DEVELOPMENTAL GENETIC STUDIES ON THIOBACILLUS FERROOXIDANS

by

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submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Faculty of Science

University of Cape Town

CAPE TOWN

September 1988

CERTIFICATION OF SUPERVISORS

In terms of paragraph 9 of "General regulations for the degree of Ph.D", University of Cape Town, we, as supervisors of the candidate R.S. Ramesar, certify that we approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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ACKNOWLEDGEMENTS

I gratefully acknowledge:

Professor Douglas E Rawlings, my supervisor, whose advice, unstinting guidance and professional critique served to distil my thoughts and to channel my energy

Professor David R Woods, co-supervisor of this project, whose constant interest and contageous enthusiasm added impetus to this study

Dr Valerie Abbratt, Dr Nozibele Mjoli, Eric O'Neill, Winsome Jones and Morag Kenmuir for their proficient technical assistance

Dr Joan Parker for making available affinity purified RecA antiserum

Joseph Santangelo, a kindly soul, for advice and for the unreserved use of his personal computer

Members of staff and fellow students in the Department of Microbiology, University of Cape Town, who have provided advice and assistance at one stage or another

Members of the Thiobacillus Research Unit for their cooperation

Jennifer Johnson, for her advice and assistance with various aspects of this thesis, and for the life support system that she provided during this study

The Council for Scientific and Industrial Research (CSIR, South Africa) and General Mining Union Corporation (South Africa) whose financial support has made this study possible.

ABBREVIATIONS

A	adenine
Ala	L-alanine
Ap	ampicillin
Arg	L-arginine
Asn	L-asparagine
Asp	L-aspartic acid
ATCC	American Type Culture Collection
bp	base pair (s)
C	cytosine
cfu	colony forming units
Ci	Curie
CIP	calf intestinal phosphatase
Cm	chloramphenicol
cpm	counts per minute
CsCl	caesium chloride
Cys	L-cysteine
D	daltons
d	day (s)
d	(superscript) dominant
DNase	deoxyribonuclease
dsDNA	double-stranded DNA
eop	efficiency of plating
g	grams
G	Guanine
Gln	L-glutamine
Glu	L-glutamic acid
Gly	L-glycine
h	hour (s)
His	L-histidine
Ile	L-isoleucine
J	Joules
Km	kanamycin
kb	kilobase (s) or kilobase pairs
kcal	kilocalories
l	litres
LA	Luria-Bertani agar
LB	Luria-Bertani
Leu	L-leucine
Lys	L-lys
m	metre (s)
M	molar
Met	L-methionine
mg	milligram
MIC	minimum inhibitory concentration
min	minute (s)

mm	millimetre (s)
mM	millimolar
MMS	methyl methane sulfonate
mol	mole (s)
mRNA	messenger RNA
M_r	relative molecular mass
Nal	nalidixic acid
ng	nanogram
NQO	4-nitro-1-quinoline
NTG	N-methyl, N-nitro, N-nitrosoguanidine
NTP	any nucleotide triphosphate
OD_x	optical density at X nm
ORF	open reading frame
ori	origin of replication
oriT	origin of transfer
^{32}P	radioactive isotope of phosphorous
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
pfu	plaque forming units
Phe	L-phenylalanine
pmol	picomole
Pro	L-proline
r	(superscript) resistance or resistant
Rif	rifampicin
RNase	ribonuclease
rpm	revolutions per minute
s	second (s)
s	(superscript) sensitive
^{35}S	radioactive isotope of sulphur
SDS	sodium dodecyl sulphate
Ser	L-serine
Sm	streptomycin
ssDNA	single-stranded DNA
T	thymine
Tc	tetracycline
Thr	L-threonine
Trp	L-tryptophan
Tyr	L-tyrosine
u	units of enzyme activity
UV	ultraviolet
V	volts
Val	L-valine
v/v	volume per volume
v/w	volume per mass
wt	wild-type

ABSTRACT

Thiobacillus ferrooxidans is an industrially important bacterium which is used in bioleaching operations. The work reported in this investigation extends current knowledge of the genetics of this organism.

