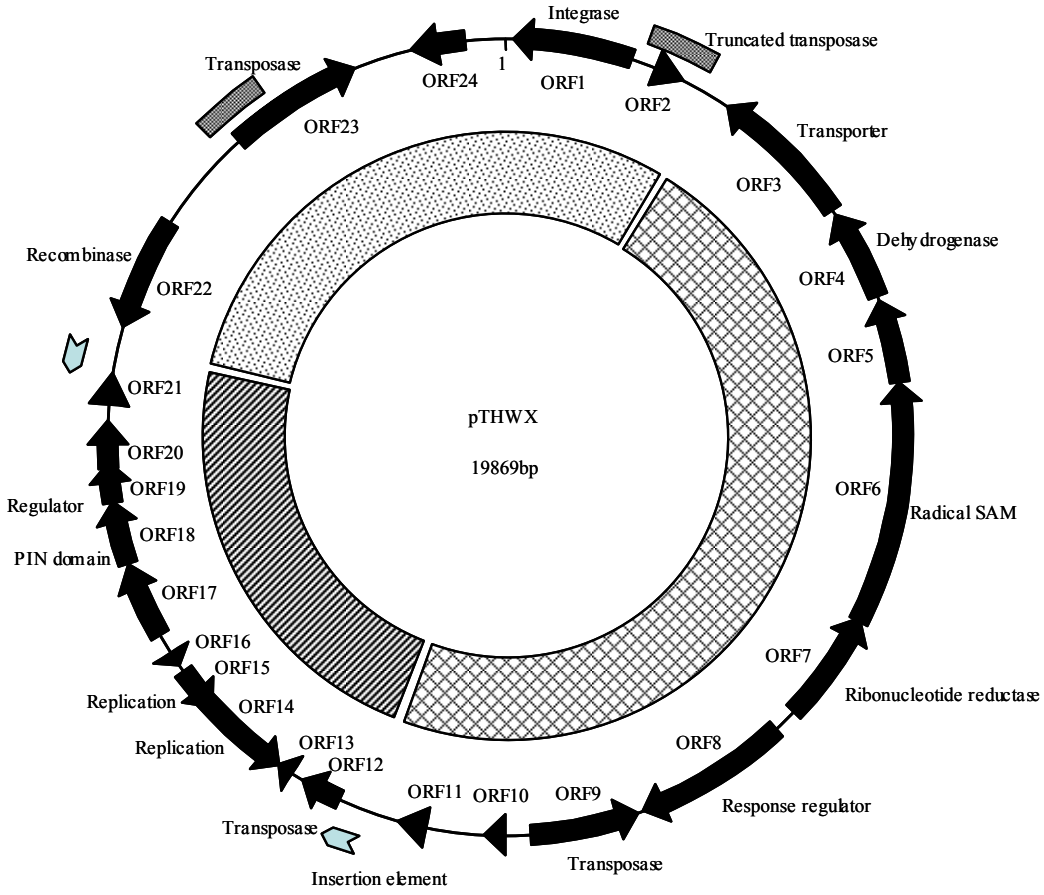

Figure 3.6 Plasmid map of pTHWX depicting potential ORFs and the regions into which the plasmid has been divided.

Open reading frames and their direction of translation are indicated with arrows. Open reading frames are numbered clockwise from an arbitrarily chosen nucleotide number 1. Function predictions are indicated outside the plasmid circle. Invert repeat. Open reading frame. DNA duplication. Replication region. Accessory region. Transposase / recombinase region.

Table 3.3 Gene predictions for pTHWX

ORF or feature [Position including stop]	No. of aa[1]	Size (kDa)	Amino acid identity to informative database match	RBS pos and seq	Additional information
ORF1 [1026-57]	322	35.1	Phage integrase, N-terminal SAM-like (326 aa) from *Frankia* sp. CcI3; 48/64% identity/similarity over 323 aa	1038-1034 GGGAG	Glimmer2 score: 99 Inverted repeats just before RBS 68.63 %GC
ORF2 [1220-1477]	85	9.7	Transposase, IS4 (420 aa) from *Pseudoalteromonas atlantica* T6c; 37/56 % identity/similarity over 67 aa	1204-1208 GAGGG	Glimmer2 score: 99 59 %GC
Term1 [1378-1390]	-	-		-	Rho independent terminator. Confidence: 100, Stem length: 4, Position of gap in stem: 0
ORF3 [3135-1876]	393 (418)	41.7	Conserved hypothetical protein (440 aa) from *Chlorobium ferrooxidans* DSM 13031 25/43% identity/similarity over 353 aa	3065-3069 AGGAG	Glimmer2 score: 99 49.57 %GC
Term1 [767-787]	-	-	Rho independent terminator of ORF1	-	Confidence: 100, Stem length: 9, Position of gap in stem: 0
ORF4 [3095-3829]	733	26.5	COG4221: Short-chain alcohol dehydrogenase (259 aa) of unknown specificity from *Nostoc punctiforme* PCC 73102; 33/50% identity/similarity over 236 aa.	AGGGG 3834-3839	Glimmer2 score: 99 48.97 %GC
ORF5 [4532-3864]	222	25.6	Conserved hypothetical protein (217 aa) from *Streptomyces ambofaciens* 29/39% identity/similarity over 209 aa	AGGAG 4540-4544	Glimmer2 score: 99 50.97 %GC
ORF6 [4529-6388]	619	70.1	Putative Radical SAM (639 aa) from *Streptomyces ambofaciens*. 41/59% identity/similarity over 623 aa	AGGTG 6396-6400	Glimmer2 score: 99, Start is a GTG 48.76 %GC Predicted signal peptide aa 1 to 19

ORF or feature [Position including stop]	No. of aa[1]	Size (kDa)	Amino acid identity to informative database match	RBS pos and seq	Additional information
Term7 6410-6449	-	-	Rho independent terminator of ORF7	-	Confidence: 100, Stem length: 17, Position of gap in stem: 0
ORF7 [6456-7376]	290	32.9	Conserved hypothetical protein (321 aa) from *Salinispora tropica* CNB-440. 30/45% identity/similarity over 188 aa.	GGGAGA 7391-7395	Glimmer2 score: 99 Start is GTG 48.96 %GC Predicted signal peptide aa 1 to 21
ORF8 [7564-8820]	418	46.7	Cellulose synthase subunit (1140 aa) from *Escherichia coli* K12 25/45% identity/similarity 258 aa	AGAAG 7526-7530	Glimmer2 score: 99 53.3 %GC
ORF9 [8857-9735]	292	31.3	Transposase IS116/IS110/IS902 (406 aa) from *Clostridium thermocellum* ATCC 27405; 38/42 % identity/similarity over 289 aa		Glimmer2 score: 99 Start is TTG, not ATG. 62.34 %GC
ORF10 [9927-10103]	58	6.33	No significant similarity		Glimmer2 score: 99 Start is TTG, not ATG 53.67 %GC
ORF11 [10569-10802]	77	8.86	IstB-like ATP-binding protein (181 aa) from *Syntrophomonas wolfei* subsp. *wolfe* str. Goettingen; 62/83 % identity/similarity over 37 aa		Glimmer2 score: 94 Start is GTG, not ATG. 53.84 %GC
ORF12 [11290-11631]	113	12.9	Tn1000-like transposase (534 aa) of *Bacillus thuringiensis*; 40/59 % identity/similarity over 62 aa		Glimmer score: 0 54.38 %GC
ORF13 [11722-11853]	43	4.6	No significant similarity found.	11683- 11687	Glimmer2 score: 51 58.33 %GC

ORF or feature [Position including stop]	No. of aa[1]	Size (kDa)	Amino acid identity to informative database match	RBS pos and seq	Additional information
ORF14 [11850-12650]	266	30.9	hypothetical protein Nham_0139 (632 aa) from *Nitrobacter hamburgensis* X14 43/57% identity/similarity over 250 aa Replication protein (488 aa) from *Bordetella bronchiseptica*; 33/49 % identity similarity over 263 aa		Glimmer2 score: 99 59.67 %GC
ORF15 [12547-12915]	122	4.92	Replication protein from *Bordetella bronchiseptica*. 31/50% identity similarity over 76 aa.		Glimmer2 score: 95 59.62 %GC
ORF16 [12970-13077]			No significant similarity.	GGAGGA 13088-13093	Glimmer2 score: 94 51.58 %GC
ORF17 [13258-13878]	206	23.5	Putative transcriptional regulator, MerR family (291 aa) from *Desulfovibrio desulfuricans* G20. 32/47% identity/similarity over 180 aa.		Glimmer2 score: 99 Start is TTG 54.58 %GC
ORF18 [13879-14388]	169	19.0	Hypothetical protein APE0890 (156 aa) from *Aeropyrum pernix* K1; 22/44 % identity/similarity over 117 aa.	GGGAG 13086-13090	Glimmer2 score: 99 49.6 %GC
ORF19 [14379-14705]	109	12.2	Putative transcriptional regulator (237 aa) from *Rhodococcus* sp. S9; 35/45 % identity/similarity over 73 aa	AGGAG 13584-13588	Glimmer2 score: 95 53.51 %GC
ORF20 [14731-15036]	101	11.5	Preprotein translocase, SecA subunit (900 aa) from *Rickettsiella grylli*. 30/50% identity/similarity over 78 aa.	AGGA 14720-14723	Glimmer2 score: 0 53.92 %GC
ORF21 [15158-15418]	86	9.60	Flagellar biosynthetic protein FlhB (360 aa) from *Caulobacter* sp. K31. 30/48% identity/similarity over 85 aa.		Glimmer2 score: 99 49.42 %GC

ORF or feature [Position including stop]	No. of aa[1]	Size (kDa)	Amino acid identity to informative database match	RBS pos and seq	Additional information
Term21 [15448-15463]	-	-	Rho independent terminator of ORF21	-	Confidence: 100, Stem length: 6, Position of gap in stem: 0
ORF22 [15789-16700]	303	34.5	Putative phage integrase/recombinase (293 aa) from *Thermus thermophilus* HB8 29/45% identity/similarity over 148 aa.	GGGCG 16708-16712	Glimmer2 score: 99 54.05 %GC
ORF23 [17547-18641]	364	42.0	Transposase, IS4 (402 aa) from *Pseudoalteromonas atlantica* T6c; 30/45 % identity/similarity over 358 aa	GGGAG 17533-17537	Glimmer2 score: 99 61.18 %GC
ORF24 [19108-19539]	147	16.8	Hypothetical protein BXB0096 (124 aa) from *Bacillus anthracis* str. A2012; 31/48 % identity/similarity over 123 aa	GGAGA 19552-19556	Glimmer2 score: 93 47.29 %GC

[1] aa, amino acids

Unfortunately, the *S. thermosulfidooxidans* strain from which pTHWX was isolated has been lost, precluding any further molecular analysis of the plasmid. It is uncertain whether the sequences that are adjacent within the identified regions truly have a common ancestry, because this plasmid may have undergone transposition and rearrangement events catalysed by the predicted transposases encoded in the plasmid DNA.

3.4.3.2. Replication region of pTHWX

The putative replication region is contained within a transposon-like structure, flanked by 16 bp inverted repeats (CTCTTGACCAAATACAATCA), probably associated with ORF12, the product of which is 113 amino acids long and shares 40% identity over 62 amino acids with a TN1000-like transposase (534 aa) from *Bacillus thuringiensis*, the region of identity corresponding only to the active site of the *Bacillus* transposase.

The putative replication region includes the only sequences (ORF14, ORF15 and ORF19) encoded by pTHWX that can be linked to plasmid replication functions. ORF14 and ORF15 appear to be a single ORF that has been disrupted by a frameshift. The product of ORF14 (266 aa) is 33% identical to 263 amino acids at the carboxy terminal end of an uncharacterised plasmid replication initiation protein from *Bordetella bronchiseptica* (488 aa). The product of ORF15 (41 aa) shares 31% identity with the amino-terminal end of the same replication protein. The same applies when the ORF14 and ORF15 products are aligned with a hypothetical protein (Nham_0139) from *Nitrobacter hamburgensis* X14, with the amino-terminal end of the ORF15 product aligning with the amino-terminal end of Nham_0139, while the carboxy-terminal end of the ORF14 product aligns with the carboxy-terminal end of Nham_0139. It is possible that the truncated ORF14 might still be functional, or that the frameshift is the result of a sequencing error rather than a biological mutation.

Also contained within the inverted repeats of the transposon region are two sequences encoding transcriptional regulator-like proteins (ORF17 and ORF19), a PIN-domain containing protein, (ORF18) and three proteins (ORF13, ORF16 and ORF20) with insufficient similarity to characterised proteins to enable function predictions.

ORF17 encodes a 206 aa protein that is similar (32/47% identity/similarity over 180 aa) to a 291 aa putative transcriptional regulator of the MerR family from *Desulfovibrio desulfuricans* G20. MerR type regulators have a common N-terminal motif with a DNA binding helix-turn-

helix region, but differ at the C-terminal end where binding to a specific activator takes place. This allows MerR transcriptional regulators to activate gene transcription in reaction to a specific stimulus such as oxidative stress or the presence of heavy metal ions (Brown *et al*., 2003). The product of ORF19 is most similar to a putative transcriptional regulator from *Rhodococcus* sp. S9, although the similarity (35/45% identity/similarity over 73 aa) encompasses only the winged helix DNA-binding domain.

The 169 aa product of ORF18 is 22% identical (over 117aa) to a 156 aa PIN domain containing hypothetical protein APE0890 from *Aeropyrum pernix* K1. PIN domains are small protein domains of approximately 140 amino acids, that have five highly conserved acidic residues and either a serine or threonine residue at a sixth conserved position. This type of domain occurs in enzymes, such as nucleases, that bind divalent cations and have a predicted exonuclease function. In prokaryotes, PIN domains are also found in the toxin component of toxin-antitoxin systems, where they act to retard cell growth under stressful conditions or, if resident on a plasmid, in the event of plasmid loss (Anantharaman and Aravind, 2003; Arcus *et al*., 2005). Therefore, ORF18 may play a role in plasmid stability or control of replication.

3.4.3.3. Accessory region of pTHWX

The second plasmid region, the accessory region, stretches from ORF3 to ORF11 (Figure 3.6) and appears to encode proteins that constitute a metabolic and transport pathway. When the amino acid sequences of ORF3, ORF4, ORF5, ORF6 and ORF7 were compared to public databases using BLAST, the best matches always included chromosomal genes of actinomycetes *Salinispora tropica* and *Frankia* spp. Cci3, suggesting that these genes may have originated as a mobile genetic unit from the chromosome of a bacterium related to the actinomycetes.

The amino acid sequence of ORF3 (393 aa) contains several predicted transmembrane regions and amino acids 1 to 39 are predicted to be a signal peptide, indicating that this protein is probably targeted to the cell membrane. This protein also contains the conserved domains pfam05977 and DUF894. The amino acid sequence of ORF3 is 25% identical (over 323 aa) is to a conserved hypothetical protein from *Chlorobium ferrooxidans* DSM 13031 and is also similar to many other proteins annotated as permeases or transporters.

ORF4 encodes a 244 aa protein that contains the short-chain alcohol dehydrogenase domain COG4221 and shares 33% identity over 235 aa with a putative short chain alcohol dehydrogenase (259 aa) of unknown specificity from *Nostoc punctiforme* PCC 73102.

The amino acid sequence of ORF6 has clear similarity (41/59% identity/similarity over 623 aa) to a putative Radical SAM protein (639 aa) from *Streptomyces ambofaciens* and contains the Fe-S oxidoreductase domain COG1032 involved in energy production and conversion. Radical SAM type proteins catalyse a variety of reactions through a common mechanism involving the production of radical species by the reductive cleavage of S-adenosylmethionine. All of these proteins have an unusual Fe-S cluster (Sofia *et al.,* 2001).

The amino acid sequence of ORF7 is 30% identical to a conserved hypothetical protein similar to ribonucleotide reductases and ferritin like proteins from *Salinispora tropica* CNB-440.

The amino acid sequence of ORF8 (418 aa) was most similar (25/45% identity/similarity 258 aa) to a cellulose synthase subunit (1140 aa) from *Escherichia coli* K12. The amino acid sequence of ORF8 was predicted to contain a helix turn helix motif (cd00093) and a tetratricopeptide repeat domain (cd00189) with four repeats. The helix-turn helix motif is associated with prokaryotic DNA binding proteins belonging to the xenobiotic response element family of transcriptional regulators. The tetratricopeptide repeat domain typically mediates protein-protein interactions. As the product of ORF8 is much smaller than the *E. coli* protein, the ORF8 protein is unlikely to be a cellulose synthase. The observed similarity stems only from the DNA and protein binding domains that are common to the two proteins. The product of this ORF may be involved in regulation of expression of the accessory genes encoded in this region.

When the predicted functions of ORF3 to ORF8 are considered together, they are most likely to be involved in degradation or modification of a compound via the action of the radical SAM, ribonucleotide reductase and alcohol dehydrogenase enzymes and transport of the compound across the cell membrane via the permease-like protein. Whether the compound is imported and modified, or exported after modification is unknown.

Two predicted protein products in the accessory region of pTHWX contain amino acid sequences that are similar to predicted transposases. ORF9 is highly similar (38/42%

identity/similarity over 289 aa) to a predicted transposase from *Clostridium thermocellum* ATCC 27405 and contains the conserved pfam02371 domain associated with the transposase IS116/IS110/IS902 family. The predicted amino acid sequence of ORF11 contains the conserved domain pfam01695 associated with the IS21 family of insertion sequences. These transposases may no longer be functional, as no cognate invert or direct repeats are evident that could be recognised by the encoded transposases of ORF9 or ORF11.

ORF10 showed no good match to known sequences but is included because of the significant Glimmer2 score.

3.4.3.4. Transposase / recombinase region of pTHWX

The third region of pTHWX region has three ORFs that encode proteins similar to integrases, recombinases or transposases (ORF22, ORF23 and ORF1).

The product of ORF22 (303 aa) shares 29/45% identity/similarity over 148 aa with a putative phage integrase/recombinase (293 aa) from *Thermus thermophilus* HB8

ORF23 encodes a 364 aa protein that shares 30% identity over 358 aa with a 406 aa transposase from *Pseudoalteromonas atlantica*. A ±500 bp region of DNA at the 5' end of ORF23 is duplicated between ORF1 and ORF3. ORF2, a truncated homolog of ORF23, falls within this duplicated region. ORF2 may have become truncated during a recombination event between the duplicated areas of DNA. No direct or inverted DNA repeat sequences were found that could be recognised as insertion sites for transposable elements, therefore the transposases encoded in this region, like those in the accessory region of pTHWX, may no longer be functional. The same kind of recombination event that lead to the truncation of ORF2, may have uncoupled these transposase-like genes from their recognition sites.

The 322 aa product of ORF1 shared 48% identity over 323 aa with a 326 aa phage integrase from *Frankia* sp. CcI3.

The closest database match (31% identity over 123 aa) for ORF24 is to a hypothetical protein GBAA_pXO2_0096 from *Bacillus anthracis* str. 'Ames Ancestor', which is conserved within the *Bacillus cereus* group. The function of this protein is unknown.

In summary, plasmid pTHWX has a core replication region contained within a transposon structure. The method of plasmid replication remains unknown. Outside of the replication region, multiple transposition and recombination events have occurred. The accessory genes of pTHWX appear to have a chromosomal origin and may be involved in the response to, modification and transport across the cell membrane of an unknown substance. Whether this confers an adaptive advantage to the plasmid host is uncertain, although the continued integrity of the accessory gene cluster in the face of DNA rearrangements in the rest of the plasmid sequence provides evidence for selective pressures at work.

The nucleotide base composition of this plasmid is not uniform, with the average GC ratio of open reading frames varying from 50% for the accessory region to 54% for the transposon replication region and 60% for the ORFs encoding transposase-like sequences.

3.5. Conclusions

3.5.1. Plasmid gene prediction with Glimmer2

As the Glimmer software (version2) generated different gene predictions for the plasmids depending on where the sequence start was defined, it can be concluded that Glimmer2 is not as reliable at predicting open reading frames for smaller plasmids as it is for predicting chromosomal genes. This is to be expected, as the training sets used to create the Markov model used for predictions become severely limited for small plasmids. Glimmer was originally designed as a whole genome annotation tool, where the training sets are much larger. It is therefore unwise to rely too much on the predictive ability of this software for smaller plasmids.

Also, Glimmer is designed to predict genes that are typical of a given genome. Genes acquired by horizontal transfer can be missed because they do not resemble the native genes of the host (plasmid or chromosome) genome. One example where this has occurred is in the case of the pTHWX ORF12 which is probably a real gene, as it is similar to a Tn1000-like transposase, and yet receives a Glimmer score of 0. This is probably because it is not typical of other pTHWX genes, having been acquired through transposition at a later time. A newer version of the Glimmer software, Glimmer3, is now available. The major improvements of this version are greater accuracy in predicting translation initiation sites and reducing false

positives (Delcher *et al.,* 2007). This does not improve gene prediction with small training sets.

3.5.2. Sequence diversity of Sulfobacillus plasmids

Plasmids of sulfobacilli have considerable sequence diversity. No sequence similarity between the three plasmids studied here could be detected. The genetic load of the plasmids differ markedly, from pSulfBC1 which encodes only a minimal replication function and one putative chaperone-like protein, to pKara, which carries three genes with functions related to cell-envelope biogenesis, and pTHWX which seems to contain DNA from various sources, encoding mobile genetic elements and proteins involved in modification and membrane transport of substances. These three plasmids represent only a fraction of the diversity of *Sulfobacillus* plasmids, as plasmids of greater than 50 kb have been detected in almost all surveyed strains (Chapter 2).

From the diversity of transposons and transposon remnants present on pTHWX it can be concluded that the potential for the movement of genetic material within a *Sulfobacillus* genome is considerable. The differing G+C ratios of the ORFs within accessory and replication regions of pTHWX provide evidence for divergent origins of these regions, and for the occurrence of horizontal transfer events to sulfobacilli.

3.5.3. Replication mechanisms of Sulfobacillus plasmids

The replication mechanisms of pKara, pSulfBC1, and pTHWX are unrelated. The replication region of pKara contains iterons and complex secondary structures, but no highly conserved replication initiation protein, suggesting that it is reliant on host factors for replication initiation, although the exact mechanism remains unknown.

The replication region of pSulfBC1 is highly similar to that of rolling circle replicating plasmids of the pC194 family, which encode all the factors necessary for initiation of leading strand replication. Plasmids of this family have been used successfully as cloning vectors in other bacterial lineages, introducing the possibility of using existing cloning vectors of this type to transform strains of *Sulfobacillus*.

The replication region of pTHWX seems to be contained within a transposon structure. Although *cis* acting DNA sequences involved in replication initiation could not be defined, and the RepA-like initiation protein has possibly been disrupted by a frameshift mutation, this replicon seems able to support a larger DNA load, and may yet be a better candidate for the construction of a cloning vector.

CHAPTER FOUR
TRANSFORMATION BY ELECTROPORATION

4.1. Aim

The aims of this part of the study were to determine appropriate antibiotics for selection of *Sulfobacillus* transformants; to construct transformation vectors; to determine whether sulfobacilli can be transformed by electroporation and to investigate the effects of various factors on electroporation efficiency of *Sulfobacillus*.

4.2. Introduction

The cell membrane is an efficient barrier to the entry of macromolecules such as DNA into cells. Electroporation overcomes this barrier by application of a brief, high intensity electrical pulse, thereby creating temporary pores in the membrane, allowing DNA to cross the lipophilic membrane via aqueous conduits.