Conjugation was attempted as a means for plasmid DNA transfer to *T. ferrooxidans*. Recombinant *T. ferrooxidans* plasmids, pDER401 and pDER405, were shown to code for mobilization and replication functions in *Escherichia coli* and *Thiobacillus novellus* strains. The plasmids were mobilizable at high frequency by the IncP plasmid, R68.45. Attempts to transfer the *T. ferrooxidans* recombinant plasmids directly from *E. coli* to *T. ferrooxidans* were unsuccessful. In multistage mating experiments, plasmid DNA was transferred from *E. coli* to *T. novellus*, and from *T. novellus* to *Thiobacillus intermedius*. However, in subsequent matings, plasmid transfer from these thiobacilli to *T. ferrooxidans* could not be shown.

A genomic library of *T. ferrooxidans* ATCC 33020 was constructed in the plasmid vector, pEcoR251, for the purpose of cloning a *recA*-like gene from this organism. The library consisted of approximately $1,78 \times 10^4$ clones carrying chromosomal DNA fragments of about 3-12 kilobases (kb). The library was successfully screened for functional complementation of *E. coli* auxotrophic mutants. Clones that conferred resistance to methyl methane sulfonate (MMS), a

DNA-damaging agent, were isolated in an E. coli recA$^-$ mutant. In an attempt to clone a homologous marker, T. ferrooxidans ATCC 33020 was mutated to rifampicin resistance (Rif^r) and DNA from the mutant strain was used in the construction of plasmid- and cosmid-based libraries. The plasmid library contained approximately 1,35 X 10^4 clones with inserts of about 1-13 kb. The cosmid library consisted of approximately 8.2 X 10^3 colonies, 4.0 X 10^4 in vitro packaged cosmids, and an amplified in vivo-packaged cosmid lysate containing approximately 1.82 X 10^{11} infectious particles, carrying inserts of about 35-55 kb. Complementation of E. coli auxotrophic mutants was observed with the plasmid and cosmid library of the T. ferrooxidans Rif^r strain. Screening both libraries for a Rif^r marker was unsuccessful.

Three recombinant plasmids, pRSR100, pRSR101, and pRSR102, each containing the functional analogue of the E. coli recA gene, were isolated from the plasmid-based genomic library of T. ferrooxidans ATCC 33020. The plasmid, pRSR100, was used for further characterization of the cloned recA-like gene. pRSR100 complemented defects in DNA repair and homologous recombination in an E. coli recA$^-$ strain. Antiserum raised against E. coli RecA protein reacted with two protein bands with an apparent M_r of approximately 40 000 and 38 000 in extracts of the recA deletion mutant, E. coli JK696, containing pRSR100. A single band with an apparent M_r of approximately 40 000 was detected in

T. ferrooxidans cell extracts with the *E. coli* RecA antiserum.

The nucleotide sequence of the *T. ferrooxidans* *recA* gene has been determined. No SOS box characteristic of LexA-regulated promoters could be identified in the 196-bp region upstream of the coding region. The *T. ferrooxidans* *recA* gene specifies a protein of 346 amino acids that has 66% and 69% homology to the RecA proteins of *E. coli* and *P. aeruginosa*, respectively. Most amino acids that have been identified as being of functional importance in the *E. coli* RecA protein are conserved in the *T. ferrooxidans* RecA protein. Although some amino acids that have been associated with ATPase and constitutive protease activity have been substituted, the cloned protein has retained these activities. The cloned *recA* gene was expressed in *E. coli* from both the λ P_r and *lac* promoters. However, no expression from the 2.2 kb *T. ferrooxidans* DNA preceding the gene was evident.

CHAPTER 1

GENERAL INTRODUCTION

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1.1 Plan of this thesis

A condensed review of aspects of microbial leaching, and the organisms involved in this process is presented in this, the general introduction. An overview of reported genetic studies relating to T. ferrooxidans is provided as a measure of the genetics of this organism, and to provide perspective for the present investigations.

The body of the thesis is an account of the experimental work towards understanding specific aspects of the genetics of T. ferrooxidans. Each of the four chapters that constitute the body of this dissertation includes an independent introduction providing background information with regard to the specific phenomenon or subject of the investigation in that chapter. The final chapter comprises general conclusions; an assessment of the findings of this study and includes prospects for extending the research reported here.

1.2 Bioleaching

Bioleaching involves the dissolution of metals from ores by the oxidative action of bacteria. Interest in microbial

leaching to complement conventional metal recovery technologies is for several reasons.