Electrotransformation has become the first recourse for transforming bacterial species, due to its wide applicability, technical simplicity, reproducibility and efficiency. Electroporation has been applied to the transformation of representatives of all domains of life: from archaea, to Gram-negative and Gram-positive bacteria (Table 4.1), and single-celled and multicellular eukaryotes (Mitchell *et al.,* 1998; Osumi and Inoue, 2001; Thomas, 2003). An acidophilic, Gram-negative, iron and sulphur oxidising biomining bacterium, *Acidithiobacillus ferrooxidans* has been transformed by electroporation, although the transformation frequencies were very low (200 colonies per microgram DNA) and only one strain of 30 could be transformed (Kusano *et al.,* 1992). Strains of the Gram-negative acidophilic heterotroph, *Acidiphilium*, have also been transformed by electroporation (Glen *et al.*, 1992).

Cells do not normally require any special treatment (such as production of protoplasts) before or after application of the electrical pulse, minimising the technical skill required and reducing variability. Under optimal conditions, electroporation efficiency for *E. coli* can be as high as 10^9 to 10^{10} transformants per microgram of DNA (Calvin and Hanawalt, 1988; Dower *et al*., 1988).

Not all species or strains are equally amenable to transformation by electroporation (Buckley *et al.,* 1999; Turgeon *et al.,* 2006). The efficiency of electrotransformation is highly strain-

dependent (Buckley *et al.,* 1999; Turgeon *et al.,* 2006; other references in Table 4.1) and parameters need to be independently optimised for each strain. Gram-positive bacteria are less efficiently transformed by electroporation than Gram-negative bacteria (Trevors *et al.,* 1992), and some species remain untransformable despite considerable optimization efforts. Optimal electroporation parameters can vary considerably and knowledge of optimal conditions for one bacterial strain is not necessarily transferable to related strains.

There are many factors affecting electroporation efficiency. These include the nature and preparation of cells; the vector(s) employed; electroporation pulse parameters; electroporation buffer; recovery medium and selection of transformants after electroporation.

4.2.1. Nature and preparation of cells

The thick cell walls of Gram-positive bacteria present a physical barrier to entry of DNA and may also prevent the efficient formation of membrane pores. Various strategies have been used to reduce the hindrance of thick cell walls. The addition of compounds that interfere with cell wall synthesis (penicillin, glycine or threonine) or membrane synthesis (isoniacin) to the growth medium; treatment of cells with lytic enzymes (lysozyme or mutanolysin) or multiple freeze-thaw cycles have been reported to increase electrotransformation frequencies (Holo and Nes, 1989; Brigidi *et al.,* 1990; and other references in Table 4.1). This occurs at the cost of greater fragility of cells.

When glycine or threonine is incorporated into the cell wall, it results in decreased cross-linking of fibres and thus weakening of the structure. This improves electroporation in many cases, but also leads to impairment of cell growth and produces fragile cells that are difficult to work with (Buckley *et al.,* 1999). Sometimes the benefits of cell-wall weakening agents can be offset by the greater fragility of the cells, actually decreasing the electrotransformation efficiency at high field strengths (Turgeon *et al.,* 2006). One way to offset the disadvantages of glycine treatment is to add an osmotic stabilizer such as sucrose to the growth medium and / or electroporation medium (Holo and Nes, 1989; Xue *et al.,* 1999; Ito and Nagane, 2001; and other references in Table 4.1). The osmotic stabilizer protects cells against lysis and improves growth relative to that achieved in a hypotonic solution. Another way to manage the growth inhibition caused by glycine treatment is to add glycine to cells already in the exponential phase of growth (Turgeon *et al.,* 2006).

Table 4.1 Electroporation of a selection of Gram-positive bacteria.

Species	Maximum electroporation efficiency and optimum pulse parameters	DNA	Reference
Freshwater and marine caulobacters *Caulobacter crescentus*	10^8 transformants per µg plasmid DNA 2.5kV/cm, 4.2 ms time constant. Cells grown to mid-log phase, resuspended in 10% glycerol as electroporation medium, high cell density 10^{11} cells per ml. 2.5 kV, 25 µF	1.25 µl of pKT230 (100 ng/µl) added to 50 µl of cells	Gilchrist and Smit, 1991
Bacillus cereus ATCC 14579	2 x 10^9 cfu $\mu g^{-1} ml^{-1}$ 25 µF, 200 Ω, 20 kV/cm. Efficiency declined at higher volts Glycine added to exponential phase cultures	pC194, RCR replication, 2.9 kb, medium copy number	Turgeon *et al.,* 2006.
B. cereus ATCC 14579	2 x 10^6 cfu $\mu g^{-1} ml^{-1}$ 25 µF, 200 Ω, 20 kV/cm. Efficiency declined at higher volts Glycine added to exponential phase cultures.	pLS1, RCR replication, 4.4 kb, low copy number.	Turgeon *et al.,* 2006
B. cereus ATCC 14579	8 x 10^6 cfu $\mu g^{-1} ml^{-1}$ 25 µF, 200 Ω, 20 kV/cm. Efficiency declined at higher volts Glycine added to exponential phase cultures.	pT181, RCR replication, 4.4 kb, high copy number.	Turgeon *et al.,* 2006
B. cereus ATCC 14579	1 x 10^8 cfu $\mu g^{-1} ml^{-1}$ 25 µF, 200 Ω, 20 kV/cm. Efficiency declined at higher voltages. Glycine added to exponential phase cultures.	pMTL500Eres. Theta replication, 7.1 kb, low copy number	Turgeon *et al.,* 2006
Lactococcus lactis subsp. *cremoris* BC101	2.7 x 10^7 transformants per 0.8 µg 25 µF, 200 Ω, 2.0 kV, 2 mm electrode gap. 4.5-5.0 ms time constant. Cells grown in presence of glycine (up to 4%) and sucrose (0.5 M)	pIL253 pSa	Holo and Nes, 1989

Species	Maximum electroporation efficiency and optimum pulse parameters	DNA	Reference
Actinomyces viscosus *A. naeslundii*	$10^2 – 10^7$ transformants per µg plasmid) 2.5 kV, 25 µF 400 Ω, 2mm electrode gap. Glycine or threonine in growth medium did not affect transformation.	pJRD215, derived from RSF1010	Yeung and Kozelsky, 1994
B. subtilis PB1424	2.8 x 10^4 transformants per µg plasmid. 25 µF 6250 V/cm 0.5 µg DNA. Mid log phase cells give higher transformation efficiency. Freeze thaw cycles improved efficiency.	pVE18 (pC194 chloramphenicol marker)	Brigidi *et al.,* 1990
Rhodococcus equi	1.1 x 10^4 transformants per µg DNA 2.5 kV, 400 Ω, 25 µF 2 mm electrode gap. Mid-log phase culture, heat treated (50˚C for 9 min, cooled in ice water). Growth medium supplemented with 1% glycerol, 0.2% Tween80 and 2% glycine.	RF30, pBS305, pK4 shuttle vectors derived from *Rhodococcus* spp.	Sekizaki *et al.,* 1998
Streptococcus salivarius	10^5 transformants per microgram DNA Glycine (10%) added to exponential phase cultures. Osmotic stabilizer 0.4 M Sorbitol. 25 µF 200 Ω, 1.6 kV. Electroporation buffer pH 4.5	200 ng to 1 µg for pNZ123 and pDL278.	Buckley *et al.,* 1999
B. subtilis	1.4 x 10^6 transformants per µg DNA Hyper osmotic growth-, electroporation- and recovery media (Sorbitol and Mannitol).		Xue *et al.,* 1999
B. licheniformis	1.8 x 10^4 transformants per µg DNA. Hyper osmotic growth-, electroporation- and recovery media (Sorbitol and Mannitol).	pUBxynA	Xue *et al.,* 1999
Clavibacter xyli subsp. *cynodonti*	20% PEG 8000 in the electroporation buffer.	IncP type plasmid pLAFR3	Metzler *et al.,* 1992
Clostridium perfringens and *C. acetobutylicum*	Electroporated in the presence of 10% PEG		Dower *et al.,* 1992.

Species	Maximum electroporation efficiency and optimum pulse parameters	DNA	Reference
Thermoanaerobacterium saccharolyticum JW/SL-YS485	10 to 1000 transformants per µg DNA 1 mm cuvettes, 1.25 kV, 400 Ω, 25 µF, time constant 4-8 ms. Isoniacin (isonicotinic acid hydrazide) was added to cultures in early exponential phase at final concentration of 4 mg/ml to weaken cell walls. Autoplast formation observed via light microscopy.	Replicating vectors pIKM1, pRKM1 and pRUKM. Suicide vectors pUXK and pUXKC.	Mai *et al.,* 1997; Mai and Wiegel, 2000
Clavibacter xyli subsp. *cynodontis*	2x 10^5 transformants per µg DNA 12 ms 14-16 kV/cm. 20% PEG 8000 in electroporation buffer. Freeze thaw cycles did not improve efficiency.	IncP plasmid pLAFR3	Metzler *et al.,* 1992.
Lactobacillus plantarum L137	1.5 x 10^4 transformants per µg DNA 1% glycine added growth medium. 2 mm cuvette, 25 µF, 2.0 kV, parallel resistance 400 Ω (10 kV/cm). 40% w/v PEG 1000 and 0.5 µg/ml ssDNA (salmon sperm DNA) in electroporation medium.	pRN14	Kaneko *et al.,* 2000
Lactococcus lactis subsp. *lactis*	10^4 transformants per µg DNA Cells grown to stationary phase in defined media with 0.24% DL threonine. Electroporation medium: ddH_2O, High concentrations of DNA used in electroporation (1µg). Competent cells could be frozen	pGB301 (9.8 kb)	McIntyre and Harlander, 1989
Brevibacterium methylicum	10^5 transformants per µg DNA Highest transformation frequency when plasmid DNA had been isolated from *B. methylicum*.	pEC71, pZ6-1 (coryneform replicons) pLS5 (Streptococcus replicon) RSF1010 (Wide host range replicon)	Nesvera *et al.,* 1994
Enterococcus faecalis	6.6 x 10^3 transformants per microgram plasmid DNA 4-8% glycine added to growth media. Fragile cells handled with care – no vortexing.		Shepard and Gilmore 1995
Desulfitobacterium	12.5 kV/cm, 25 µF, 400 Ω	pIL253 (G+ theta	Smidt *et al.,* 2001

Species	Maximum electroporation efficiency and optimum pulse parameters	DNA	Reference
dehalogenans	40 mM Glycine in growth medium	replicon of pAMβ1)	
Actinobacillus actinomycetemcomitans	Early log phase culture. High cell density during electroporation (saturated at OD600 of 6). Electroporation buffer (15% glycerol, 272 mM sucrose, 2.43 mM K_2HPO_4, 0.57 mM KH_2PO_4) at pH 5.5 better than at pH 7.4. Competent cells could be frozen. 2.5 kV, 200 ohm, 25 μF	pDL279 and pDL280 (derivatives of native plasmid pVT736-1 and pUC19) pYG10 (plasmid of *A. pleuropneumaniae*)	Sreenivasan *et al.,*1991
B. cereus 569	2 x 19^{-5} transformants per viable cell. 3 μF, 3.750 V/cm	pC194	Belliveau and Trevors, 1989
Strains of *Bacillus, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Listeria, Pediococcus, Propionibacterium* and *Staphylococcus*	$10^1 – 10^5$ transformants per μg DNA	Various plasmids including pC194	Luchanski *et al.,*1988
Staphylococcus carnosus	10^6 transformants per transformation 0.5 M sucrose, 10% glycerol as electroporation medium. Electroporation at room temperature. 21 kV/cm, 1.1 ms (pulse width, NOT time constant). Rich, pH buffered recovery medium. Heat shock before electroporation to inactivate intracellular nucleases.	4 μg of DNA per electroporation	Löfblom *et al*., 2007
L. lactis spp. *lactis*	225 x 10^7 transformants per μg DNA Cells pre-treated with lithium acetate and dithiothreitol.		Papagianni *et al.,*2007
C. thermocellum	5 x 10^4 transformants per μg DNA Custom built electroporation device and cuvettes. Isoniacin in growth media (effect was strain dependent). Sample chilled. Four	Plasmid DNA was appropriately methylated to avoid restriction.	Tyurin *et al.,* 2004

Species	Maximum electroporation efficiency and optimum pulse parameters	DNA	Reference
	hour recovery.		
C. acetobutylicum DSM 792	6 x 10^2 transformants per µg DNA 1.8 kV, 50µF, 600Ω, 5.5 ms	Plasmid DNA was appropriately methylated to avoid restriction.	Nakotte *et al*., 1998

Besides the cell membrane and cell wall, another physical barrier that has been reported to interfere with electrotransformation is the S-layer (Gilchrist and Smit, 1991). An S-layer was present in cells of *S. thermosulfidooxidans* VKM-B-1269 and *S. thermosulfidooxidans* subsp. *asporogenes*, strain INMI-41 (Severina *et al.,* 1995), but was absent in *S. thermotolerans* (Bogdanova *et al.,* 2006). Transformation can be facilitated by removal of the S-layer, or by use of strains that are unable to produce a functional S-layer (Gilchrist and Smit, 1991).

The shape and size of cells influence the field strength needed for electroporation, with greater field strength required for smaller spherical cells, than for larger, rod-shaped cells (Calvin and Hanawalt, 1988; Prasanna and Panda, 1997).

The growth phase of the cells is an important factor. In general, cells in the early to mid exponential phase of growth are more easily transformed by electroporation, though exceptions occur (Brigidi *et al.,* 1990; references in Table 4.1). Sometimes higher electroporation efficiencies are achieved when cells are harvested in the stationary phase (Kawagishi *et al.,* 1994; Prasanna and Panda, 1997).

Pre-treatment of cells with lithium acetate and dithiothreitol improved the electrotransformation efficiency of *Lactococcus lactis* spp. *lactis* without affecting cell survival (Papagianni *et al.,* 2007). The mechanism by which these compounds act to increase transformation efficiency is unknown.

In most cases, electrocompetent cells must be kept cold during preparation. However, exceptions do occur where cells are more competent when prepared at room temperature (Augustin and Götz, 1990), or electrocompetence is not affected by preparation temperature (Löfblom *et al.,* 2007).

Certain species or strains harbour DNA restriction modification systems, which destroy incoming DNA in the cytoplasm. This impairment can sometimes be sidestepped by using an appropriate DNA source (see section 4.2.2, “Vectors for electrotransformation”). Often the restriction modification system needs to be neutralized in the recipient, by isolating restriction deficient mutants (Belavin *et al.,* 1988; Liebl *et al.,* 1989) or by heat treatment of cells before electroporation (Reid *et al.,* 1982). Extracellular nucleases can be removed by thorough washing of cells and / or deactivated by metal-chelating agents such as EDTA (Vischi and Marchetti, 1997).

4.2.2. Vectors for electrotransformation

Vectors must be able to replicate in the host cytoplasm or integrate in the host chromosome. Good sources for replicating vectors are natural plasmids of the bacterium in question, or wide host range plasmids (Kusano *et al*., 1992), while integrating vectors should contain DNA sequences that are highly similar to regions of chromosomal DNA.

The preparation of vectors to be used for electrotransformation should be carefully considered. Impurities in plasmid preparations can sometimes reduce transformation efficiency (Dunny *et al*., 1991), while in other cases less pure preparations can be used (Belliveau and Trevors, 1989; Holo and Nes, 1989). The standard method is to use cesium chloride gradient purified DNA.

The methylation status of the transforming DNA can be of cardinal importance, since electrotransformation is often thwarted by endogenous restriction systems (Lefrançois and Sicard, 1997; Vischi and Marchetti, 1997) which may be modulated by modification of DNA with methyl groups. Restriction modification systems consist of a restriction endonuclease and a methylase that confers immunity to it by modifying DNA at the recognition site of the restriction enzyme. When inappropriately modified DNA enters the cell the incoming DNA will be cleaved at unmodified recognition sites. Plasmid DNA is methylated in *S. thermosulfidooxidans* DSM 9293^{T} at sites that overlap the recognition sites of restriction enzymes *Cla*I (ATCGAT) and *Stu*I (AGGCCT) (Chapter 2). The *Cla*I and *Stu*I sites that were not cleaved had overlapping *dam* methylase (GATC) and *dcm* methylase (CCAGG) recognition sites respectively. Therefore, plasmid DNA for use in electroporation of *S. thermosulfidooxidans* should be prepared from *dam* and *dcm* positive hosts.

In cases where a considerable restriction barrier exists, it is more efficient to isolate transforming DNA from the same or closely related strain (Holo and Nes, 1989; Nesvera *et al*., 1994; Yeung and Kozelsky, 1994). Pre-treatment of transforming DNA by *in vitro* methylation also lends a degree of protection against host nucleases (Kwak *et al.,* 2002).

Using electroporation, the transformation frequency increases linearly for low DNA concentrations and logarithmically for high concentrations of DNA (Gilchrist and Smit, 1991; Rittich and Španová, 1996; and other references in Table 4.1). Often even small amounts of DNA can deliver transformants, but some Gram-positive species require up to a microgram or

more to enable recovery of transformants (Sreenivasan *et al*., 1991). During initial optimization, microgram quantities of plasmid DNA must be used during electroporation. Once transformants are obtained, this amount may be reduced for economic reasons.

4.2.3. Electroporation solutions

The electroporation buffer must be of low ionic strength to prevent arching during application of the electric pulse and must not cause additional stress to the cells. Electroporation buffers reported in literature range from distilled water (McIntyre and Harlander, 1989) to high osmolarity pH buffered solutions (Xue *et al*., 1999). The buffer which minimises cell death during electroporation is the best, while incorporating elements such as PEG or non-ionic surfactants which might increase electroporation efficiency by binding DNA to cells or by lowering the transmembrane potential is desirable.

In general, higher electrotransformation efficiencies are obtained with electroporation buffers at neutral pH, approximating that of the cytoplasm (Prasanna and Panda, 1997) since medium components may enter the cells though membrane pores and disturb the cellular pH. Exceptions to this rule do occur. *Actinobacillus actinomycetemcomitans* was electrotransformed at greater efficiency in slightly acidic (pH 5.5) electroporation buffer (Sreenivasan *et al*., 1991) and *Enterococcus faecalis* was transformed optimally with acidic (pH 4) electroporation buffer (Dunny *et al*., 1991).

The osmotic strength of the electroporation buffer is of significance, not only to the survival of cells, but also for secondary effects in improving electroporation efficiency. High osmolarity media are postulated to protect cells from osmotic lysis (Holo and Nes, 1989), or even to facilitate the influx of DNA into the cell when hyper osmotic solutions are employed (Xue *et al*., 1999). Importantly, the osmoticum must also be of low ionic strength, to avoid arcing, and must not be toxic or inhibitory to the organism being transformed. Glycerol, sucrose, sorbitol and mannitol have been used with success (Table 4.1). The addition of glycerol to the electroporation medium has been reported to increase time constants and reduce the risk of arcing during electroporation (Holo and Nes, 1989).

Polyethylene glycol (PEG) has been included in electroporation buffers to improve electroporation efficiency. The exact mechanism is uncertain, although it has been postulated to occur by increasing binding of DNA to cells (Metzler *et al.,* 1992) and / or by increasing

the number and size of electropores created in the cell membranes (Hood and Stachow, 1992). PEG may also prevent degradation of DNA by secreted endonucleases (Vischi and Marchetti, 1997).

The inclusion of bio-compatible surfactants may cause a higher degree of membrane permeability with a smaller electrical pulse, reducing thermal injury and other side effects of high field strengths (Troiano *et al*., 1998).

The concentration of cells in the electroporation mix is important, as the transformation efficiency increases with increasing cell concentration (Prasanna and Panda, 1997).

4.2.4. Electroporation pulse parameters

The electrical parameters determined by the electroporation device include the field strength, capacitance, parallel resistance and time constant. Increasing the field strength increases the number and size of pores created in the lipid membrane. Higher field strengths generally increase the transformation frequency, but at the cost of greater cell death. The optimum field strength is very dependent on the species and strain being electroporated (Belliveau and Trevors, 1989; Marcus *et al.,* 1990; Gilchrist and Smit, 1991; Table 4.1). Gram-positive bacteria generally require higher voltages than Gram-negative bacteria for successful electroporation.

The time constant can be varied by changing the composition of the electroporation buffer or the parallel resistor selected on the electroporator. As for field strength, the optimum time constant must be determined empirically for each type of organism, though in general a shorter time constant results in higher cell survival, but reduced transformation efficiency, while longer time constants can increase the transformation efficiency at the cost of lower survival rate (Gilchrist and Smit, 1991).

After the electrical pulse is delivered, the likely effectiveness of the pulse to create membrane pores is estimated by determining the survival rate of the cells (Table 4.2).

Table 4.2 The survival rate of cells in conditions delivering optimum transformation efficiency by electroporation.

Strain	Percentage of cells surviving electroporation	Transformants per μg DNA	Reference
Bacillus cereus 569 UM20-1	46%	2.0×10^{-5}	Belliveau and Trevors, 1989
Enterococcus faecalis	50 to 80%	2.0×10^{5}	Dunny *et al*., 1991
Caulobacter crescentus	40-50%	3×10^{8}	Gilchrist and Smit, 1991
Bacillus subtilis	15-25%	2.8×10^{4}	Brigidi *et al.,* 1990
Streptococcus salivarius	50%	10^{5}	Buckley *et al*., 1999
Lactococcus lactis spp. *lactis*	13-15%	2.2×10^{9}	Papagianni *et al*., 2007

4.2.5. Recovery media

The recovery medium must allow cells to repair damage caused by the electroporation pulse by resealing the cell membrane and compensating for cellular components leaked from the pores. This medium must also allow the expression of any selectable markers. The recovery medium does not need to be of low ionic strength and usually consists of rich growth medium, sometimes pH buffered and osmotically stabilized. A highly acidic medium is not necessarily incompatible with successful recovery of acidophilic cells (Kusano *et al*., 1992) despite the potential for disturbing the intracellular pH.

The dilution factor of cells in the recovery medium is important. McIntyre and Harlander (1989) found that the transformation efficiency increased from 382 transformants per μg DNA to 2000 transformants per μg DNA when the dilution factor was increased from 1:2 to 1:100.

4.2.6. Selection of transformants after electroporation

The antibiotic marker used to select for transformants can alter the transformation efficiency. Some antibiotics, such as tetracycline and related compounds, can result in lower transformant recovery (Steele *et al.,* 1994; Turgeon *et al.,* 2006).

Antibiotic selection for putative transformants of sulfobacilli poses special challenges, in that the optimum growth environment of sulfobacilli (low pH and high temperatures) may be

detrimental to the stability of antibiotics. Therefore appropriate antibiotics that are stable under these conditions must be chosen. Kanamycin, streptomycin, tetracycline and chloramphenicol have been demonstrated to be stable at pH 2 for at least 96h (Davidson and Summers, 1983). However, these assays were conducted with stock solutions in buffers, not with dilute solutions in bacterial growth medium. Kanamycin was stable at working concentrations in acidic growth medium (pH 4.2) for three weeks at 24˚C thereafter loss of activity was observed (Davidson and Summers, 1983). Ampicillin has been reported to be effective at pH 4.3 (Holmes *et al*., 1984).

Stability of antibiotics at high temperature at working concentrations in growth media has been assayed (Peteranderl; *et al*., 1990; Aagaard *et al*., 1994). It was shown that the suitability of antibiotics for selection of thermophilic organisms varied considerably. Carbomycin, celesticetin, chloramphenicol, puromycin, sparsomycin, tetracycline, and thiostrepton, anisomycin, clindamycin, erythromycin, kanamycin, lincomycin and spectinomycin were functional for at least five days at pH 3 and 70˚C (Aagaard *et al*., 1994). Peteranderl and co-workers (1990) reported that lasalocid, monesin, cyclohexamide, kanamycin, trimethoprim, metronidazole, amphotericin, chloramphenicol, polymyxin, bacitracin and streptomycin showed acceptable stability at 50˚C and pH 5, while neomycin, erythromycin, novobiocin, vancomycin, penicillin, G, tetracycline and ampicillin were degraded more rapidly. Importantly, the stability of antibiotics at high temperature was strongly affected by the pH of the growth medium. Therefore, care should be used in the choice of antibiotic markers for selection of thermophilic acidophilic bacteria. A suitable antibiotic must be stable and functional at low pH and high temperature, or media must be developed that allows *Sulfobacillus* spp. to grow at higher pH. Sulfobacilli can grow at 37˚C, but growth is much faster at 50˚C, especially on solid media. It would be an advantage to be able to use antibiotic selection at 50˚C.

4.3. Materials and Methods

4.3.1. Electroporation equipment

The Gene Pulser® II Electroporation System from Bio-Rad was used for all electroporation experiments. Electroporation pulse settings were 200 Ω parallel resistance and 25μF and the applied voltage was varied between 0 kV and 2.5 kV. Bio-Rad gene pulser® cuvettes with 2

mm electrode gaps, and Thermo Hybaid® cuvettes with 1 mm electrode gaps were used. Although the Bio-Rad cuvettes were designed to be used once, because of the prohibitive cost cuvettes were used several times before discarding. Cuvettes were rinsed with chloride bleach and thoroughly washed with water after each use. Cuvettes were sterilized by rinsing with 70% ethanol and dried at 50˚C.

4.3.2. Antibiotic resistance survey

Before attempts to transform sulfobacilli by electroporation could commence, a suitable selective marker had to be chosen. To test antibiotics at low pH, single colonies of the available *Sulfobacillus* strains were inoculated in 10 ml FeSYE media and grown at 37˚C overnight. One millilitre of the overnight culture was inoculated in fresh 10 ml FeSYE with or without antibiotics (ampicillin 100 µg/ml, chloramphenicol 50 µg/ml, tetracycline 10 µg/ml, streptomycin 50 µg/ml, kanamycin 50 µg/ml). Media turning reddish brown and an increase in optical density at 550 nm were considered indicators of cell growth.

Where growth inhibition was observed in liquid media, the minimum inhibitory concentration (MIC) of that antibiotic on solid media was determined. FeSo plates were prepared with a dilution series of the particular antibiotic and *Sulfobacillus* cultures were streaked to single colonies on these plates. Sensitivity was scored according to the appearance of single colonies on plates after five days of incubation at 45˚C.

To test antibiotic sensitivity of *Sulfobacillus* strains at higher pH, a buffered, low iron medium was used, where the $FeSO_4$ content is reduced from 25 mM to 2.5 mM and potassium tetrathionate was replaced with 2.5 mM sodium thiosulphate. Plates were prepared at a range of pH values and dilutions of *Sulfobacillus* cultures were plated onto low iron media at different pH values ranging from pH 2.8 to pH 5.

4.3.3. Vector construction

Once suitable antibiotics for selection were determined, vectors carrying appropriate resistance markers were obtained or constructed.

Plasmid pHV33 consists of the *Staphylococcus aureus* plasmid pC194 cloned into the single *Hin*dIII restriction site of the *E. coli* cloning vector pBR322 (Horinouchi and Weisblum,

1982). The ampicillin and tetracycline resistance markers derived from pBR322 are functional in *E. coli* but not in *B. subtilis,* while the pC194 derived chloramphenicol resistance marker is functional in many Gram-positive hosts (Aleshin *et al.,* 1999; Li *et al*., 2007; Rasko *et al*., 2007).

Candidate *E. coli-Sulfobacillus* shuttle vectors (pSulfTACm and pSulfTYCm) were based on two transposon-captured derivatives of pKara (section 3.4.1) the native plasmid isolated from *S. thermosulfidooxidans* DSM 9293^{T}. The kanamycin marker of the EZ::TN transposon was replaced with the pC194 chloramphenicol marker excised from pHV33. First, plasmid pSKCm was constructed by cloning a 1701 bp *Cla*I-*Cla*I fragment of pHV33 containing the pC194 chloramphenicol resistance marker and rolling circle replication initiation protein, into the *Cla*I digested pBluescript KS^{+} vector. This fragment was excised from pSKCm using the *Xho*I and *Eco*RI restriction sites in the multiple cloning site of pBluescript KS^{+}. pSulfTA, a transposon captured derivative of pKara (chapter 2) was digested with *Xho*I and *Eco*RI to remove the kanamycin resistance marker of the transposon. The *Xho*I-*Eco*RI fragment with the pC194 chloramphenicol resistance marker was ligated to the *Xho*I-*Eco*RI digested pSulfTA fragment and transformed into *E. coli* EC100D to yield pSulfTACm (Figure 4.1). The same cloning strategy was used to construct pSulfTYCm, as the *Eco*RI and *Xho*I sites fell within the transposon sequence, which was inserted into a different area of the pKara parental plasmid.

Plasmids pHV33, pSulfTACm and/or pSulfTYCm were used in all electroporation attempts.

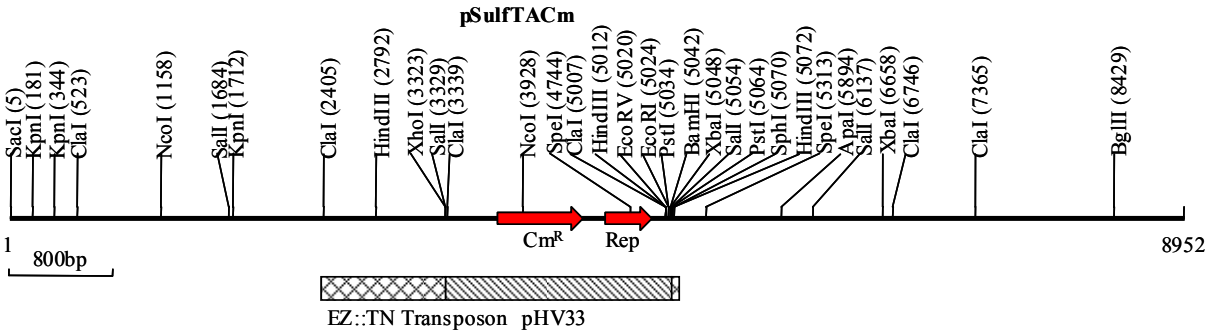

Figure 4.1 Restriction map of pSulfTACm

The positions of sequences derived from the EZ::TN transposon containing the R6Kγ origin of replication (▨) and from pHV33 encoding the chloramphenicol resistance marker and rolling circle replication initiation proteins (▨) are indicated.

4.3.4. Assay for restriction endonuclease production

Since endogenous restriction endonucleases have often been implicated in the failure of electrotransformation attempts, *S. thermosulfidooxidans* DSM 9293^T was tested for production of endonucleases.

The method of Belavin and co-workers (1988) was used to detect endonuclease activity in *Sulfobacillus*. Cells were harvested by centrifugation, washed and resuspended in extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.1 g/l lysozyme). The cell suspension was incubated for 15 min at room temperature with shaking. The resulting cloudy lysate was cleared by centrifugation for 1 min at 10 000 g. Fourteen microlitres of the lysate was added to 6 µl plasmid DNA (± 1 µg) in restriction buffer (20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM 2-mercaptoethanol). After incubation at 37˚C for 15 to 30 min, the plasmid DNA quality was evaluated by agarose gel electrophoresis.

4.3.5. Selection of strains for electroporation optimizations

Investigating the effects of many factors influencing electroporation on eleven strains would have been prohibitively expensive; therefore initial optimizations had to be done on one or two strains only with other strains added for comparison in some experiments.

4.3.6. Electroporation solutions and applied voltage

To determine which solution would be suitable for electrotransformation of sulfobacilli, four different media were investigated, 5 mM $MgCl_2$, 10% glycerol, 10% glycerol supplemented with PEG 4000 and 10% glycerol supplemented with sucrose. *Sulfobacillus* cultures were harvested and washed as normal, then washed three times in the particular electroporation medium. Electrical pulse parameters were 200 Ω parallel resistance and 25 µF capacitance, while the applied Voltage was varied from 1 kV to 2.5 kV.

4.3.7. Recovery medium

To determine which recovery medium would enhance survival of cells after electroporation, *Sulfobacillus* strain G2 was harvested from 1l of exponential phase culture, washed as normal (section 2.3.3), then three times in 10% glycerol and finally resuspended in 10% glycerol.

Cells were added to chilled electroporation cuvettes with a 2 mm electrode gap and an electrical pulse was applied to duplicate samples (2.5 kV, 200 Ω, 25 μF). Immediately after the pulse, 1 ml of the various recovery media (MilliQ water, 10% glycerol, 1X Basal salts and complete FeSYE growth medium) was added to the pulsed cells. Two samples were also placed in electroporation cuvettes and diluted with 1 ml 10% glycerol without a pulse being applied. Cells were allowed to warm to room temperature and recover overnight, and then dilutions were plated on FeSYE plates.

4.3.8. The effect of cell wall weakening agents on Sulfobacillus *cells*

Gram-positive organisms are often more efficiently transformed by electroporation after the cell wall has been weakened. The effect of three cell wall weakening agents on cells of *Sulfobacillus* was evaluated.

One millilitre of actively growing *Sulfobacillus* strain G2 was inoculated in 5 ml of FeSYE media (adapted from Johnson *et al.,* 2005) and FeSYE media supplemented with glycine (1% glycine and/or 1% glycerol) or threonine (0.5%, 1.0% and 2%) and incubated at 40˚C, shaking. Growth was quantified as optical density at 600 nm.

For lysozyme treatment, *Sulfobacillus* cells were grown and washed as normal, resuspended in 2 ml TE buffer and then split into two 1 ml aliquots. Lysozyme (0.1 μg/ml) was added to one tube and distilled water to the other. The cell suspensions were incubated at 37˚C for 15 min. Cells were monitored for protoplast formation by light microscopy. At the end of 15 min, cells were diluted to 10 ml in TE and harvested gently by centrifugation for 10 min at 2000 g.

4.3.9. Lithium chloride treatment

Treatment of cells with lithium chloride has been shown to induce electrocompetence in bacteria (Papagianni *et al.,*2007), although the mechanism by which this occurs is uncertain. The S-layer of *Sulfobacillus* cells can be removed by treatment with 5 M $LiCl_2$ (Severina *et al*., 1998). This treatment has also been used to remove the S-layer of *Caulobacter* strains before electroporation without causing significant cell death (Gilchrist and Smit, 1991).

Sulfobacillus strain YOO17 was grown to mid-log phase in one litre FeSYE media, then harvested and washed as normal. All steps were on ice or at 4˚C. Cells were resuspended in 50 ml cold 1x basal salts. This volume was split in two and cells recovered as before. One cell pellet was resuspended in 25 ml cold 1x basal salts, the other cell pellet in 25 ml cold 5 M $LiCl_2$. Cell suspensions were incubated on ice for 10 min, and then recovered by centrifugation as before. Each pellet was resuspended in 8 ml cold 10% glycerol.

One hundred microlitres of $LiCl_2$ treated and of control cells was mixed with 1 µg DNA of plasmid pSulfTACm. Cells were electroporated at 1.0 kV or 2.0 kV, with 200 Ω parallel resistance and 25 µF. One millilitre of distilled H_2O was added immediately after the electrical pulse. After 10 min, 1 ml of 2x basal salts was added and the cells were incubated overnight at 37˚C without shaking. Dilutions were plated on FeSYE solid media with or without chloramphenicol and incubated at 40˚C for five days.

4.4. Results

4.4.1. Antibiotic resistance survey

In FeSYE liquid growth medium, cell growth of all the tested *Sulfobacillus* strains was inhibited by chloramphenicol at a concentration of 50 µg/ml and by tetracycline at 10 µg/ml. Ampicillin, kanamycin and streptomycin at concentrations of 100 µg/ml, 50 µg/ml and 50 µg/ml, respectively in liquid medium had no visible effect on cell growth. An example of the growth inhibition of *S. thermosulfidooxidans* strain 611 by chloramphenicol is shown in Figure 4.2.

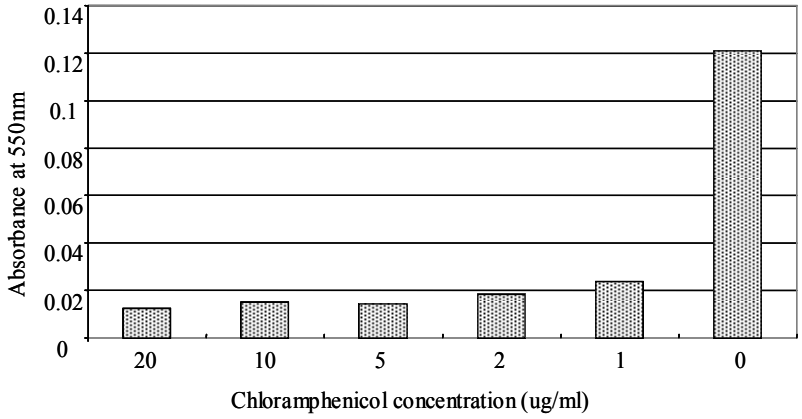

Figure 4.2 Growth inhibition of *Sulfobacillus* strain 611 by chloramphenicol in liquid medium.

Cell growth, as measured by optical density at 550 nm after overnight growth, was significantly inhibited by 1 μg/ml and higher concentrations of chloramphenicol. Other strains were similarly inhibited.

As selection of transformants would occur on solid media, the minimum inhibitory concentrations (MIC) of chloramphenicol and tetracycline towards strains of *Sulfobacillus* on FeSo solid medium were tested and found to be 0.5 μg/ml and 10 μg/ml respectively (Table 4.3).

Table 4.3 MIC of chloramphenicol and tetracycline in FeSo solid medium.

	Chloramphenicol concentration (μg/ml)							Tetracycline concentration (μg/ml)					
Strain	0	0.1	0.25	0.5	1.0	2.0	5	0	0.6	1.3	2.6	5.2	10.3
DSM 9293^T	+	+	+	-	-	-	-	+	+	+	+	+	-
YOO17	+	+	+	-	-	-	-	+	+	+	+	+	-
MT13	+	+	+	-	-	-	-	+	+	+	+	+	-
Andy	+	+	+	-	-	-	-	ND	ND	ND	ND	ND	ND
L15	+	+	+	-	-	-	-	+	+	+	+	+	-
THWX	+	ND	ND	-	-	-	-	ND	ND	ND	ND	ND	ND
G2	+	ND	ND	-	-	-	-	+	+	+	+	+	-
GG6-3	+	ND	ND	-	-	-	-	ND	ND	ND	ND	ND	ND
GSM	+	ND	ND	-	-	-	-	ND	ND	ND	ND	ND	ND
YTF1	+	ND	ND	-	-	-	-	+	+	+	+	+	-
611	+	ND	ND	-	-	-	-	+	+	+	+	-	-

[a]ND not determined; + visible single colonies; - no growth

It is possible that other antibiotics such as ampicillin or kanamycin may be inactivated at pH 2.5 therefore attempts were made to test the efficacy of these antibiotics against *Sulfobacillus* cells at a higher pH. FeSo plates could not be used to test the antibiotic resistance of

Sulfobacillus cells at higher pH, as FeSo growth media is not buffered, making adjustment of the pH difficult; also tetrathionate is unstable at higher pH. The buffered FeSYE medium, with sodium thiosulphate substituted for potassium tetrathionate, could not be adjusted to a higher pH either. At pH 4 a precipitate (probably jarosite) formed. When the $FeSO_4$ concentration of the FeSYE medium was reduced from 25 mM to 0.5 mM, formation of the precipitate at pH 4 was delayed, but still formed before significant cell growth occurred, both in liquid and solid media. Microscopic observation of the liquid medium revealed few microbial cells but copious amounts of granular precipitate. In the solid media, a crystalline precipitate at pH 3 and below was observed by stereomicroscopy. An amorphous precipitate was observed in solidified media above pH 4. When the iron source was omitted from liquid or solid media entirely, the precipitation of media components was eliminated, but cell growth of sulfobacilli is unstable in the absence of iron (Zakharchuck *et al.,* 2003). Testing of antibiotics at higher pH was therefore abandoned, as it would be impractical to perform selection of transformants on media that was not stable.

4.4.2. Assay for restriction endonuclease production

To determine whether endonucleases that would hinder transformation are produced by sulfobacilli, plasmid DNA was treated with cell extracts from *S. thermosulfidooxidans* strain DSM9293^T and fragments were separated by agarose gel electrophoresis. Degradation of plasmid DNA was evident, but no discrete bands were observed (Figure 4.3).

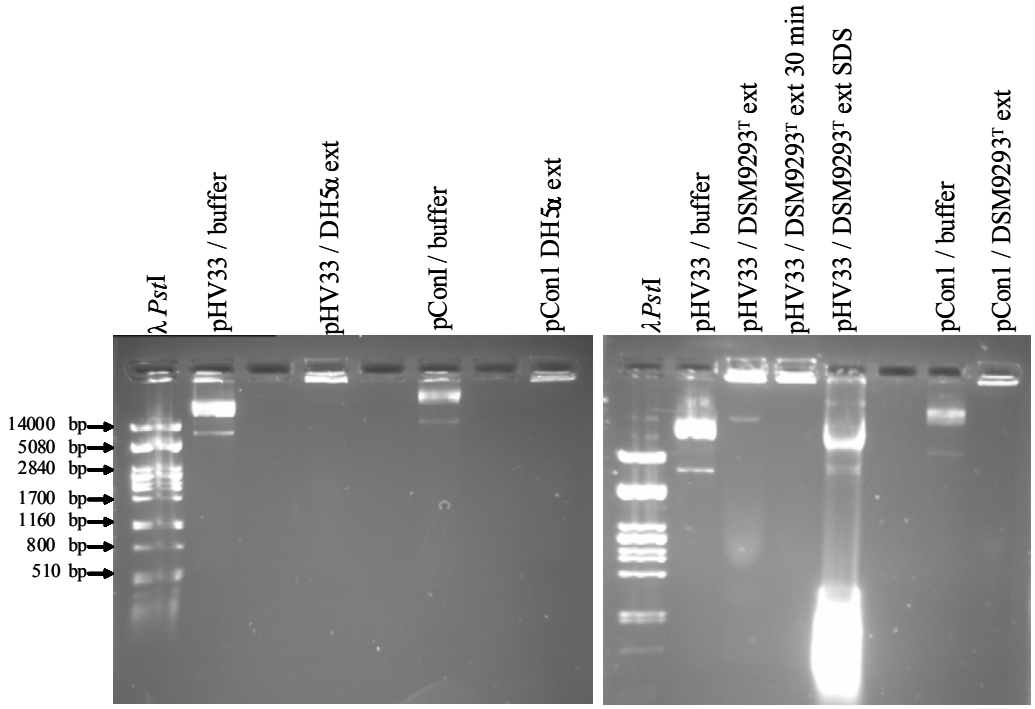

Figure 4.3 Endonuclease assay:

Agarose gel of plasmid DNA samples treated with cell extracts from *E. coli* DH5α and *Sulfobacillus* DSM9293^T. Cell extracts from *E. coli* DH5α and *Sulfobacillus* strain DSM 9293^T or extraction buffer only were added to plasmid DNA of pHV33 and pCon1 and incubated at 37˚C. All incubations were done for 15 min, unless otherwise indicated. Plasmid DNA was degraded by both extracts but more rapidly by the DH5α extract. When SDS was added, degradation of both DNA and RNA was inhibited.

The lack of discrete bands indicated that plasmid DNA was degraded in a non-specific manner, rather than by a nuclease with a specific recognition site. The plasmid DNA was added as a circular molecule, therefore the nuclease must have endonucleolytic function. The nuclease activity of the *Sulfobacillus* extract was not higher than that of the *E. coli* extract. Addition of the protein denaturing agent SDS to the extracts diminished degradation of DNA, indicating that the degradation was caused by a nuclease, and not by residual iron or other media components.

4.4.3. Selection of strains for electroporation optimizations

Of the 11 strains available, 5 exhibited cell-aggregation or relatively poor growth in FeSYE medium. Strains G2, YOO17, L15, 611, Andy and Mintek45 consistently produced good cell yields from liquid media and produced larger colonies on solid media within five days. When

strains YOO17 and L15 were electroporated, colonies recovered on solid media were very small and transparent, never becoming opaque, or attaining the "fried egg" morphology that was evident before electroporation. Strain G2 produced colonies large enough to pick from plates in a shorter time than other strains (three days rather than five). Strains 611, L15 and YOO17 harboured plasmids, which would complicate screening for transformants and might cause problems with plasmid incompatibility. Strains Andy and Mintek45 did not appear to have plasmids but took five days to produce colonies on solid media. *Sulfobacillus* strain G2 was therefore chosen for investigation of electroporation parameters.

4.4.4. Electroporation solution and applied voltage

The best electroporation solution should place no additional stress on electroporated cells, yet should promote the efficient uptake of DNA. The first electroporation solution was 5 mM $MgCl_2$ and this was evaluated using strain G2 and the type strain DSM 9293^T. In this buffer, at least 1.5 kV applied voltage was needed to permeablize a significant percentage of cells (as estimated by cell death upon application of the electric pulse) (Figure 4.4).

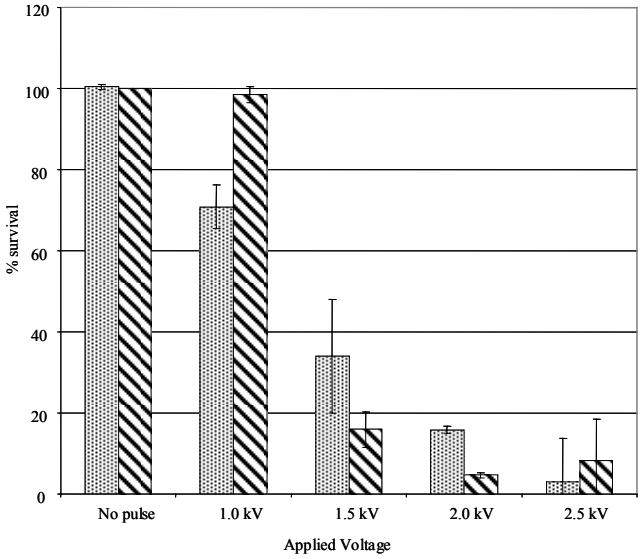

Figure 4.4 Survival of *S. thermosulfidooxidans* strains DSM9293^T (▨) and G2 (▩) after electroporation in $MgCl_2$ electroporation medium.

Application of electrical pulses of less than 1.5 kV resulted in almost no reduction in viable cell numbers for strain DSM9293^T in $MgCl_2$ electroporation solution.

Strain DSM 9293^T and $MgCl_2$ electroporation medium were not used again, as this strain already contains the plasmid which was used for the backbone of the constructed shuttle

vectors pSulfTACm and pSulfTYCm. Also frequent arcing resulted when 5 mM $MgCl_2$ electroporation solution was used. Only the results from electroporations which did not experience arcing were included in the dataset used to compile Figure 4.4. No transformants were recovered on chloramphenicol media after electroporation of DSM 9293^T in 5 mM $MgCl_2$.

The second electroporation solution to be evaluated was 10% glycerol, which has been reported to reduce arcing (Holo and Nes, 1989). Strain G2 was used for this experiment. The 10% glycerol solution resulted in a reduction in arcing but no transformants were obtained. As expected, the number of surviving cells after electroporation of sulfobacilli decreased as the voltage increased. At an applied voltage of 1 kV over a 2 mm electrode gap (5 kV/cm), there was no significant decrease in the survival of *Sulfobacillus* cells. With an applied voltage of 2.5 kV (12.5kV/cm), approximately 60% of cells survived the electric pulse (Figure 4.5). This falls within the optimum range determined empirically for other Gram-positive bacteria (Table 4.2). Therefore, the minimum applied voltage needed to produce membrane pores in a significant proportion of *Sulfobacillus* G2 cells is 2.5 kV (12.5 kV/cm) in 10% glycerol solution.