In the past, mostly high-grade ores were exploited using conventional physical and chemical processes. However, some refractory ores, even from high-grade reserves, are not efficiently extracted using these methods (Brierley, 1978; 1982). The threatened depletion of mineral deposits has served as an incentive for the development of more efficient extraction processes. Moreover, many conventional procedures for processing metal ores, particularly those requiring heat treatment, are energy intensive (Ebner, 1978). The bioleaching of ores, which is carried out under mild conditions, may provide an energy-saving alternative.

Conventional mining procedures often give rise to high levels of toxic wastes in the environment, whereas contained bioleaching operations are relatively pollution free (Holmes, 1988). Also, there are vast reserves of fossil fuels such as coal and oil in existence which contain high levels of sulphur. Burning of such fuels results in a severe impact on the environment in the form of corrosive "acid-rain". Bioleaching has the potential to remove sulphur from these fuels, and is the subject of much research (Dugan, 1984; Monticello and Finnerty, 1985; Beyer et al., 1986; Bos et al., 1986; Dogan et al., 1986).

In comparison with conventional hydrometallurgical and pyrometallurgical methods, bacterial leaching is

particularly suited to the treatment of low-grade ores and for the recovery of metals from the tailings of conventional processes (Brierley, 1978; Lundgren and Silver, 1980). The metal content of some ores is too low to be economically recovered by concentration and smelting, but not low enough to be discarded as waste. The reserves that may be exploited through bioleaching are vast. For example, the copper reserves of Chile have been estimated to be 1.5×10^8 tons, of which 4.7×10^7 tons occur in low-grade ores where recovery by conventional technology is not profitable (Gentina and Acevedo, 1985). In addition, the growth in Chile's copper industry has produced large quantities of acid-leached ores and tailings which contain residual metal. In the U.S.A., up to 25% of all copper production results from microbial leaching of low-grade ores (Torma, 1986).

Currently, bacterial leaching is practised in many countries including Australia, Canada, Chile, Mexico, Peru, Spain, the U.S.A., the U.S.S.R. (Hutchins et al., 1986) and South Africa (Holmes, 1988). Among the metals recovered are copper, uranium, nickel, cobalt, zinc, lead and gold (Brierley, 1978; Gentina and Acevedo, 1985; Hutchins et al., 1986). Most countries have developed bioleaching processes for the extraction of base metals. However, in South Africa primary interest in bioleaching is for the recovery of gold.

In some gold-bearing ores, the metal is encapsulated by iron-sulphide minerals. Such ores are particularly refractory to conventional extraction methods (Brierley,

1982). Microbial pretreatment results in the removal of the encasing material, rendering the ore amenable to cyanidation. In a demonstration plant in the U.S.A., 95,6% of available gold was recovered from refractory ores after pretreatment with bacteria, whereas using cyanide alone, only 65,7% of the gold was recovered (Holmes, 1988). In addition, microbial pretreatment is an environmentally superior alternative to roasting, which is part of the conventional process used to remove the iron sulphide from ores. Roasting generates air pollutants such as sulphur dioxide, volatile arsenic compounds and other harmful chemicals (Brierley, 1982).

1.3 Brief description of the leaching process

The predominant commercial methods used in bacterial extraction of ores are dump, heap, *in situ* and vat leaching (Brierley, 1978; Lundgren and Silver, 1980). Microbial leaching may occur directly by the metabolism of microorganisms, or indirectly by the products of their metabolism (Brierley, 1978; Hutchins *et al.*, 1986). Direct attack is initiated by bacterial attachment to the ore surface; the organisms then enzymatically attack the oxidizable ferrous (Fe^{2+}) iron or sulphide components in the ore (Brierley, 1978). The iron or sulphide compounds oxidized in this way are solubilized thereby exposing or releasing the target metal.

In the indirect mechanism of microbial leaching, physical contact between the bacteria and the ore to be leached is not necessary. The bacteria are used to oxidize soluble ferrous iron to ferric iron (Fe^{3+}), which is the primary oxidant in the indirect process (Silverman, 1967; Lundgren and Silver, 1980). When sulphuric acid is present, the ferric iron reacts with the metal sulphide in a metal sulphide conglomerate, which in turn is oxidized to a soluble form. During this catalysis, the oxidant (ferric iron) is reduced to ferrous iron, whereupon it is rapidly oxidized by the bacteria, thus establishing a cyclic process (Silverman, 1967; Lundgren and Silver, 1980).