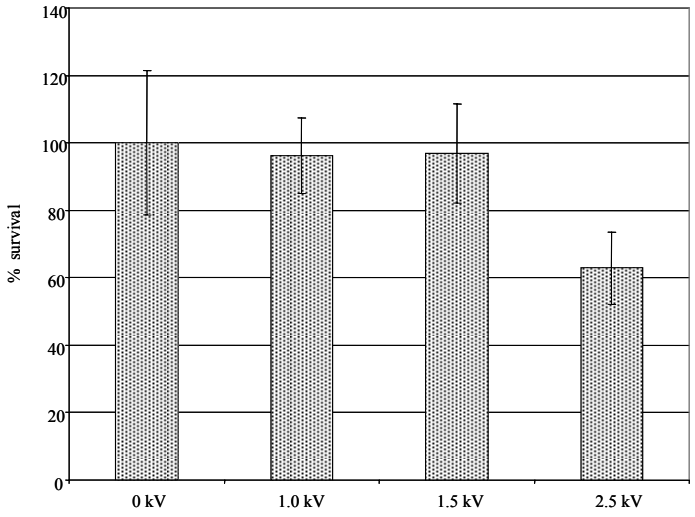

Figure 4.5 Survival of *Sulfobacillus* strain G2 after electroporation in 10% glycerol electroporation medium.

Application of electrical pulses of less than 2.5 kV resulted in almost no reduction in viable cell numbers for strain G2 in 10% glycerol electroporation solution. Capacitance 25 μF, Resistance 200 Ω, Applied voltage was varied between 0 and 2.5 kV.

To investigate whether the addition of another osmoticum would increase the survivability during electroporation, sucrose was added to the electroporation medium, but this did not have a significant positive effect (Figure 4.6), and no transformants were recovered on chloramphenicol media.

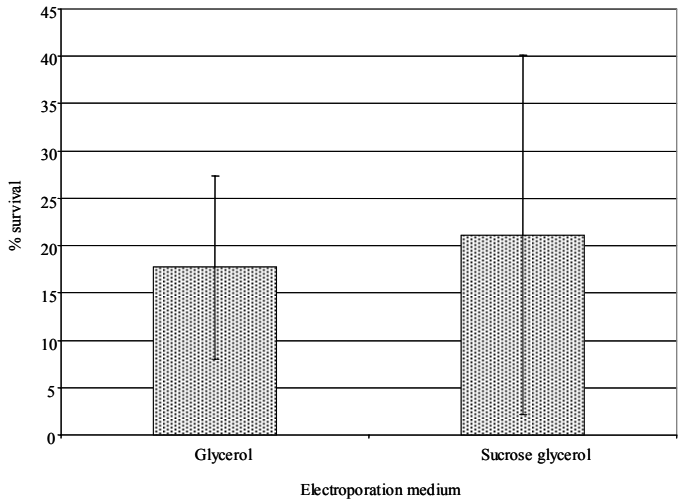

Figure 4.6 Survival of *Sulfobacillus* G2 cells after electroporation in 10% glycerol and 10% glycerol supplemented with sucrose.

The addition of sucrose to the electroporation solution did not confer a significant advantage above the use of glycerol alone.

Next, the effect of polyethylene glycol (PEG) was investigated. Addition of PEG 4000 to the electroporation medium caused a slight decrease in survivability during electroporation relative to 10% glycerol alone (Figure 4.7), but no transformants were recovered.

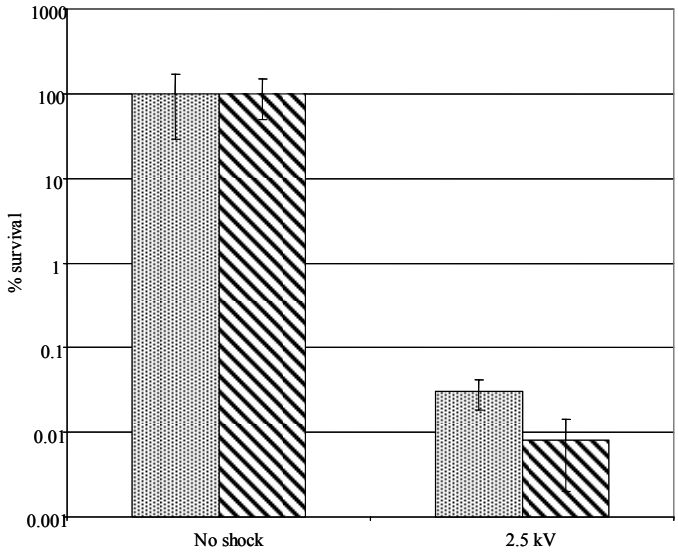

Figure 4.7 The effect of PEG 4000 in the electroporation medium on the survival of *Sulfobacillus* strain G2.

The electroporation medium was 10% glycerol (▨), or 10% glycerol supplemented with 10% PEG 4000 (▧).

4.4.5. Recovery medium

The recovery medium should allow cells to repair damage to cellular membranes and to express any selectable markers introduced. Recovery media are usually nutritionally rich growth media, buffered at neutral pH and which allow rapid growth of the bacterium being transformed. Four recovery media were investigated: complete FeSYE medium, 1x basal salts, 10% glycerol and distilled water (MilliQ). Surprisingly, for cells of *Sulfobacillus* strain G2, distilled water enabled the best recovery rate after electroporation (Figure 4.8).

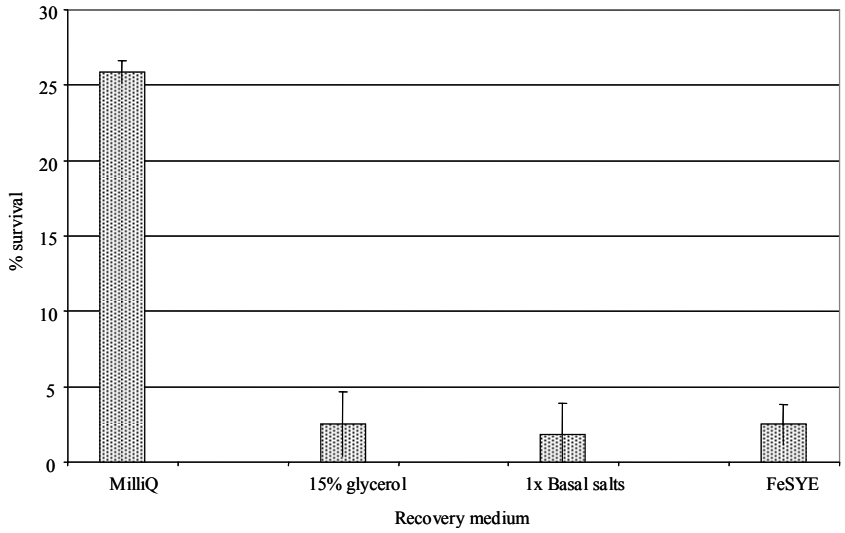

Figure 4.8 The effect of different recovery media on survival of *Sulfobacillus* G2 cells after electroporation.

Cells were harvested and washed as normal, then 100 µl aliquots were electroporated at 2.5 kV, 25 µF, 200 Ω. Nine hundred microlitres of the respective recovery media were added immediately after electroporation and the cells were allowed to recover overnight at room temperature, before plating dilutions on FeSYE solid media. Distilled water (MilliQ) allowed the best recovery.

4.4.6. The effect of cell wall weakening agents on Sulfobacillus

Three cell wall weakening agents were tried, glycine, threonine and lysozyme. Glycine could not be used in FeSYE growth medium to weaken the cell walls of sulfobacilli. An unknown compound produced during growth of sulfobacilli in FeSYE medium reacted with the glycine. When a 1 in 10 inoculum of actively growing cells was transferred from FeSYE medium to FeSYE+ glycine (4%, 2% or 1%), the media immediately turned reddish brown and a precipitate formed. To determine the cause of the unwanted reaction, various medium components were added separately to glycine solutions. Added separately, basal salts, potassium tetrathionate and iron sulphate had no effect. The addition of an excess of trace elements solution caused a slight blue colour to develop. The addition of complete FeSYE to 2% glycine caused slight precipitation, but no brown colour developed. When washed *Sulfobacillus* cells were added, a slight precipitation resulted, but no brown colour. Spent, cell free FeSYE medium and spent medium with cells caused the development of a reddish brown colour and the formation of a precipitate. The addition of cells and spent medium of

Acidithiobacillus caldus, a Gram-negative acidophilic iron and sulphur oxidiser did not cause precipitation or colour development. When *Sulfobacillus* cells from an actively growing culture were harvested and washed and used as an inoculum for glycine-containing FeSYE medium, a precipitate resulted after overnight growth.

Threonine was more compatible with the FeSYE growth medium. No discolouration or precipitation formed on addition of threonine (0.5%, 1% or 2%) to the growth medium, or after growth of cells in the medium. The growth of *Sulfobacillus* cells was not significantly inhibited by the presence of threonine in the medium.

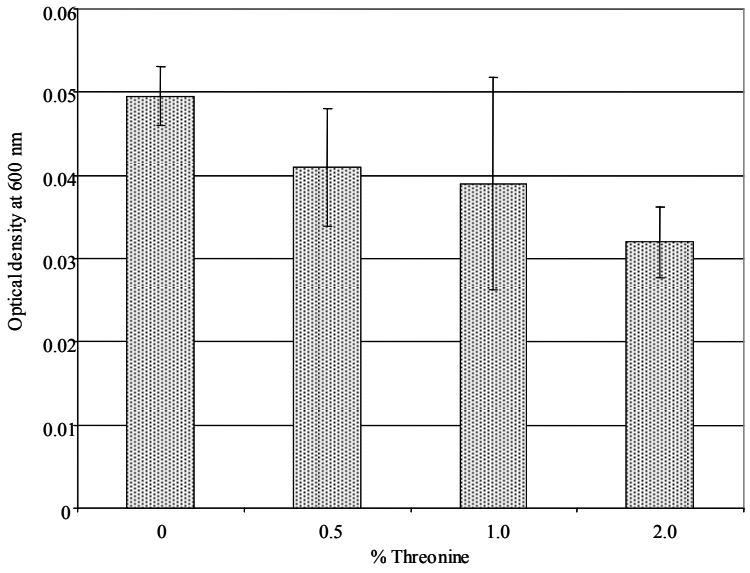

Figure 4.9 The effect of threonine on the growth of *Sulfobacillus* strain G2 in FeSYE medium.

Threonine slightly inhibited growth of *Sulfobacillus* strain G2.

Lysozyme treatment caused chains of *Sulfobacillus* cells to break up into single cells, although spheroplasts were not observed by light microscopy. The survival of lysozyme treated cells after electroporation was reduced to less than 10% relative to untreated cells (Figure 4.10).

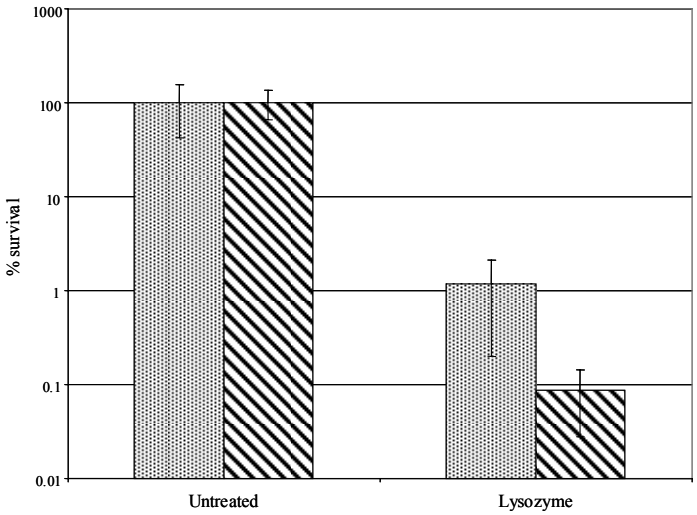

Figure 4.10 The effect of lysozyme treatment on survival of *Sulfobacillus* cells after electroporation.

Cells of *Sulfobacillus* strain G2 (▨) and YOO17 (▨) were treated with 0.1 µg/ml lysozyme for 15 min at 37˚C before resuspending in 10% glycerol solution for electroporation. Less than one percent of cells survived electroporation after lysozyme treatment.

4.4.7. Lithium chloride treatment

Lithium chloride treatment of *Sulfobacillus* cells did not have a significant effect on survival after electroporation (Figure 4.11), and neither did it allow transformation – no colonies were observed on FeSYE plates with chloramphenicol.

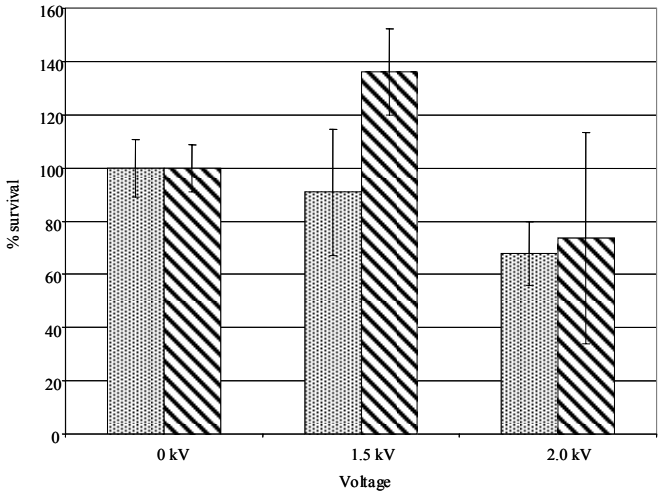

Figure 4.11 Effect of Lithium chloride treatment on the survival of *Sulfobacillus* cells after electroporation

Untreated (▒). Lithium chloride treated (▨).

4.5. Discussion

One advantage of electroporation over other transformation methods is that small cell volumes can be used. This is very useful for biomining bacteria, since these mostly have poor cell yields compared to *E. coli*. *Sulfobacillus* YOO17, a good grower compared to other strains of *Sulfobacillus* and other important biomining bacteria, yields merely 500 mg (wet weight) of cells from two litres of FeSYE medium.

In some ways, *Sulfobacillus* spp. are easier to work with than most other biomining bacteria. They grow faster and to higher cell densities than *Leptospirillum* and *Acidithiobacillus* species. Sulfobacilli readily grow on simple solid media and are not as sensitive to organic compounds.

Large variability between electrocompetence of different batches of cells may occur (Löfblom *et al*., 2007). Therefore results of electroporation experiments can only be directly compared when the same batch of cells is used.

The electroporation cuvettes used in this study are intended for single use only, but the cost of this would have been prohibitive, as large numbers of electroporation experiments were conducted. There are disadvantages to re-using cuvettes. Traces of DNA or cell debris may become stuck to the electrodes because of the strong currents. This may lead to reduced time

constants and / or greater variability in results or contamination with DNA from the previous use. The washing procedure followed was sufficient to prevent any contamination. The time constants measured during electrical pulse applications did not vary greatly, unless arcing occurred during the pulse application. However, large variability in the survival rates of sulfobacilli were found, which may be attributable to the re-use of cuvettes. This is in agreement with the variability reported by McIntyre and Harlander (1989) when re-using cuvettes. They reported successful electrotransformation of *Lactococcus*, although greater variability and less efficient transformation occurred when re-using cuvettes. Arcing was reported after approximately five uses of a cuvette.

A reliable method of selection for transformants of *Sulfobacillus* is essential for the development of a genetic system for this genus. This was complicated by the acidophilic and moderately thermophilic nature of sulfobacilli, since many antibiotics are sensitive to extremes of pH and temperature. An antibiotic resistance survey was conducted to determine the susceptibility of sulfobacilli to various antibiotics.

Both optical density at 550 nm and visible browning of the medium were considered as indicators for growth of sulfobacilli. An increase in optical density correlated well with observation of the medium turning brown and with the number of cells visible in a wet mount. However, chemical, rather than enzymatic, reactions also cause the growth medium to turn brown after an extended period, and spectrophotometry is confounded by the formation of jarositic precipitates, which do not always correlate with cell numbers. Therefore, appearance of single colonies on solid media was deemed to be a more reliable test for antibiotic resistance.

Sulfobacillus strains were naturally resistant to ampicillin, kanamycin and streptomycin, while they were inhibited by chloramphenicol (0.5 μg/ml) and tetracycline (10 μg/ml) (Table 4.3). Chloramphenicol and tetracycline have been reported to be adequately stable at low pH and high temperature (Davidson and Summers, 1983; Peteranderl *et al*., 1990; Aagaard *et al*., 1994), however tetracycline is slightly less stable and has been implicated in reduced transformant recovery (Steele *et al.,* 1994; Turgeon *et al.,* 2006). Therefore chloramphenicol was a better choice for transformant selection in *Sulfobacillus* strains.

It is possible that more antibiotics may be effective against sulfobacilli at slightly higher pH. However, there was some difficulty in devising a suitable higher pH growth medium for

sulfobacilli. Although it has been reported that the sulfobacilli are nutritionally versatile, able to grow autotrophically, heterotrophically and mixotrophically, with various energy sources, (Tsaplina *et al.,* 1991) in practice growth without iron is not stable and can only be maintained for a few subcultures (Tsaplina *et al.,* 2000; Zakharchuck *et al.,* 2003). Raising the pH of iron-containing medium above pH 3 caused the rapid formation of precipitates in liquid and solid media.

Vectors for electroporation were based on an indigenous plasmid of *Sulfobacillus* strain DSM 9293^T. The availability of a library of random transposon insertions in the natural *Sulfobacillus* plasmid allowed the easy construction of vectors with foreign sequences inserted at different locations in the natural plasmid. Insertion of DNA into the putative replication region of pKara was avoided.

The source of the transforming DNA is often important for electroporation, as restriction of transforming DNA may be avoided by proper modification of the DNA. *Sulfobacillus* strain DSM 9293^T methylates both *dam* and *dcm* sites on pKara (Figure 2.14), therefore the vectors used for electroporation were prepared in a *dam* and *dcm* methylation positive *E. coli* host.

It has not been reported whether any *Sulfobacillus* strains possess restriction-modification systems. The method of Belavin *et al.,*(1988) may provide a rapid, if rough indication of which strains may have reduced DNA restriction activity. It would be beneficial to concentrate transformation attempts on such a strain, both because of greater chances of initial success, and because this strain may then be used to passage any DNA that must be transformed into another strain, enabling transformation of restriction-positive strains.

Surprisingly, degradation of plasmid DNA was more extensive with the *E. coli* DH5α cell extract than with cell extracts from *Sulfobacillus* cells (Figure 4.3). Since DH5α is highly transformable, this may indicate that restriction endonucleases will not be a major barrier to transformation in *Sulfobacillus* strain DSM 9293^T. However, it may be that nucleases are simply extracted with greater efficiency from cells of *E. coli* than from thick walled *Sulfobacillus* cells. Plasmid DNA was completely degraded by the *Sulfobacillus* strain DSM 9293^T cell extract and no definite banding pattern emerged, indicating that a rather non-specific nuclease is active in this strain. This assay could be improved by standardising the protein content of the extracts before testing the nuclease activity. Screening of more *Sulfobacillus* strains may reveal a strain with reduced nuclease activity.

The different electroporation solutions tested had little impact on survival of *Sulfobacillus* cells. Problems with arcing during electroporation were reduced by using a simple 10% glycerol solution. A slight increase in survival when using 10% glycerol was noted relative to using magnesium chloride electroporation buffer. This should be interpreted with caution, because a different batch of *Sulfobacillus* cells was used for the two experiments. Addition of PEG to the electroporation solution reduced cell survival only slightly. This is encouraging, since inclusion of PEG in electroporation buffers has been shown to improve electroporation efficiency in several Gram-positive species (Dower *et al.,* 1992; Metzler *et al.,* 1992; Kaneko *et al.,* 2000).

Sulfobacillus spp. present an interesting problem with respect to the electroporation recovery medium, since their preferred growth medium could be considered to be rather harsh in comparison to other reported recovery media, being highly acidic and containing high concentrations of iron. One other biomining bacterium has been transformed by electroporation. Kusano *et al.* (1992) used an acidic, iron-rich medium (9K) for recovery of the Gram-negative acidophile *Acidithiobacillus ferrooxidans*. Thus recovering cells are not necessarily damaged by acid or high concentrations of metal ions, although it must be noted that the Gram-positive sulfobacilli are very different from acidithiobacilli.

The best recovery medium for *Sulfobacillus* strain G2 seemed to be distilled water, and not its optimal growth medium (Figure 4.8). This is in contrast to literature, where rich growth medium, often supplemented with an osmotic stabiliser and buffered at neutral pH, is most often used (Table 4.1 and references therein).

The effect of cell wall weakening agents on *Sulfobacillus* cells varied. Glycine reacted with an unknown substance produced during growth of sulfobacilli in FeSYE medium, causing the media to change from a clear colourless solution to red-brown, and the immediate precipitation of unknown media components. Therefore, weakening of the cell wall by growing sulfobacilli in the presence of high concentrations of glycine seems impractical.

Threonine slightly inhibited *Sulfobacillus* cell growth but is compatible with the growth medium and metabolites produced by *Sulfobacillus*. The effect of this cell wall weakening agent on electroporation should be tested further.

Enzymatic removal of cell walls with lysozyme was effective. *Sulfobacillus* cells are clearly affected by treatment with this enzyme. Lysozyme treatment caused chains of cells to break up and also caused greater fragility of cells during electroporation, indicating that the cells walls of sulfobacilli could be weakened by this treatment, therefore reducing the barrier posed by the cell wall, however the survival of lysozyme treated cells after electroporation is too low. This would decrease chances of recovering transformants. Therefore lysozyme treatment should be tested in combination with osmotic stabilizers in the electroporation medium to try and improve survivability.

Pre-treatment of cells with lithium chloride has been reported to increase electrotransformation frequency of *Lactococcus lactis* spp. *lactis* (Papagianni *et al.,*2007) and to remove the S-layer, which impaired electroporation efficiency from *Caulobacter* cells (Gilchrist and Smit, 1991). Lithium chloride treatment did not greatly reduce viability of *Sulfobacillus* cells but neither did it result in recovery of transformants. The effectiveness of this treatment to remove S-layers from sulfobacilli (if present) was not investigated.

Although plasmids PSulfTACm, pSulfTYCm and/or pHV33 were included in all electroporations no transformants were recovered under any of the conditions tested. The vectors all carry the pC194 (Horinouchi and Weisblum, 1982) chloramphenicol resistance gene. The chloramphenicol resistance gene has been shown to function in a wide array of Gram-positive and Gram-negative bacterial hosts (Luchanski *et al.,* 1988; Belliveau and Trevors, 1989; Brigidi *et al.,* 1990; Aleshin *et al.,* 1999; Turgeon *et al.,* 2006).

Plasmids pSulfTACm and pSulfTYCm are based on a native plasmid of *Sulfobacillus* strain DSM 9293^{T} (pKara) and therefore have a good chance of being able to replicate in other strains of *Sulfobacillus thermosulfidooxidans*. Since the minimal replicon of the original pKara is uncharacterized except as a tentative prediction from sequence data, two shuttle vectors were constructed, with the *E. coli* replicon and selectable marker inserted at different locations.

Despite the care taken in choosing the plasmid backbone and selectable marker for the electroporation vectors, no transformants were obtained in any electroporation experiments. There are many possible causes for this failure.

Insufficient permeabilization of the membranes of sulfobacilli could have precluded entry of DNA molecules. An argument against this hypothesis is that the degree of cell death caused by application of voltages higher than 2.5 kV falls within the range reported for successful electrotransformation of other Gram-positive bacteria (Table 4.2). Although *Sulfobacillus* cell walls were susceptible to lysozyme treatment, this treatment was insufficient to allow transformation. The effectiveness of PEG treatment and lithium chloride treatment could not be evaluated. Since no transformants were recovered in any experiments, an increase or decrease in transformation frequency by PEG or lithium chloride could not be quantified. One way to determine whether sufficient permeabilization of the cell envelope occurred would be to monitor the uptake of fluorescent dextrans (Han *et al.,* 2007) or other easily detected macromolecular substances by electroporated cells and this should be included in future studies.

A second cause for failure to recover transformants may be the inability of the vectors to replicate in sulfobacilli. Two of the vectors were based on a native *Sulfobacillus* plasmid, a strategy that has proven effective for the construction of vectors for many bacterial species. However, an essential replication region may have been interrupted by the insertion of the selectable marker and *E. coli* replicon. The third vector contained the replicon of pC194, a plasmid with an exceptionally wide host range (Goursot *et al.,* 1982). A plasmid with a similar replicon has been isolated from *Sulfobacillus* strain BC1 (Section 3.4.2.2). On this plasmid, a rolling-circle replication initiation protein and *nick* site of the pC194-type is conserved, indicating that at least a theoretical possibility exists for the pC194 replicon to function in *Sulfobacillus* hosts. Since a large library of transposon captured derivatives of pKara is available, construction of vectors that do not have an interrupted replicon should be a trivial, if labour intensive exercise.

A third cause for the failure to recover transformants of sulfobacilli may be the lack of expression, translation, proper folding or functioning of the chloramphenicol resistance determinant, although this marker originates from another Gram-positive organism (*Staphylococcus aureus*) and has been shown to function in a wide variety of hosts.

A fourth difficulty may be destruction of plasmid DNA by endogenous nucleases of sulfobacilli. Since plasmid DNA in *Sulfobacillus* strain DSM 9292^{T} was methylated at *dam* and *dcm* sites (section 2.4.6.2), vectors for electrotransformation were isolated from *dam* and

dcm methylase positive *E. coli* strains. Also, strain DSM 9293^T did not seem to harbour more efficient nucleases than a highly transformable *E. coli* strain (section 4.4.2). Therefore interference of nucleases was not expected to be a problem. However, methylation and restriction systems may vary between strains of the same species (Acetto *et al*., 2005); therefore nucleases cannot be eliminated as a cause of transformation failure. Temporary inactivation of host restriction systems has been reported by heating of cells before electroporation. This option may be explored for future electroporation attempts, possibly at higher temperatures than reported in literature for other bacteria, since sulfobacilli are moderately thermophilic and their nucleases may be expected to be heat resistant. Another option for circumventing inactivation of vectors by nucleases may be to use an integrative vector. Circular replicating vectors may be inactivated by as little as one nick introduced in the DNA. However, an integrative vector may be linearised or even cut into a few pieces and may still integrate in the host chromosome. Therefore, inclusion of *Sulfobacillus* genomic sequences on the vector might enable linearised plasmid to integrate in the chromosome. The *Sulfobacillus* arsenic resistance operon (van der Merwe, 2007) could be used for this purpose.

4.6. Conclusions

Chloramphenicol or tetracycline can be used in low pH media to select for *Sulfobacillus* transformants.

The most effective method for mitigating the physical barrier to DNA entry imposed by the thick Gram-positive type cell wall of sulfobacilli is treatment with lysozyme, although this severely reduces survivability during electroporation.

Applied voltages must be equal to or greater than or 2.5 kV over a 2 mm electrode gap (5 kV/cm) for sufficient permeabilization of *Sulfobacillus* membranes.

The best medium for recovery of cells after electroporation is distilled water.

No transformants were obtained by electroporation with *Sulfobacillus* cells with plasmids pHV33, pSulfTACm or pSulfTYCm. Whether this is due to inadequate permeabilization of the cell envelope, destruction of transforming DNA by endogenous nucleases, or failure of the plasmid replicons or selectable marker to function in cells of *Sulfobacillus* is unknown.

CHAPTER FIVE
CONJUGATION

5.1. Aim

The aims of this part of the study were to construct vectors suitable for conjugative transfer from *Escherichia coli* to sulfobacilli; to design a mating medium that would support both donor and recipient cells; and to determine whether sulfobacilli can be transformed by conjugation.

5.2. Introduction

Conjugation is a natural process whereby a plasmid (or transposon) is transferred between two cells in close contact. Conjugation has been exploited in molecular biology for transfer of vectors into many different bacteria, including Gram-positive and acidophilic bacteria (Table 5.1, Table 5.2) and conjugative transfer of plasmids has been achieved between organisms that are phylogenetically very distant from one another (Table 2.1).

Conjugative systems can be divided into three functional elements. The first is a DNA sequence element, the origin of transfer (*oriT*) that must be present in *cis*. The other two elements, the mating pair formation (mpf) system and the relaxosome, are plasmid encoded protein complexes that can be provided in *trans* (Zechner *et al*., 2000; Yang *et al.*, 2007). The two protein complexes are connected via a plasmid-encoded coupling protein (Grohmann *et al.*, 2003). The mating pair formation system establishes cell contact and forms a mating bridge with the recipient. The relaxosome cleaves and unwinds the plasmid, starting at the *oriT*. The single stranded DNA intermediate is transferred to the recipient via the mating bridge.

Conjugative plasmids encode all the elements necessary for their own transfer from a donor cell to a recipient cell and are thus self-transmissible. Most mobilizable plasmids encode the genetic information necessary for relaxosome formation and DNA processing, but must rely on the mating pair formation system of a conjugative plasmid resident in the same cell. Sometimes the only element needed in *cis* for a plasmid to be mobilizable is the origin of transfer, provided the transfer proteins are provided in *trans.*

When using conjugation as a transformation system, plasmid vectors can initially be introduced into an easily transformable bacterium and then transferred to other organisms that are challenging to transform by simpler methods such as electroporation. Unlike transformation by natural transformation and transduction, where a very limited range of recipients can be transformed with a specific system, DNA transfer by conjugation with some conjugation systems seems to have a very broad application range.

The greatest differences between conjugation in Gram-positive and Gram-negative bacteria are found in the initial establishment of cell contact. In Gram-negative systems, this contact is mediated by a conjugative pilus which binds to the recipient and then retracts by depolymerization, bringing the cells together (Curtiss, 1969; Ou and Sanderson, 1970). In most Gram-positive systems, the mechanisms of cell contact establishment are less well characterized or unknown (Grohmann *et al.*, 2003). The known Gram-positive systems encode protein aggregation factors by which cell contact is established (Grohman *et al*., 2003). Conjugative pili encoded by Gram-positive plasmids have not been observed (Prozorov, 2003). This lack of knowledge does not preclude the use of conjugation to transfer vectors from Gram-negative to Gram-positive bacteria, as Gram-negative conjugative pili seem to be able to mediate contact with Gram-positive cells and all the molecular mechanisms for conjugation mediated by Gram-negative conjugation systems are supplied by the donor cell. It appears that there is little hindrance to the intergeneric transfer of DNA by conjugation, but successful transformation is limited by the ability of the transferred plasmid to become established in the recipient cell.

There are several advantages to using conjugation as a transformation tool. Conjugation frequencies are often very high. For example, up to 75% of *Bacillus thuringiensis* recipient cells were transformed by intra-species conjugation (Prozorov, 2003) and the frequency of transfer of a large plasmid between cells of *B. subtilis* 19 (Poluektova *et al*., 2004) and from *E. coli* to *Magnetospirillum gryphiswaldense* (Schultheiss and Schüler, 2003) approached 100% .

Very large DNA fragments, up to 160.5 kb, can be transferred without a decrease in conjugation efficiency (Kuroki *et al.,* 2007). This is in contrast to other commonly used transformation methods such as heat shock transformation of chemically competent cells or

PEG transformation of protoplasts, where the transformation efficiency rapidly declines with increasing plasmid size.

Salyers *et al.* (2000) suggested that plasmids may be afforded a degree of protection when entering a nuclease laden recipient cell during conjugation since a single stranded DNA molecule is transferred to the recipient and most nucleases act on double stranded DNA. However, several studies have shown that conjugated DNA's are still susceptible to attack by different types of resident nucleases. Type II restriction enzymes in *Clostridium difficile* may attack the introduced DNA after it has been converted to double stranded form but before methylation of the DNA has been completed (Minton *et al.*, 2004). A methyl-specific restriction system in *Streptomyces coelicolor* also cleaved DNA transferred by conjugation (Flett *et al.*, 1997). This restriction system could be circumvented by using a methylation deficient mutant *E. coli* donor strain, which yielded 10^4 more transformants than methylation positive S17-1 donors. Therefore plasmid transfer by conjugation can be still be hindered by restriction systems, especially transfer of large plasmids that contain many restriction sites (Poluektova *et al*., 2004).

Some complications arise when using conjugation as DNA delivery mechanism. A suitable donor strain must be found or constructed. This strain must be able to deliver the plasmid in question, and therefore must be able to survive at least for a time alongside the recipient strain. Secondly, the donor and recipient strains must be distinguishable after mating. This requires a positive selection mechanism for the recipient and donor strains. In some cases this can be simple – for instance, incubating plates at 50˚C to select for *B. subtilis* and against *E. coli*. However, when organisms with similar growth requirements are used it may be necessary to use antibiotic markers. This can be very problematic if conjugation is being used as the means to develop a genetic system for a recipient that cannot be transformed by other means (Salyers *et al*., 2000).

The conjugative transfer of plasmids from *E. coli* (a neutrophile) to various Gram-negative acidophilic organisms has been reported (Table 5.2). A few examples of the transformation of Gram-negative biomining bacteria are given below.

Wide host range plasmids from the IncQ, IncP and IncW group have been transferred from *E. coli* to strains of *Acidithiobacillus ferrooxidans* and *At. thiooxidans* by conjugation (Jin *et al.,* 1992; Peng *et al.,* 1994*;* Liu *et al.,* 2001). The transfer frequency was affected by a

number of factors, including the growth medium in which the donor and recipient were prepared before mating, the pH of the mating medium, the concentration of thiosulphate and diaminopimelic acid (a cell wall component that improved growth of acidithiobacilli on solid media) in the mating medium and the donor to recipient ratio (Liu *et al.,* 2001).

Matings between the acidophile *Acidithiobacillus acidophilus* AYCC 27807 and *E. coli* were not successful. However, plasmid DNA could be transferred from *E. coli* to *Thiobacillus novellus* and then from *T. novellus* to *At. acidophilus*. Plasmid DNA could not be transferred from *At. acidophilus* to *E. coli*, but could be transferred from *At. acidophilus* to *Thiomonas perometabolis* and from *T. perometabolis* back to *E. coli* (Davidson and Summers, 1983). *T. novellus* and *T. perometabolis* and *At. acidophilus* were included in the genus *Thiobacillus* spp. but were later reclassified (Kelly and Wood, 2000). Plasmid RP4 has been transferred by conjugation from *E. coli* to *Acidithiobacillus thiooxidans* and from *At. thiooxidans* to *E. coli* (Jin et al., 1992).

A plasmid carrying a transposon-borne arsenic resistance operon was successfully transferred from *E. coli* to *Acidithiobacillus caldus* strain C-SH12 by conjugation, but *At. caldus* strains BC13 and KU were not similarly transformed (Kotze, 2006). The transconjugant *At. caldus* C-SH12 was more resistant to arsenite than the parental strain that had only the chromosomal arsenic resistance transposon.

The IncP type broad host range conjugative plasmid RP4 has proven to be a highly effective tool in the transformation of many organisms. An *E. coli* strain (S17-1) has been engineered with the *trans* acting elements of the RP4 mating system integrated in the chromosome. This strain can thus mobilize a plasmid that has the RP4 *cis* acting origin of transfer or another compatible *oriT* and has been used for the transformation of a great number of bacteria. Using this donor strain eliminates the complexity of having two plasmids, the conjugative and the mobilizable plasmid, present in the donor strain. The pilus produced by IncP type plasmids (like RP4) is rigid and does not allow mating in liquid medium therefore mating experiments with this donor strain must be conducted on semi-solid media.

Table 5.1 Examples of conjugation between Gram-positive and Gram-negative organisms

Plasmid(s)	Donor(s)	Recipient(s)	Comment	Reference
pIP823	Strains of : *Listeria monocytogenes, Enterococcus faecalis* and *Staphylococcus aureus*	Strains of *Listeria monocytogenes, Enterococcus faecalis, Staphylococcus aureus, Bacillus subtilis*, and *E. coli*	$<10^{-9}$ to10^{-1} transconjugants per donor.	Charpentier *et al.,* 1999
pAT18	*E. coli*	*Staphylococcus aureus,* and *Listeria monocytogenes*	Subinhibitory concentrations of penicillins increased transfer frequency	Trieu-Cuot *et al.,* 1993.
Vectors based on the *Lactobacillus fermentum* plasmid pLF1311 and containing the RP4 *oriT*	*E. coli*	Strains of *Lactobacillus*, *Lactococcus, Enterococcus* and *Bacillus*		Aleshin *et al.,* 2000
pECM1	*E. coli* S17-1	*E. coli* MM294. Coryneform bacteria of genera *Arthrobacter*, *Brevibacterium* and *Corynebacterium*	10-^{8} and 10^{-2} transconjugants per donor. Improved by heat treatment of recipient cells	Schäfer *et al.,* 1990
Derivatives of pK18mob.	*E. coli* S17-1	*Brevibacterium lactofermentum* R31	Frequency not given	Ramos *et al.,* 2003.
pTO1	*E. coli* S17-1	Strains of the genera *Actinomadura, Arthrobacter, Micromonospora, Nocardia, Rhodococcus* and *Streptomyces.*	10^{-3} to 10^{-5} transconjugants per recipient spore.	Voeykova *et al.,* 1998
RSF1010	*E. coli* S17-1	*Streptomyces lividans* *Mycobacterium smegmatis*	Transconjugants per recipient: 10^{-6} for *S. lividans*. 10^{-2} for *M. smegmatis*.	Gormley and Davies, 1991.

Table 5.1 Continued: Examples of conjugation between Gram-positive and Gram-negative organisms

Plasmid(s)	Donor(s)	Recipient(s)	Comment	Reference
pIP501	*Enterococcus faecalis*	*Streptomyces lividans* *Escherichia coli*		Kurenbach *et al.,* 2003
pAT191 (pBR322, with transfer functions of streptococcal plasmid pAMβ1)	*Enterococcus faecalis*	*E. coli*	5×10^{-9} transconjugants per donor.	Trieu-Cuot *et al.,* 1988
pKT240, pKT240::czc, pML10 (all IncQ plasmids)	*E. coli*	Strains of *Arthrobacter* spp.		Margesin and Schinner, 1997.
pSUP1021, pSUP2021	*E. coli* S17-1	*Corynebacterium glutamicum* and *Brevibacterium lactofermentum*	5×10^{-8} transconjugants per donor. Vectors integrated in chromosome	Mateos *et al*., 1996
Vectors based on pAMB1, pCB101 and pWV01 with cloned *oriT* of RK2	*E. coli*	*Clostridium acetobutylicum,*		William *et al*., 1990

Table 5.2 Examples of conjugation between acidophilic and neutrophilic organisms.

Donor strains and plasmids	Recipient(s)	Comment	Reference
E. coli C600 *Acidithiobacillus thiooxidans* Tt-3 with plasmids RP4, R68.45. RP1::Tn*501* and pUB307	*Acidithiobacillus thiooxidans* Tt-3, *At. thiooxidans* Tt-7 and *E. coli* HB101	Transfer frequencies ranging between 10^{-7} and 10^{-3}	Jin *et al.,* 1992
E. coli strains HB101, MOS blue, *E. coli* S17-1 and MC10161 with plasmids RP4, pKT240 and pJRD215.	*At. ferrooxidan*s ATCC 33020	Apparent transfer frequency ranging between $<10^{-8}$ and 10^{-7} depending on the donor strain plasmid. (method of Peng *et al.,* 1994)	Liu *et al.,* 2001
E. coli S17-1(pJRD215)	*At. ferrooxidans* ATCC 19859	Apparent transfer frequency 10^{-4} (method of Peng *et al.,* 1994)	Liu *et al.,* 2001
E. coli S17-1(pJRD215) IncQ plasmid. Stable in *At. ferrooxidans*.	*At. ferrooxidans* strains ATCC 33020, ATCC 19859, BRGM1 and Tf-49.	Apparent transfer frequency from 4.4 x 10^{-5} to 2.5 x 10^{-3} depending on the recipient strain. Optimised method of Liu *et al.,* 2001	Liu *et al.,* 2001
E. coli S17-1(pJB3Km1) IncP plasmid. Unstable in *At. ferrooxidans*.	*At. ferrooxidans* strains ATCC 33020, ATCC 19859, BRGM1 and Tf-49.	Apparent transfer frequency from 6.2 x 10^{-7} to 1.3 x 10^{-5} depending on the recipient strain. Optimised method of Liu *et al.,*2001	Liu *et al.,* 2001
E. coli S17-1(pUFR034) IncW plasmid. Unstable in *At. ferrooxidans*.	*At. ferrooxidans* strains ATCC 33020, ATCC 19859, BRGM1 and Tf-49.	Apparent transfer frequency ranging from 1 x 10^{-6} to 6.8 x 10^{-6} Optimised method of Liu *et al.,* 2001	Liu *et al.,* 2001
E. coli S17-1 (pRK415) *E. coli* S17-1 (pLAFR3)	Various *Acidiphilium* strains	10^{-5} to 10^{-9} transconjugants per recipient, dependent on recipient strain	Glenn *et al.,* 1992

Donor strains and plasmids	Recipient(s)	Comment	Reference
E. coli K12J53(RP4) *E. coli* S17-1(pVK101) *E. coli* CSH52(pSUP106) *Pseudomonas putida*(pPGH11)	*Acidiphilium cryptum*	1.0×10^{-5} to 1.8×10^{-2} transconjugants per recipient depending on plasmid and donor	Quentmeier and Frederich, 1994

Table 5.2 Continued: Examples of conjugation between acidophilic and neutrophilic organisms.

Donor strains and plasmids	Recipient(s)	Comment	Reference
E. coli K12J53(RP4) *E. coli* S17-1(pVK101) *Pseudomonas putida*(pPGH11)	*Acidobacterium capsulatum* 161	$< 10^{-8}$ to 1.0×10^{-3} transconjugants per recipient, depending on plasmid and donor	Quentmeier and Frederich, 1994
E. coli K12J53(RP4) *E. coli* S17-1(pVK101) *E. coli* CSH52(pSUP106) *Pseudomonas putida*(pPGH11)	*Thiobacillus acidophilus* DSM 700	$<10^{-8}$ to 1.1×10^{-5} transconjugants per recipient, depending on plasmid and donor	Quentmeier and Frederich, 1994
pSDK-1 containing the *E. coli* phosphofructokinase gene (*pfkA*)	*Acidithiobacillus thiooxidans* Tt-7		Tian *et al.,* 2003

5.3. Materials and Methods

5.3.1. Strains and constructs used in conjugation experiments

E. coli S17-1 or *E. coli* S17-1 *pir*$^{+}$ (Simon *et al.,* 1983) served as the donor strains for all mobilization experiments. These *E. coli* strains have the conjugative plasmid RP4 integrated in the chromosome and thereby provide mobilization functions in *trans*. Competent cells of *E. coli* S17-1 and S17-1 *pir*$^{+}$ were prepared by the standard $CaCl_2$ / $MgCl_2$ method (Seidman *et al.,* 1997) and transformed by heat shock (5 min 37˚C). *E. coli* CSH56, and *Sulfobacillus* strains Riv14, 611, L15, Y0017 and DSM 9293^{T} were used as recipients.

Mobilizable vectors used for conjugation experiments were based on wide host range mobilizable plasmids and on a native plasmid of *Sulfobacillus* strain DSM 9293^{T}. The vectors included the broad-host-range mobilizable IncQ plasmid, pTC-F14cm (Gardner *et al*., 2001) and three vectors constructed during this study – pCon1, pSulfTACmOriT and pSulfTYCmOriT.

A broad-host range mobilizable plasmid, pCon1 (Figure 5.1) was constructed by cloning the Gram-positive chloramphenicol marker of pC194 onto pDR412 - a chloramphenicol resistant derivative of the broad host range plasmid pTF-FC2 (Rawlings *et al.,* 1984). The chloramphenicol acetyl transferase (*cat*) gene of pC194 was excised from the *E. coli* – *B. subtilis* shuttle vector pHV33 (Horinouchi and Weisblum, 1982) as a 1668 bp *Cla*I-*Cla*I fragment which also contained the pC194 *rep* and *ori*. This 1668 bp fragment was blunted with T4 polymerase and then digested with *Spe*I, thus removing most of the pC194 *rep* gene and the pC194 origin of replication. Digestion of pDR412 with *Xba*I and *Stu*I yielded compatible ends for ligation with the *Spe*I-blunt fragment containing the pC194 *cat* gene.

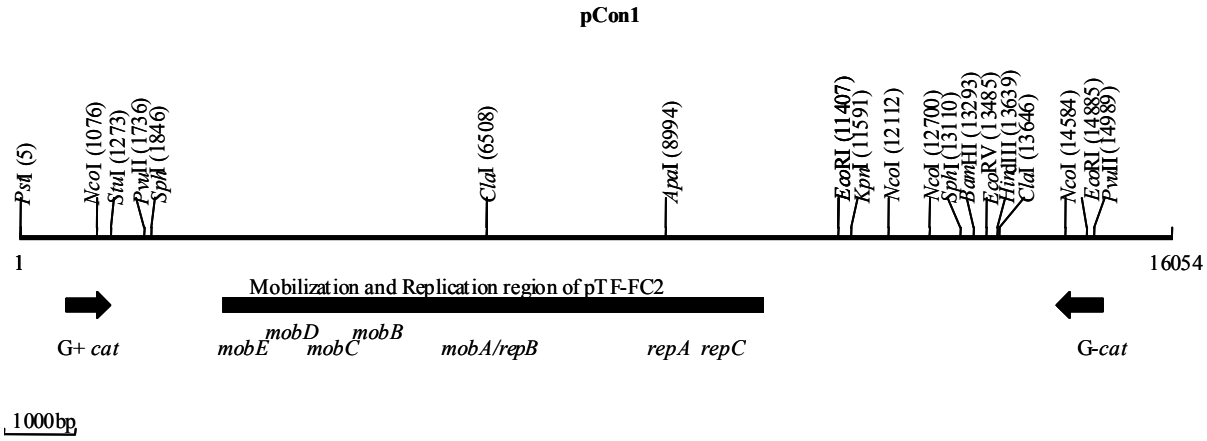

Figure 5.1 Plasmid map of pCon1

The Gram-negative (G+ *cat*) and Gram-positive (G- *cat*) chloramphenicol resistance markers are indicated with arrows. The mobilization and replication region from pTF-FC2 is indicated with a solid bar beneath the restriction map. The products of the *mob*A to *mob*E genes are involved in plasmid mobilization. The products of *rep*A, *rep*B and *rep*C are involved in plasmid replication.

Mobilizable vectors based on pKara, the native plasmid of *Sulfobacillus* strain DSM 9293^T, were constructed by cloning the RP4 origin of transfer (*oriT*) into two transposon-captured versions of pKara, pSulfTACm and pSulfTYCm (section 4.3.3). To enable the mobilization of these constructs, the RP4 origin of transfer was cloned next to the *cat* gene of pSulfTACm and pSulfTYCm as an *Eco*RI-*Eco*RI fragment excised from a cloned PCR product kindly provided by L. van Zyl. The mobilizable derivatives of pKara were named pSulfTACmOriT and pSulfTYCmOriT respectively. A plasmid map of pSulfTACmOriT is shown in Figure 5.2. The location of the transposon insertion in pSulfTYCmOriT is also indicated.