In the presence of ferrous iron, both direct and indirect mechanisms of bacterial attack are likely to contribute to the leaching of mineral ores.

Apart from direct and indirect leaching, galvanic conversion may contribute to the dissolution of some metals in ores (Berry and Murr, 1978). This mechanism involves contact between dissimilar metal sulphides in an electrolyte to create a galvanic cell. This arrangement results in the dissolution of the metal sulphide with the lower relative electrical potential. Bacteria may aid galvanic conversion by oxidizing the film of elemental sulphur on the disintegrating electrode. The quantitative contribution by galvanic conversion to the leaching process is not known.

1.4 Leaching bacteria

Conditions within leach structures, such as dumps or heaps, may vary markedly with regard to nutrients, pH, temperature, and aeration (Brierley, 1978; Hutchins et al., 1986). Because the leaching environment represents a wide variety of ecological niches, a diverse population of organisms that contribute to leaching may be isolated.

Most organisms isolated from leaching sites and characterized to date are mesophiles, growing best in the temperature range 20^0C - 35^0C (Kelly et al., 1979; Hutchins et al., 1986). Those most important to leaching processes thrive in acidic solutions and obtain energy by oxidizing inorganic substrates. These include Thiobacillus ferrooxidans, Thiobacillus thiooxidans and Leptospirillum ferrooxidans (Brierley, 1982).

Several organisms have been reported to be capable of leaching at temperatures of 50^0C - 55^0C. These moderate thermophiles include Thiobacillus-like THI strains (Brierley and Le Roux, 1977). Unlike T. ferrooxidans, these organisms require small amounts of yeast extract, cysteine or glutathione for growth. A Gram-positive, acidophilic bacterium has also been reported to play a role in leaching (Golovacheva and Karavaiko, 1978; Karavaiko et al., 1987). This organism, designated Sulfobacillus, actively oxidizes sulphur, pyrite minerals and ferrous iron at pH 1.6 - 2.4, and between 45^0C - 50^0C, in the presence of small amounts of

organic compounds (Karavaiko et al., 1987). Sporulation in the sulfobacilli has been described (Karavaiko et al., 1987). The ability of some Sulfobacillus strains to oxidize sulphur compounds has been disputed (Hutchins et al., 1986).

At still higher temperatures Sulfolobus sp. may be the most important leaching organisms. Norris and Parrott (1986) reported that, under laboratory conditions, strains of Sulfolobus are capable of high rates of iron and sulphur oxidation. These organisms enhanced yields from some recalcitrant ores, and could operate at higher pulp densities than T. ferrooxidans. Sulfolobus sp. have been shown to leach metals from a variety of ores at between 60^0C - 80^0C (Norris and Parrott, 1986; Hutchins et al., 1986).

Heterotrophic bacteria, including non-iron-oxidizing members of the genus Thiobacillus, and fungi also occur in leaching environments, but their contribution to metal extraction is not known (Kelly et al., 1979). Mixed populations of autotrophic and heterotrophic bacteria have been reported to be more efficient at leaching some minerals than pure cultures of individual organisms (Kelly et al., 1979).

Undoubtedly only a fraction of the organisms in leaching operations have been isolated and characterized. Additional useful organisms may yet be discovered, and attention is currently being given to screening new isolates for enhanced metal oxidation and metal-binding capabilities (Holmes, 1988).

1.4.1 T. ferrooxidans. T. ferrooxidans, the most studied of leaching bacteria, is approximately 1 um long and 0,5 um in diameter, Gram negative and rod-shaped (Brierley, 1978; Hutchins et al., 1986). The organism is well adapted to its inorganic acidic niche. Optimum growth occurs in an acidic medium (pH 1,5 to pH 2,5) and the organism derives its energy from the oxidation of ferrous iron to ferric iron, and reduced forms of sulphur to sulphuric acid. Oxygen usually acts as the final electron acceptor although, when grown on elemental sulphur (S^0), ferric iron may act as an alternate electron acceptor (Sugio et al., 1985). A chemiosmotic gradient generated by the electron transport chain is used to synthesize adenosine triphosphate (ATP) (Apel et al., 1980).

T. ferrooxidans is an obligate chemoautotroph and obtains carbon through the fixation of atmospheric carbon dioxide. All T. ferrooxidans strains that have been tested are also able to fix atmospheric nitrogen (Mackintosh, 1978; Pretorius et al., 1986).

1.5 Strain improvement: the role of the molecular biologist

The slowness of the microbial leaching process, compared with conventional physical and chemical methods, has been regarded as its major drawback. In leaching operations, conditions such as temperature, pH, aeration, particle size, pulp density and other parameters may be manipulated to

create a combination that will result in an optimum rate of bioleaching (Brierley, 1978).

However, it is the metabolic activity of the bacteria themselves that is likely to be a major rate-limiting step in biological leaching processes. It is unlikely that the activity of a given organism can be improved beyond a certain level, irrespective of physiochemical manipulation. This level may be regarded as the ceiling activity and is limited by the organism's genetic constitution. For this reason, the genetic modification of leaching organisms, using mutation and selection, and recombinant DNA technology, has been considered.

Mutation and selection is an empirical approach of strain improvement and requires no knowledge of the genetics or physiology of the organism. It involves the application of chemical or physical stress on a population of organisms, which results in the selection of survivors that are most tolerant or resistant to such pressure. As with penicillin and amino acid production (Ikeda and Beppu, 1983), mutation and selection may markedly improve an organism's productivity. However, mutation and selection is limited in that it involves the modification of proteins and regulatory mechanisms that are already present in the cell.

Random mutation and selection techniques to enhance the capability of T. ferrooxidans have been carried out by Groudeva and Groudev (1980). Mutant populations capable of

increased rates of ferrous-iron oxidation were obtained. Gencor of South Africa has successfully used spontaneous mutation and selection in adapting strains of T. ferrooxidans to grow in the presence of arsenic (Gencor Group Laboratory Communication, Gencor, Johannesburg, South Africa). These arsenic-tolerant strains have been shown to be useful in the pretreatment of arsenopyrite ores.

Unlike mutation and selection, the application of recombinant-DNA technology for strain improvement requires much information regarding the genetics of an organism, and in turn provides a potentially powerful means for altering its genetic constitution. Using this technology there is the potential to introduce desirable, extraneous genetic material into a leaching organism. The introduced genetic material, once established in the cell, may allow improvements in the performance of leaching bacteria that cannot be achieved through mutation and selection.

New or modified genetic material may be introduced on plasmids of which there may be many copies per cell. Through this process of gene amplification, it may be possible to identify enzymes whose activities act as metabolic "bottlenecks". This knowledge would provide new insights on how to improve the growth rate and leaching performance of bacteria.

A wide range of potential improvements by gene transfer to leaching bacteria have been proposed (Chakrabarty, 1978;

Holmes, 1988). The enhancement of growth rate, and the rate of iron and sulphide oxidation by bacteria as a means of enhancing the leach rate is desirable. However, little is known about the genetic mechanisms controlling these processes. An improvement in the tolerance of leaching organisms to certain heavy metals, and decreased sensitivity to organic compounds, cyanide, surfactants and inhibitory ions encountered in a leaching environment are all of practical importance. However, the introduction of genes into these bacteria requires a means for their delivery into the organism.

1.6 The genetics of *T. ferrooxidans*

More is known about the general physiology and metabolism of *T. ferrooxidans* than any other organism associated with metal leaching. In addition, *T. ferrooxidans* is considered the major leaching microorganism of economic importance (Hutchins *et al.*, 1986). For these reasons *T. ferrooxidans* was chosen from among the leaching organisms for genetic study and manipulation. However, genetic manipulation of other strains may be required, if their contribution to leaching operations is shown to be of significance. The ability to genetically manipulate *T. ferrooxidans* is partly dependent on an understanding of the molecular biology of the organism. Although its unique metabolism has been extensively studied (Brierley, 1978; Murr *et al.*, 1978), little is known about the genetics of *T. ferrooxidans*.

T. ferrooxidans has a unique physiology, it occupies an ecological niche that would have kept it physically separated from most other Eubacteria (Lane et al., 1985). It is possible that the organism has undergone substantial changes in its genetic material during evolution to its acidophilic, chemoautolithotrophic way of life. This may be illustrated by the size of the T. ferrooxidans genome which is 2.8 X 10^6 base pairs (bp) compared with 4 X 10^6 bp of Escherichia coli (Yates and Holmes, 1987). The extent of genetic drift between T. ferrooxidans and other organisms is important when considering the genetic manipulation of this bacterium. For optimal expression of foreign DNA in bacteria, it is important that the donor DNA is derived from organisms that are genetically related. Therefore, for the introduction of foreign DNA into T. ferrooxidans it is necessary to identify sources of genes that are compatible with its transcription and translation machinery.