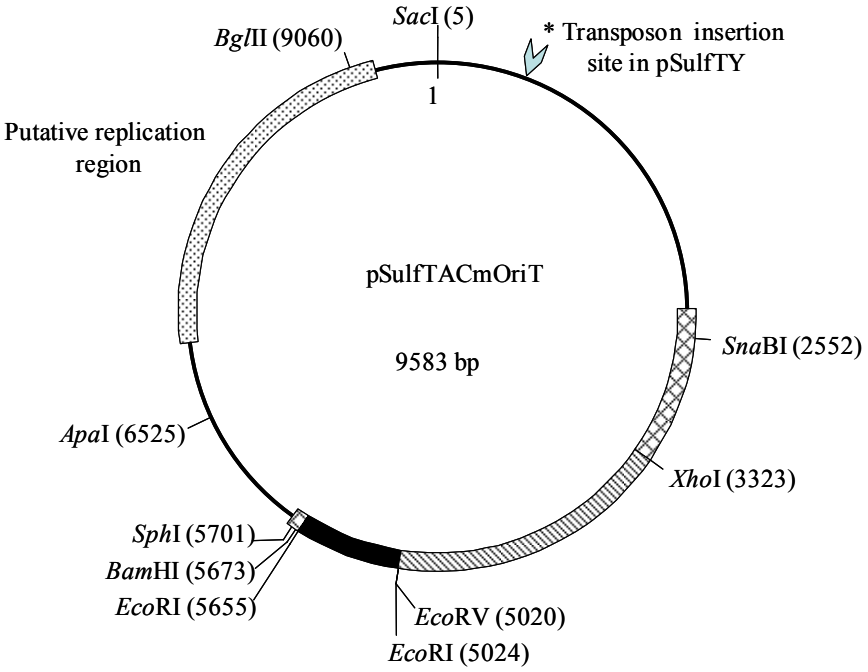

Figure 5.2 Plasmid map of pSulfTACmOriT with relevant DNA elements indicated.

The EZ:TN Transposon derived sequences encoding an *E. coli* origin of replication are indicated with crossed lines (⊠), pC194 derived sequences encoding Gram-positive chloramphenicol resistance are indicated with diagonal lines (▨), RP4 derived sequences encoding an origin of conjugative transfer are indicated by solid black (■). All the foreign DNA elements were inserted outside of the putative replication region of pKara (▩). The insertion site of the abovementioned elements in another derivative of pKara, pSulfTYCmOriT, is indicated with an arrow (➲).

5.3.2. Mating media

Three types of mating media were investigated. Mating medium 1 was Luria agar. Mating medium 2 was 1x T.F. basal salts (section 8.1.5) supplemented with 0.25 g/l yeast extract, 0.1 g/l sodium thiosulphate and 5 g/l agarose and adjusted to pH 4. Mating medium 3 was 1x T.F. basal salts supplemented with 0.25 g/l yeast extract, 0.1 g/l sodium thiosulphate and 5 g/l agarose and adjusted to pH 4.5.

5.3.3. Mating methods

Membrane filters (Supor ® Membrane Disc filters, Gelman Laboratory) were sterilized by autoclaving and placed on the surface of the mating media.

E. coli donors and recipients were cultured in Luria broth for mating experiments on Luria agar. For matings on mating medium 2 and mating medium 3, *E. coli* donors and recipients were cultured in 1 x T.F. basal salts supplemented with 0.25 g/l yeast extract (pH 4.5). *Sulfobacillus* strains were grown and washed as normal (section 2.3.3), with an additional washing step in 1 x T.F. basal salts (pH 4.5). The donor and recipient strains were mixed in a 1:1 ratio and collected by centrifugation at 6000 rpm for 2 min in a bench-top centrifuge. The supernatant was removed by aspiration and the resulting slurry of cells was dripped onto membrane filters on the surface of mating media plates. The plates were placed upright at 37˚C overnight to allow conjugation to take place. Cells were washed from the membrane filters by vortexing and dilutions were plated on selective media. To select for transformants of *E. coli,* cells were plated on Luria agar supplemented with 5 µg/ml chloramphenicol. For selection of transformants of *Sulfobacillus,* cells were plated on FeSo plates (section 8.1.8) supplemented with 1 µg/ml chloramphenicol. Nalidixic acid (50 µg/ml) was used to select for *E. coli* CSH56 recipients. Streptomycin (50µl/ml) was used to select for *E. coli* S17-1 donors.

5.4. Results

Conjugative mobilization of plasmids pTC-F14cm, pCon1, pSulfTACmOriT and pSulfTYCmOriT between *E. coli* strains occurred at a high frequency (between 10^{-1} and 10^{-4} transconjugants per donor) on Luria agar medium (Table 5.3). The same set of matings was carried out between the four *E. coli* donor strains, and *Sulfobacillus* strains DSM 9293^{T}, G2, L15, YOO17, 611(2005), MT13 and Andy but no recipients or transconjugants of *Sulfobacillus* strains could be recovered after overnight matings on Luria agar.

Table 5.3 Conjugation on Luria agar medium

Donors	Recipient *E. coli*	Transconjugants per donor*	Standard deviation
S17-1 (pCon1)	CSH56	2.06×10^{-1}	1.3×10^{-1}
S17-1 (pTC-F14cm)	CSH56	8.20×10^{-4}	3.6×10^{-5}
S17-1 *pir*$^{+}$ (pSulfTACmOriT)	CSH56	2.05×10^{-2}	1.2×10^{-3}
S17-1 *pir*$^{+}$ (pSulfTYCmOriT)	CSH56	2.57×10^{-2}	7.1×10^{-3}

* No transconjugants were observed for any strain of *Sulfobacillus*. These results are not shown.

Mating medium 2, at pH 4, allowed survival of *Sulfobacillus* recipient cells, but very few *E. coli* donor cells survived the mating period on this medium and no transconjugants of *E. coli* CHS56 or any strains of *Sulfobacillus* were observed.

Both *E. coli* and *Sulfobacillus* cells survived the overnight mating period on mating medium 3 (pH 4.5). Mobilizable plasmids were transferred to *E. coli* CSH56 recipients at high frequencies (between 10^{-1} and 10^{-3} transconjugants per donor) (Table 5.4). The transfer frequencies of all the mobilizable plasmids were similar on Luria agar and mating medium 3 (Figure 5.3). In agreement with published observations (van Zyl *et al.*, 2003), the pTF-FC2 based plasmid (pCon1) was mobilized at a higher frequency than the pTC-F14 based plasmid (pTC-F14cm).

Table 5.4 Conjugation frequencies on mating medium 3

Donor	Recipient *E. coli*	Transconjugants per donor*	Standard deviation
S17-1 (pCon1)	CSH56	2.44×10^{-1}	6.4×10^{-2}
S17-1 (pTC-F14cm)	CSH56	2.54×10^{-3}	8.7×10^{-4}
S17-1 *pir*$^{+}$ (pSulfTACmOriT)	CSH56	1.25×10^{-2}	2.8×10^{-3}
S17-1 *pir*$^{+}$ (pSulfTYCmOriT)	CSH56	3.03×10^{-2}	4.8×10^{-3}

* No transconjugants were observed for any strain of *Sulfobacillus*. These results are not shown.

Despite the demonstrated survival of both *E. coli* donors and *Sulfobacillus* recipients, and the successful mobilization of plasmids between *E. coli* strains on mating medium 3, no transconjugants of any of the tested strains of *Sulfobacillus* were observed

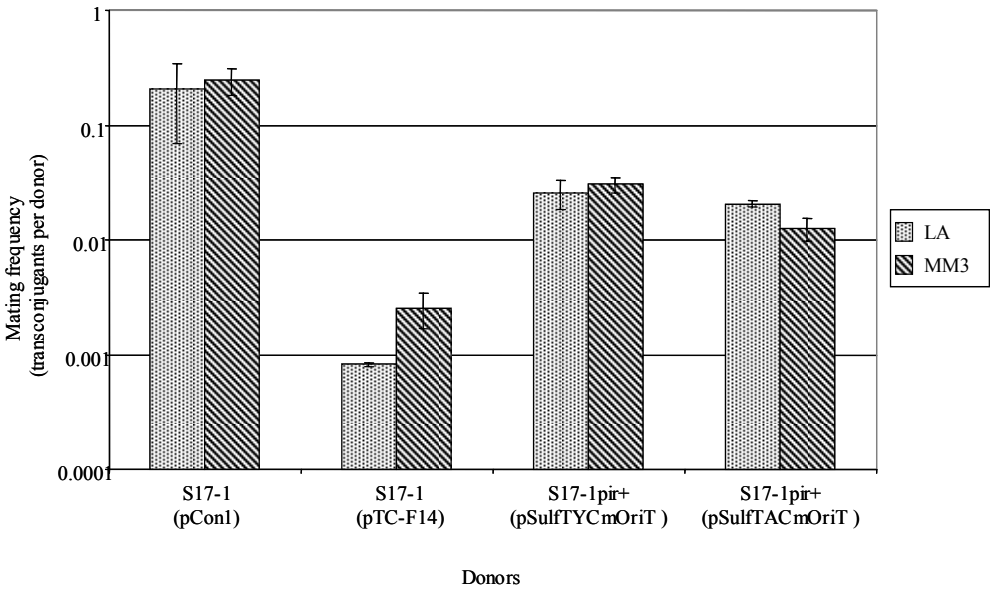

Figure 5.3 Comparison of transfer frequency of different vectors between strains of *E. coli* on Luria agar and mating medium 3

The differences in plasmid transfer frequencies on different mating media, Luria agar (▒ LA) and mating medium 3 (▒ MM3), were not significant.

5.5. Discussion.

Many genera of Gram-positive bacteria (Table 5.1) and acidophilic bacteria (Table 5.2) have been transformed by conjugation using broad host range mobilizable plasmids. Although electroporation is an attractive option for bacterial transformation due to its simplicity, the method of conjugation has been applied successfully more often (Salyers *et al*., 2000). The potential for plasmid transfer by the conjugative apparatus of IncP-type plasmids seems almost limitless, with examples of intergeneric and even inter-kingdom transfer documented (Table 2.1). Two major impediments to successful conjugative transformation of bacteria remain: finding a suitable medium for the establishment of cell-to-cell contact and formation of mating bridges, and ensuring establishment of the transferred DNA in the new host cell.

An absolute requirement for establishment of mating bridges is a mating medium that allows the survival of both donor and recipient cells. Since the growth requirements of *E. coli* and sulfobacilli differ widely, it was uncertain whether any one medium could be found to support both donors and recipients. However, a suitable mating medium was found, consisting of

Sulfobacillus basal salts supplemented with yeast extract and buffered at pH 4.5. Although both *E. coli* donors and *Sulfobacillus* recipients survive together, it is not known whether cell-to-cell contact and the establishment of mating bridges is successfully initiated. Electron-microscopic inspection of mating cells could provide an answer to this question. Further treatment of *Sulfobacillus* cells to weaken the cell wall (Trieu-Cuot *et al*., 1993) may improve the chances of mating bridge establishment.

In an effort to ensure the establishment of any transferred plasmid DNA in *Sulfobacillus* recipients, wide host range plasmids (based on the IncQ plasmids pTC-F14 and pTF-FC2), and plasmids based on an indigenous *Sulfobacillus* plasmid (pKara) were used for conjugation attempts. It is possible that the host range of the IncQ plasmids may not include *Sulfobacillus* species or that the replication functions of pKara may have been inactivated by insertion of foreign DNA. This could be one reason for the failure to observe transconjugants of sulfobacilli. Other types of broad host range plasmids, such as the Gram-positive plasmid, pAMβ, and more derivatives of pKara should be evaluated as mobilizable vectors for sulfobacilli.

DNA transferred to a *Sulfobacillus* recipient may have been destroyed by endogenous nucleases. Strain DSM 9293^{T}, which displayed less nuclease activity than a highly transformable *E. coli* strain (Figure 4.3), was included in the panel of *Sulfobacillus* strains that were used in mating experiments but transformants were not detected. Application of a heat shock (Schäfer *et al*., 1990) to *Sulfobacillus* recipient cells just before the mating period might alleviate the restriction barrier to transformation.

It is possible that plasmid DNA was transferred to cells of sulfobacilli and was successfully established in the recipient cells and yet transconjugants were not detected because the chloramphenicol marker did not function correctly in *Sulfobacillus* cells. This could be caused by failure of transcription, translation or proper folding of the protein. To ensure expression of an antibiotic resistant phenotype, two main strategies could be attempted. The chloramphenicol acetyl transferase (*cat*) gene could be cloned behind the promoter of a gene known to be expressed in sulfobacilli, for instance that of the co-transcribed *ars*R and *ars*B genes (van der Merwe, 2007). The pC194 cat gene has been reported to confer resistance to chloramphenicol at high temperature (up to 68˚C) (Soutschek-Bauer *et al*., 1987).

Other chloramphenicol or tetracycline resistance genes could be substituted for the pC194 chloramphenicol marker.

5.5.1. Conclusions

Cells of *E. coli* and *Sulfobacillus* can survive on the same medium for a theoretically sufficient time to allow mating. The conjugative transfer of plasmids between *E. coli* strains can take place on this medium.

I believe that conjugative transfer of plasmids from *E. coli* to *Sulfobacillus* is possible, but that alternative mobilizing plasmids and resistance markers need to be investigated. Also, screening of *Sulfobacillus* strains for reduced nuclease activity and / or methods of temporarily inactivating nucleases need to be implemented.

CHAPTER SIX
GENERAL DISCUSSION AND FUTURE WORK

Sulfobacillus spp. form an important part of the microbial consortia involved in mineral oxidation within biomining processes operating in the 50˚C temperature range (Rawlings *et al*., 2003) and yet very little is known about these industrially important organisms.

While studies have been undertaken to gather information about the nature and regulation of enzymatic pathways of carbon metabolism (Zakharchuk *et al*., 1994; Tsaplina *et al*., 2000; Zakharchuk *et al*., 2003) and sulphur metabolism (Krasil'nikova *et al*., 1998; 2004), these studies have been hampered by the lack of a gene cloning and inactivation system for any *Sulfobacillus* strain. When biochemical and genetic studies are used in compliment, much faster progress usually results (Salyers *et al.*, 2000).

To date, little progress has been made towards the development of genetic systems for the genus *Sulfobacillus*. As part of my honours project, a small plasmid from *Sulfobacillus* strain DSM 9293^T was isolated and partially sequenced. The sequence was completed and analized during this study. Two plasmids have also been isolated and sequenced by the research group of Prof Paul Norris and a few chromosomal genes of sulfobacilli have been cloned and sequenced (Ghauri *et al.,* 2003; van der Merwe, 2007), but at this time no reports exist of the successful transfer of genetic material to any strain of *Sulfobacillus*.

This study did not succeed in introducing novel DNA sequences in sulfobacilli. Although this lack of success is discouraging, the project should not be abandoned, as the development of genetic systems for novel bacteria often takes many years (Salyers *et al.*, 2000). Some progress has been made towards the ultimate goal of producing a transgenic strain of *Sulfobacillus* and several possibilities remain to be investigated.

Two antibiotics, tetracycline and chloramphenicol, that are suitable for the selection of *Sulfobacillus* transformants have been identified. Although this is a useful starting point, the list of antibiotics that was tested during this study is far from exhaustive and further testing may reveal other useful antibiotics. Resistance determinants to metal ions also have potential as selective markers, especially in the low pH environment in which sulfobacilli thrive. Although some data pertaining to heavy metal sensitivity are available for a few strains of *Sulfobacillus* (Vartanyan *et al*., 1990; Sampson and Phillips, 2001), a more systematic study, including a greater number of strains and a greater variety of metals would be of benefit.

Only one chloramphenicol resistance determinant was used in this study. Obtaining and testing a greater range of Gram-positive chloramphenicol resistance genes could increase the chances of a successful transformation. Cloning of resistance determinants behind promoters that are known to be expressed in *Sulfobacillus* (van der Merwe, 2007) to ensure expression of the gene is another strategy that must be investigated.

Secondly, a large diversity of plasmids was detected in strains of *Sulfobacillus*, and all were different from the three small plasmids that were previously known. These newly discovered plasmids constitute a useful and as yet unexploited resource. If these plasmids could be cloned and propagated in *Escherichia coli*, they could serve as the basis for the development of autonomously replicating *Sulfobacillus* cloning vectors; and may be screened for the presence of metal resistance determinants that might be useful markers for the selection of *Sulfobacillus* transformants.

An option that was not investigated in this study is the use of integrating DNA elements as vectors, rather than autonomously replicating elements. Such vectors would require the presence of DNA sequences that are highly similar to *Sulfobacillus* chromosomal sequences, or transposable elements that function in sulfobacilli. An arsenic resistance operon has recently been cloned and sequenced (van der Merwe, 2007), presenting the possibility of creating an arsenic gene knockout by homologous recombination, providing that an efficient DNA delivery system can be developed. Furthermore, integrons, genetic elements possessing an integrase enzyme which facilitates site-specific recombination events, have been detected in *Sulfobacillus thermosulfidooxidans* (Ghauri *et al*., 2003) implying that integron-mediated genetic exchange may take place in acidophilic environments. Integrons could be used to deliver DNA packaged within the *att* recognition sites to the chromosomes of sulfobacilli.

One of the most promising findings of this study was that cells of *Sulfobacillus* and *E. coli* can survive together for many hours on the same growth substrate, and that plasmid transfer between strains of *E. coli* is possible on this medium. Since the ability of wide host range conjugative systems to mobilize plasmids seems to be almost universal, I believe that further attempts to transform sulfobacilli should concentrate on this method of DNA delivery. The occurrence of DNA restriction systems is widespread in environmental isolates of bacteria, therefore many more strains of sulfobacilli should be isolated and / or obtained from other researchers and used for transformation attempts. Also, temporary neutralization of

endogenous restriction systems, by heating or introduction of antirestriction systems (Belogurov *et al.,*1992) on vectors should be performed before transformation attempts.

A genetic system comprises at least a suitable vector, a method for obtaining single colonies, a selectable marker, a DNA delivery system and a DNA extraction protocol. Of these prerequisites, four have been wholly or partially attained during this study: single colonies are easily grown on solid media; a number of plasmids have been observed and one has been sequenced and propagated in *E. coli*; two antibiotics, chloramphenicol and tetracycline, have been found to inhibit growth of sulfobacilli, and a simple method for the isolation of small plasmids from sulfobacilli is available.

What remains to be achieved are the following: demonstration of successful introduction of DNA into cells of *Sulfobacillus*; expression and functioning of a selectable marker in *Sulfobacillus*, and demonstration of successful replication or integration of a vector in *Sulfobacillus*.

To transform a bacterium, the parameters affecting transformation must all be correct at the same instant. Therefore the construction of a novel genetic system cannot proceed in a simple progression, but must rely on a lot of hard work, and a certain amount of luck – at least until the first transformant has been obtained. From this point forward experiments can be rationally designed to improve the transformation frequency.

APPENDIX 1: STRAINS AND CONSTRUCTS

7.1. Plasmids and strains used in this study

Table 7.1 Plasmids used in this study

Name	Description	Reference or source
pUC19	Amp^R, *Lac*Z', ColE1 replicon, Cloning vector *E. coli*.	Yanisch-Perron *et al.,* 1985
pUCBM21	Amp^R, *Lac*Z', ColE1 replicon, Cloning vector *E. coli*.	Boehringer Mannheim
pBluescript KS^+	Amp^R, *Lac*Z', ColE replicon	Stratagene
pHV33	Amp^r, Cm^r in *E. coli*, Cm^r in *B. subtilis. E. coli – B. subtilis* shuttle vector.	(Horinouchi and Weisblum, 1982)
pKara	Natural plasmid of Sulfobacillus strain	
pSulfTA	Transposon EZ::TN<R6K γ oriKAN-2> jumped into pKara at pos 2375 relative to *Sac*I	Honours Study, Joubert, 2002 University of Stellenbosch
pSulfTACm	Cm^r, mobilizable, The kanamycin marker of the R6K transposon replaced by the Chloramphenicol marker taken from pSKCm	This study
pSulfTACmOriT	Cm^r, mobilizable, The origin of transfer of RP4 cloned into the single *Eco*RI site of pSulfTACm.	This study
pSulfTY	Transposon EZ::TN<R6K γ oriKAN-2> jumped into pKara at pos 478 relative to *Sac*I	Honours Study, Joubert, 2002 University of Stellenbosch
RP4	Ap^r, Km^r, Tc^r, IncPα, Conjugative plasmid.	
pDR412	Cm^r, mobilizable, Natural pTF-FC2 plasmid with Chloramphenicol resistance gene cloned into the TN*5467* transposon.	Rawlings *et al.,* 1984
pTC-F14cm	Cm^r, mobilizable. Natural pTC-F14 plasmid with Chloramphenicol resistance gene cloned into pTC-F14 at	Gardner *et al.,* 2001

Name	Description	Reference or source
pCon1	single *Bam*HI site Cm^r, mobilizable. The chloramphenicol marker from pHV33 cloned as a *Spe*I – blunted *Cla*I fragment into a *Stu*I-*Xba*I cut pDR412	This study
R6K *oriT*	Cloned PCR product of R6K *oriT*	L. van Zyl, University of Stellenbosch

Table 7.2 Bacterial strains used in this study

Strain	Description	Reference or source
E. coli S17-1	*recA pro hsd*R (RP4-2 Tc::Mu Km::Tn7)	Simon *et al.,* 1983
E. coli S17-1 *pir*$^+$	λ *pir, recA, thi, pro, hsd*R^- (RP4:2-Tc:Mu:Km $Tn7Tp^RSm^R$)	Simon *et al.,* 1983
E. coli S17-1 *pir*$^+$ (Tn11, pUCBM21)	Harbours plasmids Tn11 and pUCBM21	L. van Zyl, University of Stellenbosch
E. coli CSH56	F- *ara* Δ*(lac pro) supD nalA thi*	Cold Spring Harbour, NY
E. coli DH5α	F*'/ end*A1 *hsdR17*($r_K^-m_K^+$) *supE44 thi-1 recA1 gyrA*(Nalr) *relA1* Δ*(lacZYA-argF) U169 (Φ80dlac*Δ*(lacZ)M15)*	Promega Corp., Madison, WI
E. coli EC100D *pir*$^+$	F^- mcrAΔ (mrr –hsdRMS-mcrBC) Φ80d lacZ ΔM15 Δlacx74 recA1 endA1 araD139 Δ (ara, leu) 7697 galU galK λ^- rpsL nupG *pir*$^+$ (DHFR).	
E. coli GM41	*Hfr-H dam*-3 *thi*-1 *rel*-1	Valarie Mizrahi (University of Witwatersrand)
E. coli HB101 (RP4)	*supE*44 *hsdS*20*(r_B^- m_B^-)* *recA*13 *ara*-14 *proA*2 *lacY*1 *galK*2 *rpsL*20 *xyl*-5 *mtl*-1 *leuB*6 *thi*-1	

Strain	Description	Reference or source
	Harbours RP4 plasmid.	
Sulfobacillus thermosulfidooxidans DSM9293^T	Also known as strain Kara or strain VKM B-1269 Russia	Golovacheva and Karavaiko (1978)
Sulfobacillus strain L15		Prof. D.B. Johnson
Sulfobacillus strain Y0017		Prof. D.B. Johnson
Sulfobacillus strain G2		Prof. D.B. Johnson
Sulfobacillus strain YTF1		Prof. D.B. Johnson
Sulfobacillus strain TH1		Prof. D.B. Johnson
Sulfobacillus strain MT13	Isolated from a chalcopyrite bioleachate; South Africa	Prof. D.B. Johnson
Sulfobacillus acidophilus strain ALV		Prof. D.B. Johnson
Sulfobacillus strain GG6-3		Prof. D.B. Johnson
Sulfobacillus strain THWX		Prof. D.B. Johnson
Sulfobacillus strain 611(2005)	Isolated from same sample as strain 611(Schutte, 2004), probably the same organism	This study
Sulfobacillus strain Andy	BHP Billiton mesophilic inoculum	This study
Sulfobacillus strain Mintek45	Mintek culture maintained on pyrite-arsenopyrite concentrate	This study

7.2. Primers and probes used in this study

Table 7.3 Primers used in this study

Name	Sequence	Source or reference
fDD2	5′-CCGGATCCGTCGACAGAGTTTGATCITGGCTCAG-3′	Rawlings, 1995
rPP2	5′-CCAAGCTTCTAGACGGITACCTTGTTACGACTT-3′	Rawlings, 1995

APPENDIX 2: MEDIA AND BUFFERS

8.1. Growth media for *Sulfobacillus* spp.

8.1.1. 5 M $FeSO_4$ stock

$FeSO_4.7H_2O$	139.0 g/l

pH < 1.3 with H_2SO_4. Autoclave.