The transfer of foreign DNA into T. ferrooxidans and an assay for an acquired phenotype is the most direct way of assessing usable genetic material in the organism. However, a means to introduce DNA into T. ferrooxidans has not yet been discovered. Foreign genes for potential use in T. ferrooxidans may be assessed indirectly. A means for studying T. ferrooxidans genetics is through the complementation of mutant strains of E. coli with cloned T. ferrooxidans genomic DNA. This provides a surrogate means of studying function and regulation of T. ferrooxidans genes. Comparison of the sequence of T. ferrooxidans genes,

their control regions and the derived amino acid sequences of their proteins with analogous genes and proteins of other bacteria is a means of determining the most suitable sources of donor DNA.

1.6.1 Characterization of *T. ferrooxidans* chromosomal genes

1.6.1.1 The glutamine synthetase (GS) gene (*glnA*).

GS plays a central role in nitrogen metabolism in most organisms. The enzyme catalyzes the assimilation of ammonia by glutamate to form glutamine (Tyler, 1978). In *E. coli*, GS is regulated at the levels of synthesis and activity. The covalent linking of an adenine residue to GS curtails the activity of this enzyme in the cell. At the genetic level, transcription of the GS gene, *glnA*, is under the control of two promoters immediately upstream of the gene (Reitzer and Magasanik, 1985). One of these promoters, *glnA*pI, is responsible for low level synthesis of GS and requires cAMP for expression. Expression from the second promoter, *glnA*pII, is regulated by nitrogen and requires the product of the *ntrC* gene for optimal expression. In the rhizobia and bradyrhizobia, two types of GS, called GSI and GSII are present. It has been shown that in *Bradyrhizobium japonicum*, GSI and GSII are controlled by different promoters (Carlson *et al.*, 1987). GSI is regulated by a *glnA*pI-type promoter and GSII is controlled by the *glnA*pII-type promoter

In other Gram-negative bacteria, the glnA gene is linked to and expressed together with two other regulatory genes. These are the ntrB and ntrC genes (McFarland et al., 1981).

The T. ferrooxidans glnA gene. The glnA gene from T. ferrooxidans has been identified by functional complementation of an E. coli glnA mutant (Barros et al., 1985). The cloned GS showed high levels of activity and was subject to inactivation by adenylation. In the Enterobacteriaceae, the nitrogen regulatory genes, ntrB and ntrC, are located immediately downstream of the glnA gene. Preliminary evidence indicates that the situation in T. ferrooxidans is different (D. Berger, personal communication). Although an ORF immediately downstream of glnA is evident, there is no homology at the nucleotide or derived amino acid level with the ntrB genes of other bacteria (Rawlings et al., 1987).

The T. ferrooxidans glnA gene was expressed in E. coli from a promoter on the T. ferrooxidans DNA. Although not confirmed as the glnA promoter, this was the first indication of a promoter of a structural gene from T. ferrooxidans being functional in E. coli. Only a glnApI-type promoter was evident on the nucleotide sequence associated with glnA (Rawlings et al., 1987). No evidence of a glnApII-type promoter was apparent. It is not known whether T. ferrooxidans has a GSII enzyme or a glnApII-type promoter.

1.6.1.2 The nitrogenase genes (*nifH*, *nifD* and *nifK*)

The process of nitrogen-fixation in bacteria is catalyzed by the enzyme nitrogenase, the product of the *nifH*, *nifD* and *nifK* genes (Postgate, 1982). The *nifHDK* genes are known to be highly conserved between different genera of nitrogen-fixing organisms. These genes are located in a single copy on the chromosome of most nitrogen-fixing bacteria, and are read as a single transcriptional unit (Postgate, 1982).

However, there are exceptions to this trend. In the fast-growing rhizobia, genes for nitrogen-fixation are located on large, 200 - 300 kilobase pair (kb) plasmids (Rosenberg *et al.*, 1981; Barbour *et al.*, 1985). Separation of the *nifHDK* genes into transcriptional units has been shown in the bradyrhizobia (Kaluza and Hennecke, 1984). In the cyanobacteria, intervening DNA between the *nifHD* and *nifK* genes is excised before the genes are transcribed (Golden *et al.*, 1985). Robson *et al.* (1986) reported that among the azotobacters, more than one copy of some of the nitrogenase genes may be located on the chromosome.