8.1.2. 0.5 M Potassium tetrathionate stock

$K_2S_4O_6$	151.3 g/l

Filter sterilise keep at 4˚C for less than three weeks.

8.1.3. 1000X Trace elements solution

$ZnSO_4.7H_2O$	10.0 g/l
$CuSO_4.5H_2O$	1.0 g/l
$MnSO_4.4H_2O$	1.0 g/l
$CoCl_2.6H_2O$	0.5 g/l
$Cr_2(SO_4)_3.15H_2O$	0.5 g/l
$Na_2B_4O_7.10H_2O$	0.5 g/l
Vanadyl Sulphate	0.1 g/l

Add 530 µl H_2SO_4. Autoclave.

8.1.4. 10X Basal salts

$(NH_4)_2SO_4$	12.5 g/l
$MgSO_4.7H_2O$	5.0 g/l

pH to 2.5 with H_2SO_4. Autoclave.

8.1.5. 10X T.F. Basal salts

$(NH_4)_2SO_4$	30 g/l
KCl	1 g/l
K_2HPO_4	5 g/l
$MgSO_4.7H_2O$	5 g/l
$Ca(NO_3)_2.4H_2O$	0.143 g/l
Na_2SO_4	14.52 g/l

pH 2.5, autoclave

8.1.6. FeSo growth medium

10X Basal salts	100 ml
Tryptone soy broth	0.25g
H_2O	850 ml

pH 2.5, Autoclave

Allow to cool, then add:

5 M $FeSO_4$	50 ml
0.5 M Potassium tetrathionate	5 ml
Trace elements solution	1 ml

8.1.7. FeSYE growth medium for Sulfobacillus

Iron-sulphur-yeast extract medium, adapted from FeSo medium (Johnson, 1995).

In this medium 10X Basal salts is replaced with 10X T.F. Basal salts and tryptone soy broth is replaced by yeast extract.

10X T.F Basal salts	100 ml
Yeast extract	0.25 g
H_2O	850 ml

pH 2.5, Autoclave

Allow to cool, then add:

5 M $FeSO_4$	50 ml
0.5 M Potassium tetrathionate	5 ml
Trace elements solution	1 ml

8.1.8. FeSo solid media modified for Sulfobacillus

Iron-sulphur-yeast extract medium, adapted from FeSo medium (Johnson, 1995).

Soln A

10 X Basal salts	100 ml
Yeast extract	0.25 g
H_2O	400 ml

Soln B

Agarose	5g
H_2O	450 ml

Solution A and B are autoclaved separately and cooled to 50˚C. The following components are then added to Solution A:

5 M $FeSO_4$	50 ml
0.5 M Potassium tetrathionate	5 ml
Trace elements solution	1 ml

Solution A and Solution B are mixed and poured into Petri dishes. The underlay of *Acidiphilum* SJH used in the original FeSo medium is not used for this medium.

8.1.9. FeSYE solid media for Sulfobacillus

Iron-sulphur-yeast extract medium, adapted from FeSo medium (Johnson, 1995).

Soln A

10 X T.F. Basal salts	100 ml
Yeast extract	0.25 g
H_2O	400 ml

Soln B

Agarose	5g
H_2O	450 ml

Solution A and B are autoclaved separately and cooled to 50˚C. The following components are then added to Solution A:

5 M $FeSO_4$	50 ml
0.5 M Potassium tetrathionate	5 ml
Trace elements solution	1 ml

Solution A and Solution B are mixed and poured into Petri dishes. The underlay of *Acidiphilum* SJH used in the original FeSo medium was not used.

8.1.10. Storage medium

For long-term storage of *Sulfobacillus* cultures, cells were frozen in 7% DMSO and / or kept in FeSo liquid medium in screw-cap bottles with ground pyrite.

8.2. Mating media

8.2.1. Mating medium1

Luria agar

8.2.2. Mating medium 2

A modification of FeSYE plates, where tetrathionate was replaced with thiosulphate and the medium pH adjusted to pH 4.

8.2.3. Mating medium 3

Soln A:

T.F. basal salts (100 ml), Yeast extract (0.25 g), H_2O (500 ml)

pH 4.5, autoclave

Soln B:

Agarose (5 g), H_2O (400 ml).

Autoclave

Cool to 50˚C mix and pour plates. pH checked with universal indicator strips (MERCK) when plates had set was still 4.5.

8.3. Media for TAFE

8.3.1. SET buffer

Tris 50 mM

EDTA,	2 mM
sucrose,	25%

pH 8.0. Autoclave)

8.3.2. 20 X TAFE buffer

Tris	24.2 g/l
EDTA (free acid)	2.9 g/l
Glacial acetic acid	5 ml/l

do not pH. Autoclave

8.3.3. TE_{50} buffer

Tris	0.360 g	(30 mM)
EDTA(Na_2)	1.68g	(50 mM)
dH_2O to	100ml	

pH to 7.6. Autoclave

8.3.4. ES buffer

EDTA (free acid)	16.8 g
Na-Lauryl Sarcosine	1.0 g
H_2O to	100 ml

pH to 8 with NaOH. Autoclave

8.3.5. ESP buffer

ES buffer + 1 µg/ml proteinase K

8.3.6. Soln A (λ concats)

	Final concentration	Volume
PEG (8000) in H_20	2%	160 µl
100 mM ATP frozen stock	2 mM	40 µl
1 M DTT stock	2 mM	4 µl
H_2O		1796 µl
Final volume		2 ml

8.3.7. Soln B (λ concats)

1 M $MgCl_2$	20 mM	200 µl
1 M Tris (pH 8)	100 mM	1 ml
H_2O		8800 µl
Final volume		10 ml

8.3.8. Soln C (λ concats)

Mix 1:1 SolnA(λ concats) with Soln B(λ concats)

8.3.9. 20 X SSC

NaCl	175.3 g/l
Sodium citrate	88.2 g/l

pH to7.4 with NaOH. Autoclave

8.3.10. Barton buffer (S1 nuclease treatment of TAFE plugs)

NaCl	50 mM
Sodium acetate	30 mM
$ZnSO_4$	5 mM

pH to 4.5. Autoclave

APPENDIX 3: DNA SEQUENCES

9.1.1. Mintek45 rDNA

The sequence of a single DNA strand is shown

```
1      TATAGAATAC TCAAGCTATG CATCCAACGC GTTGGGAGCT CTCCCATATG GTCGACCTGC
61     AGGCGGCCGC GAATTCACTA GTGATTCCGG ATCCGTCGAC AGAGTTTGAT CGTGGCTCAG
121    GACGAACGCT GGCGGCGTGC GTAATACATG CAAGTCGAGC GGACCTTCGG GTCAGCGGCG
181    GACGGGTGAG GAACACGTGA GTCATCGGGC TGTGAGTGGG GGATATCGGG CCGAAAGGCG
241    CGGCAATCCC GCATACGTTC CGGGGAACCG GAAGAAAGCT TGGCAACAGG CGCTCACAGG
301    GGAGCTCGCG GCCCATTAGC TAGTTGGGGG GGGTAATGGC CTCCCAAGGC GGCGATGGGT
361    AGCCGGCCTG AGAGGGTGAA CGGCCACACT GGGACTGAGA CACGGCCCAG ACTCCTACGG
421    GAGGCAGCAG TAGGGAATCT TCCACAATGG GCGCAAGCCT GATGG
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9.1.2. pKara

The sequence of both DNA strands is shown:

```
1     GAGCTCGGCAAGGGCGCGAGGCGGCCAACCGTTCCTCCCGCCAAGACCTCCCCGAACCGC
      CTCGAGCCGTTCCCGCGCTCCGCCGGTTGGCAAGGAGGGCGGTTCTGGAGGGGCTTGGCG

61    CTTGTTCTGCCTCAGCCCGCCTTGATCGACCTTGATGGAAGACGGCAAAGGTAGGTCTAA
      GAACAAGACGGAGTCGGGCGGAACTAGCTGGAACTACCTTCTGCCGTTTCCATCCAGATT


121   ATTCCCCGCCGGCCCACACGAAAAACAGTTATCCGGGGGAGGGGGGTATATGCCCTGGTA
      TAAGGGGCGGCCGGGTGTGCTTTTTGTCAATAGGCCCCCTCCCCCCATATACGGGACCAT


181   CCATTTTGGTACGAGGCAGATTCCGTAAAACCTGCAACGACACGTCCCATGCGGGGTTTC
      GGTAAAACCATGCTCCGTCTAAGGCATTTTGGACGTTGCTGTGCAGGGTACGCCCCAAAG

241   ATATCCGCCCAATCCGGCCATGATTTTGACTAAAGGGAGACTCCTTGTACGACTTCGGGT
      TATAGGCGGGTTAGGCCGGTACTAAAACTGATTTCCCTCTGAGGAACATGCTGAAGCCCA

301   TGCCATGCACCCCAAAATTAAGCAAAAAAGAGTCTGCCTGGTACCACCAAGGCAGGGAAG
      ACGGTACGTGGGGTTTTAATTCGTTTTTTCTCAGACGGACCATGGTGGTTCCGTCCCTTC

361   CTCCTTGTCGATTCTTGGGCCTTTTTCGAGCGGCGTGGGAATCCCTCCCTAGAGACGGCA
      GAGGAACAGCTAAGAACCCGGAAAAAGCTCGCCGCACCCTTAGGGAGGGATCTCTGCCGT

421   ACTTCCCCGCCTAATCTCGGATGGGGTTATTCTCGAAATAACCCCGCGCGGTCCTCTTTC
      TGAAGGGGCGGATTAGAGCCTACCCCAATAAGAGCTTTATTGGGGCGCGCCAGGAGAAAG

                                          RBS       Start ORF1
481   ACCTCCTTGCCCCGCGCGGGGAAACAAGGGAA**GGGAGGA**AAATCG**ATG**GCCGACGAACAA
      TGGAGGAACGGGGCGCGCCCCTTTGTTCCCTTCCCTCCTTTTAGCTACCGGCTGCTTGTT
161                                                 M  A  D  E  Q

541   CGCCTTGACAACGATCAAGAAATTTTTGTGCAAGCGGATGGCGAAGTGGCGATTAATGCG
      GCGGAACTGTTGCTAGTTCTTTAAAAACACGTTCGCCTACCGCTTCACCGCTAATTACGC
       R  L  D  N  D  Q  E  I  F  V  Q  A  D  G  E  V  A  I  N  A

601   GTTATGTCGCCCAAAGCCTTTGCCCAATTGTTGCGAGAATTGAATGCCCGGAAAATGGAT
      CAATACAGCGGGTTTCGGAAACGGGTTAACAACGCTCTTAACTTACGGGCCTTTTACCTA
       V  M  S  P  K  A  F  A  Q  L  L  R  E  L  N  A  R  K  M  D

                                                      Stop ORF1
661   ATGACGGAAATCCCAGACCAAGAAGGACAGGAGCGTGCGCCGCAATGGGAA**TAG**CCGACC
      TACTGCCTTTAGGGTCTGGTTCTTCCTGTCCTCGCACGCGGCGTTACCCTTATCGGCTGG
       M  T  E  I  P  D  Q  E  G  Q  E  R  A  P  Q  W  E

721   GTACTTTGGACCTTGATTCTTTTCGTGCAGGCAAAAAAAAATTTACGGCCCACCCCGCCT
      CATGAAACCTGGAACTAAGAAAAGCACGTCCGTTTTTTTTTAAATGCCGGGTGGGGCGGA

781   CGTTCGAAGAATTTGCCCGGTGGGAACGCGCCCGAACGTTGTTTGGCGGTTTTGGGCCTT
      GCAAGCTTCTTAAACGGGCCACCCTTGCGCGGGCTTGCAACAAACCGCCAAAACCCGGAA

841   ACGTGCGCAAACTTGAGCAGGCTTTGAAAGACCAAGAACAGGGCGGTTTTCCGTGGATCA
      TGCACGCGTTTGAACTCGTCCGAAACTTTCTGGTTCTTGTCCCGCCAAAAGGCACCTAGT
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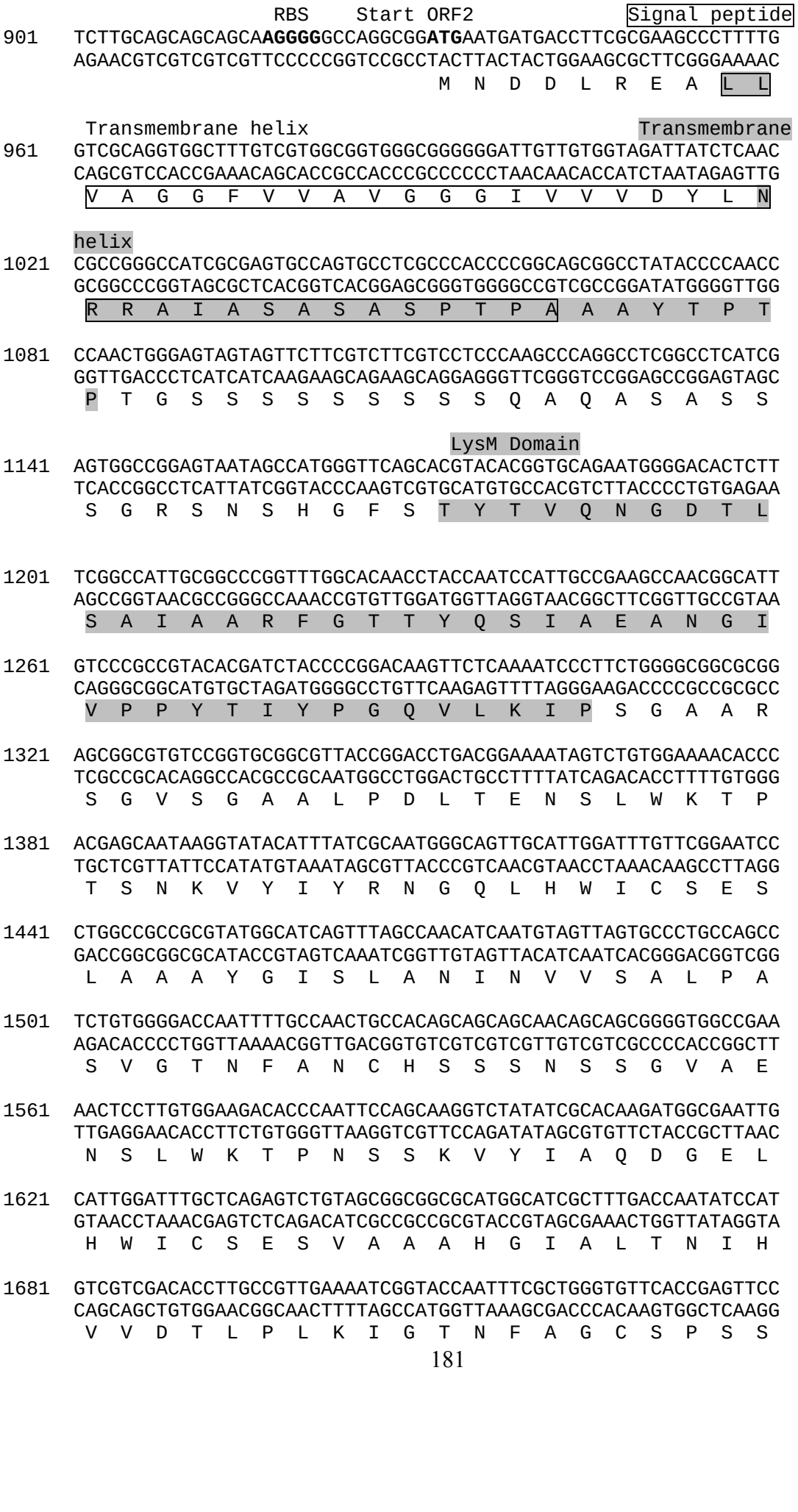
RBS Start ORF2 Signal peptide
901 TCTTGCAGCAGCAGCAAGGGGGCCAGGCGGATGAATGATGACCTTCGCGAAGCCCTTTTG
AGAACGTCGTCGTCGTTCCCCCGGTCCGCCTACTTACTACTGGAAGCGCTTCGGGAAAAC
M N D D L R E A L L
Transmembrane helix Transmembrane
961 GTCGCAGGTGGCTTTGTCGTGGCGGTGGGCGGGGGGATTGTTGTGGTAGATTATCTCAAC
CAGCGTCCACCGAAACAGCACCGCCACCCGCCCCCCTAACAACACCATCTAATAGAGTTG
V A G G F V V A V G G G I V V V D Y L N
helix
1021 CGCCGGGCCATCGCGAGTGCCAGTGCCTCGCCCACCCCGGCAGCGGCCTATACCCCAACC
GCGGCCCGGTAGCGCTCACGGTCACGGAGCGGGTGGGGCCGTCGCCGGATATGGGGTTGG
R R A I A S A S A S P T P A A A Y T P T
1081 CCAACTGGGAGTAGTAGTTCTTCGTCTTCGTCCTCCCAAGCCCAGGCCTCGGCCTCATCG
GGTTGACCCTCATCATCAAGAAGCAGAAGCAGGAGGGTTCGGGTCCGGAGCCGGAGTAGC
P T G S S S S S S S S Q A Q A S A S S
LysM Domain
1141 AGTGGCCGGAGTAATAGCCATGGGTTCAGCACGTACACGGTGCAGAATGGGGACACTCTT
TCACCGGCCTCATTATCGGTACCCAAGTCGTGCATGTGCCACGTCTTACCCCTGTGAGAA
S G R S N S H G F S T Y T V Q N G D T L
1201 TCGGCCATTGCGGCCCGGTTTGGCACAACCTACCAATCCATTGCCGAAGCCAACGGCATT
AGCCGGTAACGCCGGGCCAAACCGTGTTGGATGGTTAGGTAACGGCTTCGGTTGCCGTAA
S A I A A R F G T T Y Q S I A E A N G I
1261 GTCCCGCCGTACACGATCTACCCCGGACAAGTTCTCAAAATCCCTTCTGGGGCGGCGCGG
CAGGGCGGCATGTGCTAGATGGGGCCTGTTCAAGAGTTTTAGGGAAGACCCCGCCGCGCC
V P P Y T I Y P G Q V L K I P S G A A R
1321 AGCGGCGTGTCCGGTGCGGCGTTACCGGACCTGACGGAAAATAGTCTGTGGAAAACACCC
TCGCCGCACAGGCCACGCCGCAATGGCCTGGACTGCCTTTTATCAGACACCTTTTGTGGG
S G V S G A A L P D L T E N S L W K T P
1381 ACGAGCAATAAGGTATACATTTATCGCAATGGGCAGTTGCATTGGATTTGTTCGGAATCC
TGCTCGTTATTCCATATGTAAATAGCGTTACCCGTCAACGTAACCTAAACAAGCCTTAGG
T S N K V Y I Y R N G Q L H W I C S E S
1441 CTGGCCGCCGCGTATGGCATCAGTTTAGCCAACATCAATGTAGTTAGTGCCCTGCCAGCC
GACCGGCGGCGCATACCGTAGTCAAATCGGTTGTAGTTACATCAATCACGGGACGGTCGG
L A A A Y G I S L A N I N V V S A L P A
1501 TCTGTGGGGACCAATTTTGCCAACTGCCACAGCAGCAGCAACAGCAGCGGGGTGGCCGAA
AGACACCCCTGGTTAAAACGGTTGACGGTGTCGTCGTCGTTGTCGTCGCCCCACCGGCTT
S V G T N F A N C H S S S N S S G V A E
1561 AACTCCTTGTGGAAGACACCCAATTCCAGCAAGGTCTATATCGCACAAGATGGCGAATTG
TTGAGGAACACCTTCTGTGGGTTAAGGTCGTTCCAGATATAGCGTGTTCTACCGCTTAAC
N S L W K T P N S S K V Y I A Q D G E L
1621 CATTGGATTTGCTCAGAGTCTGTAGCGGCGGCGCATGGCATCGCTTTGACCAATATCCAT
GTAACCTAAACGAGTCTCAGACATCGCCGCCGCGTACCGTAGCGAAACTGGTTATAGGTA
H W I C S E S V A A A H G I A L T N I H
1681 GTCGTCGACACCTTGCCGTTGAAAATCGGTACCAATTTCGCTGGGTGTTCACCGAGTTCC
CAGCAGCTGTGGAACGGCAACTTTTAGCCATGGTTAAAGCGACCCACAAGTGGCTCAAGG
V V D T L P L K I G T N F A G C S P S S

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1741  AGTCCATTATCGTCAGGATCGGGATCTACCCCATTTGGCCGGCCGCCTTATGGCTTCTTT
      TCAGGTAATAGCAGTCCTAGCCCTAGATGGGGTAAACCGGCCGGCGGAATACCGAAGAAA
       S  P  L  S  S  G  S  G  S  T  P  F  G  R  P  P  Y  G  F  F

1801  TACATCGGATTTCATTGCGGGTTGCCGTTGTTTGTCCCTGCCATCCCGCACTTGTGGCCG
      ATGTAGCCTAAAGTAACGCCCAACGGCAACAAACAGGGACGGTAGGGCGTGAACACCGGC
       Y  I  G  F  H  C  G  L  P  L  F  V  P  A  I  P  H  L  W  P

1861  TCTACGTCCCCCATCCCCGCCGCCGGCACCAACCAAGTGGCTTGGGTACGAATCACCGGC
      AGATGCAGGGGGTAGGGGCGGCGGCCGTGGTTGGTTCACCGAACCCATGCTTAGTGGCCG
       S  T  S  P  I  P  A  A  G  T  N  Q  V  A  W  V  R  I  T  G

1921  CCCAAAAACGCTCCCGGGGGCGGATTGTGGGCCGTGGACGCCCGGACCAAAGCCCACACG
      GGGTTTTTGCGAGGGCCCCCGCCTAACACCCGGCACCTGCGGGCCTGGTTTCGGGTGTGC
       P  K  N  A  P  G  G  G  L  W  A  V  D  A  R  T  K  A  H  T

1981  ACGTTGGTGGTCATCGCCAGCTATGACCAAAACGGGCAATGGACGGGCAGTTGGGCCGTG
      TGCAACCACCAGTAGCGGTCGATACTGGTTTTGCCCGTTACCTGCCCGTCAACCCGGCAC
       T  L  V  V  I  A  S  Y  D  Q  N  G  Q  W  T  G  S  W  A  V

2041  GTGCGCAACCAATTTGGGACAAAACCCTTTTTGGGCAACCCCAACCCTGCCGCCAATCAG
      CACGCGTTGGTTAAACCCTGTTTTGGGAAAAACCCGTTGGGGTTGGGACGGCGGTTAGTC
       V  R  N  Q  F  G  T  K  P  F  L  G  N  P  N  P  A  A  N  Q

2101  GGGTGGGAATGTGTGTTTTGCGAGTTTCCCGGGAAACCCAAGCGTATTCTAATAACCACA
      CCCACCCTTACACACAAAACGCTCAAAGGGCCCTTTGGGTTCGCATAAGATTATTGGTGT
       G  W  E  C  V  F  C  E  F  P  G  K  P  K  R  I  L  I  T  T

                End ORF2                          RBS       Start ORF3
2161  GATTACCATTTTTGGTAATGGAATTGGTAATTGTCCCTAGGAGGCACCCGGGATGTTTAC
      CTAATGGTAAAAACCATTACCTTAACCATTAACAGGGATCCTCCGTGGGCCCTACAAATG
       D  Y  H  F  W  *
                                                          M  F  T

      Signal peptide                        Transmembrane region1
2221  TCCTGATAAATCCTTTTGGCAGGTCGCCCAAGCGATTGTCTTGGCCTTGGGCGCTCCGCT
      AGGACTATTTAGGAAAACCGTCCAGCGGGTTCGCTAACAGAACCGGAACCCGCGAGGCGA
        P  D  K  S  F  W  Q  V  A  Q  A  I  V  L  A  L  G  A  P  L

2281  CACGGTCGCCACTCTCAAAATGGTGGCGGCTTGGTCCTATTGCGAAAAGCCTCATGCGAC
      GTGCCAGCGGTGAGAGTTTTACCACCGCCGAACCAGGATAACGCTTTTCGGAGTACGCTG
        T  V  A  T  L  K  M  V  A  A  W  S  Y  C  E  K  P  H  A  T

2341  GGGCCAAGCGTGGCAGTGGAACAATCCCCTCAACACGACGCGGGCGTGTTGTGGATGGAT
      CCCGGTTCGCACCGTCACCTTGTTAGGGGAGTTGTGCTGCGCCCGCACAACACCTACCTA
        G  Q  A  W  Q  W  N  N  P  L  N  T  T  R  A  C  C  G  W  I

2401  CGGCGAGGCGAATAGCATCGGCGTCAAAATTTATCCCTCTCGCCGGGCCGGGGTTGTTGC
      GCCGCTCCGCTTATCGTAGCCGCAGTTTTAAATAGGGAGAGCGGCCCGGCCCCAACAACG
        G  E  A  N  S  I  G  V  K  I  Y  P  S  R  R  A  G  V  V  A

2461  GACCATTACCACACTCCAAAATGGGGATTACCCCACGTTGGTGGCCGGCCTGATGGCGAG
      CTGGTAATGGTGTGAGGTTTTACCCCTAATGGGGTGCAACCACCGGCCGGACTACCGCTC
        T  I  T  T  L  Q  N  G  D  Y  P  T  L  V  A  G  L  M  A  S

                Transmembrane region2
2521  CAATCCGACTCTCTTTTTTAGTGCGGCGGGCGCGGCGGAAATGGCCACCTGGGGCACTAG
      GTTAGGCTGAGAGAAAAAATCACGCCGCCCGCGCCGCCTTTACCGGTGGACCCCGTGATC
        N  P  T  L  F  F  S  A  A  G  A  A  E  M  A  T  W  G  T  S
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                                                    Transmembrane region3
2581  TATGGCTTGTGTGGCCAGTGACTTTGCCACTATGACCGACCCACCGCCGGCGTATTTAGC
      ATACCGAACACACCGGTCACTGAAACGGTGATACTGGCTGGGTGGCGGCCGCATAAATCG
        M  A  C  V  A  S  D  F  A  T  M  T  D  P  P  P  A  Y  L  A

                                                   Transmembrane region4
2641  GCCCGCTGCCGTGGCCTCGTCTGCCCCTGGTGCTCCCGCTTGGTTCTATCTAGCCCTGGG
      CGGGCGACGGCACCGGAGCAGACGGGGACCACGAGGGCGAACCAAGATAGATCGGGACCC
        P  A  A  V  A  S  S  A  P  G  A  P  A  W  F  Y  L  A  L  G

2701  GGGCCTCGGCATTGTCCTGGGCGGCGTGGCCTTGGTCGTGGCGGTCAACCCTCGGGACTG
      CCCGGAGCCGTAACAGGACCCGCCGCACCGGAACCAGCACCGCCAGTTGGGAGCCCTGAC
        G  L  G  I  V  L  G  G  V  A  L  V  V  A  V  N  P  R  D  W

2761  GACCGCCTTTGCCCAATGGGAACGGGAAGAAGTCCATACCAAAATTCAAATTCGCCGGGC
      CTGGCGGAAACGGGTTACCCTTGCCCTTCTTCAGGTATGGTTTTAAGTTTAAGCGGCCCG
        T  A  F  A  Q  W  E  R  E  E  V  H  T  K  I  Q  I  R  R  A

                                 Stop ORF3
2821  TTTTCGGTCTGGCAGAACGTTCCATCTT**TAG**AACTGGACATGGTATTTTTGGAAGAGAAA
      AAAAGCCAGACCGTCTTGCAAGGTAGAAATCTTGACCTGTACCATAAAAACCTTCTCTTT
        F  R  S  G  R  T  F  H  L  *

              RBS          Start ORF4 PGU1 domain
2881  GCCAAGTA**AGGAG**GGCGCGATAGC**ATG**GGCGTAGGGCCTCGCAATTCCCCGTTTTTATCC
      CGGTTCATTCCTCCCGCGCTATCGTACCCGCATCCCGGAGCGTTAAGGGGCAAAAATAGG
                               M  G  V  G  P  R  N  S  P  F  L  S

2941  GAAAATTTTGGGTTTTGGCAGACACAAATGAATACCTTCAACATCAAAACTTTTGGAGCC
      CTTTTAAAACCCAAAACCGTCTGTGTTTACTTATGGAAGTTGTAGTTTTGAAAACCTCGG
        E  N  F  G  F  W  Q  T  Q  M  N  T  F  N  I  K  T  F  G  A

3001  GTAGGGGACGGGGTGCATAACGATGCGCCCGCCATTCAAGCGGCTATTGACGCCGCCGCC
      CATCCCCTGCCCCACGTATTGCTACGCGGGCGGTAAGTTCGCCGATAACTGCGGCGGCGG
        V  G  D  G  V  H  N  D  A  P  A  I  Q  A  A  I  D  A  A  A

3061  CGCCAAGGTGGGGGAACCGTGTATATGCCGGTTGGCCGTTATGCTTTGCATCAAGGGTTA
      GCGGTTCCACCCCCTTGGCACATATACGGCCAACCGGCAATACGAAACGTAGTTCCCAAT
        R  Q  G  G  G  T  V  Y  M  P  V  G  R  Y  A  L  H  Q  G  L

3121  ACGTTAGCCAGCGGCATTGCCCTGGTGGGCGCGGGCCCGTGGGGGCGGGGGGCCACAGGA
      TGCAATCGGTCGCCGTAACGGGACCACCCGCGCCCGGGCACCCCCGCCCCCCGGTGTCCT
        T  L  A  S  G  I  A  L  V  G  A  G  P  W  G  R  G  A  T  G

3181  CGGGGCACCACATTGGTCCTCACCGCCCCCACCACTACGATAGCCATGCTTTGGGGGCCA
      GCCCCGTGGTGTAACCAGGAGTGGCGGGGGTGGTGATGCTATCGGTACGAAACCCCCGGT
        R  G  T  T  L  V  L  T  A  P  T  T  T  I  A  M  L  W  G  P

3241  GTCGGTGTCATGTTTGTACGGCTAAGTCAGTTCCAACTTGATGGGCAAAAGATCGGTCAG
      CAGCCACAGTACAAACATGCCGATTCAGTCAAGGTTGAACTACCCGTTTTCTAGCCAGTC
        V  G  V  M  F  V  R  L  S  Q  F  Q  L  D  G  Q  K  I  G  Q

                                                Parallel beta helix repeat1
3301  GTTGACGGCATTCATCTGGAACGAGCCACCCAACCCCAGCTCGGGTTGTGGATCTTAGAC
      CAACTGCCGTAAGTAGACCTTGCTCGGTGGGTTGGGGTCGAGCCCAACACCTAGAATCTG
        V  D  G  I  H  L  E  R  A  T  Q  P  Q  L  G  L  W  I  L  D
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                                                  Parallel beta helix repeat2
3361  AATATTTTTGTGCATGATTGTGGGGGCAATGGGGTATACGTCGACGCAAATCGTGAAGCG
      TTATAAAAACACGTACTAACACCCCCGTTACCCCATATGCAGCTGCGTTTAGCACTTCGC
       N  I  F  V  H  D  C  G  G  N  G  V  Y  V  D  A  N  R  E  A

3421  ATCAAAATCGTGCGTTCGCAGTTCAACAGCAACGGGCTGAACGGGGTGGTTTTGAATAAT
      TAGTTTTAGCACGCAAGCGTCAAGTTGTCGTTGCCCGACTTGCCCCACCAAAACTTATTA
       I  K  I  V  R  S  Q  F  N  S  N  G  L  N  G  V  V  L  N  N

3481  TCGGATTCGTTCGTCGCCGAAAGCGAAGTCGGCTTGAACGGTGGAAGCGGGATTTGGTTG
      AGCCTAAGCAAGCAGCGGCTTTCGCTTCAGCCGAACTTGCCACCTTCGCCCTAAACCAAC
       S  D  S  F  V  A  E  S  E  V  G  L  N  G  G  S  G  I  W  L

3541  GGCGGCGCGGTCCAACGGGCCTGGGGCAATTCGATCTATAGCAACAGCGGGGCCGGGATC
      CCGCCGCGCCAGGTTGCCCGGACCCCGTTAAGCTAGATATCGTTGTCGCCCCGGCCCTAG
       G  G  A  V  Q  R  A  W  G  N  S  I  Y  S  N  S  G  A  G  I

            Parallel beta helix repeat3
3601  TCCATTTCCGGCCTGAGCATTGTCGCCACCAACAATGGCATCGACCGCAATGCCCAAGAA
      AGGTAAAGGCCGGACTCGTAACAGCGGTGGTTGTTACCGTAGCTGGCGTTACGGGTTCTT
       S  I  S  G  L  S  I  V  A  T  N  N  G  I  D  R  N  A  Q  E

                                Parallel beta helix repeat4
3661  GGCATTAAGGTCTGGCCGGGCGCGAGTAATGTGATCATTGCCGATAACATGCTGCACACC
      CCGTAATTCCAGACCGGCCCGCGCTCATTACACTAGTAACGGCTATTGTACGACGTGTGG
       G  I  K  V  W  P  G  A  S  N  V  I  I  A  D  N  M  L  H  T

                                                  Parallel beta helix repeat5
3721  AATTCGCAATCGGTGAATGCGGGCTATGCCCATATTGGGGTTGAGGATAATACCACTAAC
      TTAAGCGTTAGCCACTTACGCCCGATACGGGTATAACCCCAACTCCTATTATGGTGATTG
       N  S  Q  S  V  N  A  G  Y  A  H  I  G  V  E  D  N  T  T  N

3781  ATCAGCATCAGTAACAACACCTTCTGGCAAGATCCCGGCTACGCGACCGTGGCGGCCTAT
      TAGTCGTAGTCATTGTTGTGGAAGACCGTTCTAGGGCCGATGCGCTGGCACCGCCGGATA
       I  S  I  S  N  N  T  F  W  Q  D  P  G  Y  A  T  V  A  A  Y

                                Parallel beta helix repeat6
3841  GCCGTCCACATTCATCATGCGGCCACCAATGTCAGCGTGGCGAACAACACCACAACGGGC
      CGGCAGGTGTAAGTAGTACGCCGGTGGTTACAGTCGCACCGCTTGTTGTGGTGTTGCCCG
       A  V  H  I  H  H  A  A  T  N  V  S  V  A  N  N  T  T  T  G

3901  GCAGCGTATGCCACCGCCTTTCTAGAAGACGCCAGTGGGCAGGCGCGGTATGGTCCCAAT
      CGTCGCATACGGTGGCGGAAAGATCTTCTGCGGTCACCCGTCCGCGCCATACCAGGGTTA
       A  A  Y  A  T  A  F  L  E  D  A  S  G  Q  A  R  Y  G  P  N

3961  CCCGGCACGCAGCAAACCCCATTGAGCCAGCCCGGGGAAACCGCCGGATCGATCTATTGG
      GGGCCGTGCGTCGTTTGGGGTAACTCGGTCGGGCCCCTTTGGCGGCCTAGCTAGATAACC
       P  G  T  Q  Q  T  P  L  S  Q  P  G  E  T  A  G  S  I  Y  W

4021  TGGCAAGAAGTCGCCCCCAGTGGCGTGGGCAAAAAGGTCTGGGTTTATTTGGACGGCTAT
      ACCGTTCTTCAGCGGGGGTCACCGCACCCGTTTTTCCAGACCCAAATAAACCTGCCGATA
       W  Q  E  V  A  P  S  G  V  G  K  K  V  W  V  Y  L  D  G  Y

4081  GAGAACGACACGTCCACGGCGAACGTGATAACGTTCCCCTATGCCTTTCGATTCCCGCCC
      CTCTTGCTGTGCAGGTGCCGCTTGCACTATTGCAAGGGGATACGGAAAGCTAAGGGCGGG
       E  N  D  T  S  T  A  N  V  I  T  F  P  Y  A  F  R  F  P  P

4141  GTCCTCAACAACATCGTGGGTGTGGCGGGAGCCACGGTCTCGACAACGGCATTATCCATT
      CAGGAGTTGTTGTAGCACCCACACCGCCCTCGGTGCCAGAGCTGTTGCCGTAATAGGTAA
       V  L  N  N  I  V  G  V  A  G  A  T  V  S  T  T  A  L  S  I
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                                                          Stop ORF4
4201  GATCCGGACACCACCACCACCTATACAGGGTGGATTCTTGTGGAAGGGGTG**TGA**AACCTA
      CTAGGCCTGTGGTGGTGGTGGATATGTCCCACCTAAGAACACCTTCCCCACACTTTGGAT
       D  P  D  T  T  T  T  Y  T  G  W  I  L  V  E  G  V  *

4261  TGTTACTGGCATTTTTTGGCATTGGTACGTTGATGGGAGCCTTCACTTTTTTGATTTTAG
      ACAATGACCGTAAAAAACCGTAACCATGCAACTACCCTCGGAAGTGAAAAAACTAAAATC

4321  CGAGCCTGGCCCTCGGCCAGTTCCCTCGACAACCCCGTTAACGTCGAAAACATCCTCGTA
      GCTCGGACCGGGAGCCGGTCAAGGGAGCTGTTGGGGCAATTGCAGCTTTTGTAGGAGCAT

4381  GAGGCTCCCGGAGAGGACAAACATACAAAACAGGATAAATCATACGGGAGACCCATATAC
      CTCCGAGGGCCTCTCCTGTTTGTATGTTTTGTCCTATTTAGTATGCCCTCTGGGTATATG

4441  GCGCCACAGAAACAAAATATGAGGAAATGGAAGGAATGGCATTTTTTTCCAGTCATATAG
      CGCGGTGTCTTTGTTTTATACTCCTTTACCTTCCTTACCGTAAAAAAAGGTCAGTATATC

4501  GATCTGTACTCTTCTCCGAGTCTTCCCCCATATGAAAAAAATTTCAGAATTTTCCACCAT
      CTAGACATGAGAAGAGGCTCAGAAGGGGGTATACTTTTTTTAAAGTCTTAAAAGGTGGTA

4561  ATTTTTTTGTCGGTTTTGTCTCCCGGATAGGCTGCCGTAATGTCAAAAATGTGCTATTGT
      TAAAAAAACAGCCAAAACAGAGGGCCTATCCGACGGCATTACAGTTTTTACACGATAACA

4621  GGATTTATCGATCCATGCCAGGCGAAAAAATCCCGGCCTTCGGGCCGGGCGCCGATAGCA
      CCTAAATAGCTAGGTACGGTCCGCTTTTTTAGGGCCGGAAGCCCGGCCCGCGGCTATCGT

4681  CGAGAAGGATTCGTGCGCGACTTGTCGATGATGTTCCCCAACAACATCGACCACTTGGAA
      GCTCTTCCTAAGCACGCGCTGAACAGCTACTACAAGGGGTTGTTGTAGCTGGTGAACCTT

4741  AAAGTATAGCAAAAAGCAAATCAAATTCCAAGTTCGCGAGACGAATCCTCCAAAAAAACA
      TTTCATATCGTTTTTCGTTTAGTTTAAGGTTCAAGCGCTCTGCTTAGGAGGTTTTTTTGT

         RBS         Start ORF5
4801  A**AGGAGGA**TATTTTTT**ATG**TCTGATAAAAGTATAGCACACACCGAGGACCATTTGACCTA
      TTCCTCCTATAAAAAATACAGACTATTTTCATATCGTGTGTGGCTCCTGGTAAACTGGAT
                       M  S  D  K  S  I  A  H  T  E  D  H  L  T  Y

4861  TGGTGGCAACAAGTGGCAGAAAAATTGGTTTGAGGTTCCAAATTCCTTACTCACCTTCCC
      ACCACCGTTGTTCACCGTCTTTTTAACCAAACTCCAAGGTTTAAGGAATGAGTGGAAGGG
        G  G  N  K  W  Q  K  N  W  F  E  V  P  N  S  L  L  T  F  P

4921  CAATCTGAGTTGGCAGGCGCAAATGACCTATATAGTGCTGTTGCGATTTTTGGGACCCAA
      GTTAGACTCAACCGTCCGCGTTTACTGGATATATCACGACAACGCTAAAAACCCTGGGTT
        N  L  S  W  Q  A  Q  M  T  Y  I  V  L  L  R  F  L  G  P  N

                                 Winged Helix domain
4981  CCCCGATGCGTCGCGGGCCTTCCCAAGCTACGCCACGATAGCCCGCCTGGGCCGCATGTC
      GGGGCTACGCAGCGCCCGGAAGGGTTCGATGCGGTGCTATCGGGCGGACCCGGCGTACAG
        P  D  A  S  R  A  F  P  S  Y  A  T  I  A  R  L  G  R  M  S

5041  GCGCAGCTCGGCCATACGCGCGGTCAAAGAACTGGAACGGAACGGACTCGTGCAAAAAGA
      CGCGTCGAGCCGGTATGCGCGCCAGTTTCTTGACCTTGCCTTGCCTGAGCACGTTTTTCT
        R  S  S  A  I  R  A  V  K  E  L  E  R  N  G  L  V  Q  K  E

5101  GGTGCGGCACAAGGATGCCTCCTTTTCCTCCAATGTGTATGCGTTGATCCATCCGGAAGA
      CCACGCCGTGTTCCTACGGAGGAAAAGGAGGTTACACATACGCAACTAGGTAGGCCTTCT
        V  R  H  K  D  A  S  F  S  S  N  V  Y  A  L  I  H  P  E  E
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                                          Iteron1A          Iteron1B
5161  GGAAGGAGTGGGGTCAGAGGGACACCAGGGTAGTGTCAGAGAGACACTACCTAGTGTCAG
      CCTTCCTCACCCCAGTCTCCCTGTGGTCCCATCACAGTCTCTCTGTGATGGATCACAGTC
        E   G   V   G   S   E   G   H   Q   G   S   V   R   E   T   L   P   S   V   R

                  Iteron2A                Iteron2B
5221  AGAGACACTACCCCTAGTGTCACAGGGACACCACCCTAGTGTCAGGGAGACACCTTATAA
      TCTCTGTGATGGGGATCACAGTGTCCCTGTGGTGGGATCACAGTCCCTCTGTGGAATATT
        E   T   L   P   L   V   S   Q   G   H   H   P   S   V   R   E   T   P   Y   K

5281  AAACAAAATTAAGAATACTAATATTAAGAACAACAAGAAAAAGGATGTTGTTGAGGATTT
      TTTGTTTTAATTCTTATGATTATAATTCTTGTTGTTCTTTTTCCTACAACAACTCCTAAA
        N   K   I   K   N   T   N   I   K   N   N   K   K   K   D   V   V   E   D   F

5341  TCCAAAAACCGACAAACCCCAACACGATCCCTCTCGGTCTGCTTTAGGAACCGTGTTGCA
      AGGTTTTTGGCTGTTTGGGGTTGTGCTAGGGAGAGCCAGACGAAATCCTTGGCACAACGT
        P   K   T   D   K   P   Q   H   D   P   S   R   S   A   L   G   T   V   L   Q

5401  GGACAAGTATCAGGAGATCGCCCAAGACTTGGGGATCGAGCCGGTGTTTGACGACAATGA
      CCTGTTCATAGTCCTCTAGCGGGTTCTGAACCCCTAGCTCGGCCACAAACTGCTGTTACT
        D   K   Y   Q   E   I   A   Q   D   L   G   I   E   P   V   F   D   D   N   D

5461  CGACAATGCCGCTGCCCAACGTTTATTGGCCTTGTTGCCGGAAAAGCGACGGCCAGGCAT
      GCTGTTACGGCGACGGGTTGCAAATAACCGGAACAACGGCCTTTTCGCTGCCGGTCCGTA
        D   N   A   A   A   Q   R   L   L   A   L   L   P   E   K   R   R   P   G   M

5521  GGCCTTGGGCTTTTTACAAACCCTCGTCAAGGAATTTGGGGAAGAGAACGTGGCGGAAAA
      CCGGAACCCGAAAAATGTTTGGGAGCAGTTCCTTAAACCCCTTCTCTTGCACCGCCTTTT
        A   L   G   F   L   Q   T   L   V   K   E   F   G   E   E   N   V   A   E   K

5581  ATTGCGCCTGATGCCGCCGAGCGTCAAGAATCCCCCAGGATGGCTCAAAATCGCACTGGA
      TAACGCGGACTACGGCGGCTCGCAGTTCTTAGGGGGTCCTACCGAGTTTTAGCGTGACCT
        L   R   L   M   P   P   S   V   K   N   P   P   G   W   L   K   I   A   L   E

5641  AAAGAATTTTGTAGCGCCTGAACCCGAAAAACCGCCGGTCCGCCGCGTTGAAGATCTCCC
      TTTCTTAAAACATCGCGGACTTGGGCTTTTTGGCGGCCAGGCGGCGCAACTTCTAGAGGG
        K   N   F   V   A   P   E   P   E   K   P   P   V   R   R   V   E   D   L   P

5701  CGATCACGCCCGCCGCTTGCGCGAATTGCGCGAGTTGGGGTTTGGGCACGATCCCGAACC
      GCTAGTGCGGGCGGCGAACGCGCTTAACGCGCTCAACCCCAAACCCGTGCTAGGGCTTGG
        D   H   A   R   R   L   R   E   L   R   E   L   G   F   G   H   D   P   E   P

5761  GGCTAAGAAAATTCGAGACTTGGATGCTGAATTGGCGGCCTTGTCCCCAACCCAACGCCA
      CCGATTCTTTTAAGCTCTGAACCTACGACTTAACCGCCGGAACAGGGGTTGGGTTGCGGT
        A   K   K   I   R   D   L   D   A   E   L   A   A   L   S   P   T   Q   R   Q

5821  GCAAATCGAAGCCCAAGCCTTAGCGAAATTCCGGGAGCGCACGCGGTTGGCGGCCCCGGA
      CGTTTAGCTTCGGGTTCGGAATCGCTTTAAGGCCCTCGCGTGCGCCAACCGCCGGGGCCT
        Q   I   E   A   Q   A   L   A   K   F   R   E   R   T   R   L   A   A   P   D

5881  CGCCACGAACCCTTTCCACAAGGTCTTGATGCACGGTTTTTTGGCCTTAGCCGTCGAAGA
      GCGGTGCTTGGGAAAGGTGTTCCAGAACTACGTGCCAAAAAACCGGAATCGGCAGCTTCT
        A   T   N   P   F   H   K   V   L   M   H   G   F   L   A   L   A   V   E   E

                                  Stop ORF5
5941  AATGCGCACCATCCCCACAGAGGCTGTCTGAGGGCCTTTTGGGGATGGATAGGATCAATC
      TTACGCGTGGTAGGGGTGTCTCCGACAGACTCCCGGAAAACCCCTACCTATCCTAGTTAG
        M   R   T   I   P   T   E   A   V   *
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6001  ACCCATGACGCCCACCAAAAGGGCTTTAGAAGGCGCATATGAGGACATGAGCGAAAGGAG
      TGGGTACTGCGGGTGGTTTTCCCGAAATCTTCCGCGTATACTCCTGTACTCGCTTTCCTC

6061  AAGCGAGTATGCGAACCCCTATACACACACAATGGACACCGCAACAGGATGCTTATTGGT
      TTCGCTCATACGCTTGGGGATATGTGTGTGTTACCTGTGGCGTTGTCCTACGAATAACCA

6121  TATTAATGCTGCTAGAGAACACCATGTCTCAACAACAAGCCCAAGGCAGGGCTATGGCCG
      ATAATTACGACGATCTCTTGTGGTACAGAGTTGTTGTTCGGGTTCCGTCCCGATACCGGC

6181  GCAAGCCCTCGGGCAACGAGGCAGACACCCAAATA
      CGTTCGGGAGCCCGTTGCTCCGTCTGTGGGTTTAT
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