Nitrogenase genes in *T. ferrooxidans*. Mackintosh (1978), using ^{15}N-incorporation into cellular product, showed that *T. ferrooxidans* was able to fix atmospheric nitrogen. The presence of nitrogen-fixing genes in *T. ferrooxidans* was shown by Pretorius *et al.* (1986). These workers detected homologous sequences in all five strains of *T. ferrooxidans* that were probed with the *nifHDK* genes of *Klebsiella pneumoniae*. This indicated that the ability to

fix nitrogen is likely to be a characteristic of most T. ferrooxidans strains. Genetic drift between the five strains of T. ferrooxidans was indicated by different banding patterns of the restricted chromosomal DNA fragments that were homologous to the nifHDK probe. The T. ferrooxidans ATCC 33020 nifHDK genes were isolated and cloned into an E. coli plasmid vector (Pretorius et al., 1986).

The nifHDK genes are present in a single copy in the T. ferrooxidans genome and appear to be transcribed as a single unit (Pretorius et al., 1987). Whether the genes are located on a plasmid or on the chromosome in T. ferrooxidans is not clear. However, it has been shown that the genes are not present on either of the two small detectable plasmids present in T. ferrooxidans ATCC 33020.

Transcription of the nifHDK genes in nitrogen-fixing bacteria so far studied, requires two clearly recognizable nucleotide sequences upstream of the nifH gene (Buck et al., 1986). These are an ntrA-dependent promoter and a nifA-binding site. In T. ferrooxidans, there is an ntrA-dependent promoter and two nifA-binding sites in tandem, upstream of the nifH gene (Pretorius et al., 1987).

1.6.1.3 Sequencing studies. The T. ferrooxidans glnA gene has been sequenced (Rawlings et al., 1987) and the deduced GS amino acid sequence was compared with the GS sequences from nine other bacteria (D.E. Rawlings, personal

communication). The T. ferrooxidans GS enzyme has a similar percentage homology (58% to 64%) with the other Gram-negative bacteria for which GS sequence information is available.

A comparison of the amino acid homology between the nifHDK polypeptides from 11 bacteria indicated that the nitrogenase proteins of T. ferrooxidans were most closely related to those of the bradyrhizobia (nifH, 86%; nifD, 68%; nifK, 69% homology) than any other group of nitrogen-fixing bacteria for which nitrogenase-sequence information is available (Rawlings, 1988; D.E. Rawlings, personal communication). Conservation of amino acid sequence between the organisms was highest in the nifH encoded subunit of nitrogenase.

1.6.1.4 Cloning of rusticyanin. Rusticyanin is a small copper protein, with an apparent M_r of 16 000, induced when T. ferrooxidans is grown on ferrous iron (Cobley and Haddock, 1975). It is thought to be the initial member in a chain of electron carriers. Kulpa et al. (1986) reported the cloning of the rusticyanin gene from T. ferrooxidans. This was done by probing a T. ferrooxidans DNA library in E. coli, using antibodies raised against purified rusticyanin protein. The amino acid sequence of rusticyanin has been determined directly from the protein. Since rusticyanin in T. ferrooxidans is the only known example of this protein, an amino acid sequence comparison with similar proteins from other organisms is not possible.

1.6.1.5 Repeated DNA sequences in *T. ferrooxidans*.

Yates *et al*. (1987) reported that approximately 6% of the genome of *T. ferrooxidans* ATCC 19859 consists of repeated sequences. These workers cloned two non-homologous families of these sequences in *E. coli* and showed that they are repeated between 20 to 30 times per genome. One family of repeated sequences exhibited a high mobility within the *T. ferrooxidans* genome. Yates *et al*. (1987) suggested that movement of the repeated sequences could be mutagenic and pointed out the potential destabilizing effect that such sequences may have on genetically engineered organisms. The exploitation of these sequences to force the change of *T. ferrooxidans* to suit specific environmental conditions was also suggested.

Information regarding the structure and function of *T. ferrooxidans* chromosomal genes is steadily increasing. At the same time plasmid DNA as a means for introducing potentially beneficial extraneous DNA into *T. ferrooxidans* is being investigated.

1.6.2 Plasmids from *T. ferrooxidans*

Plasmids are circular, self-replicating pieces of DNA. They carry genes that provide mostly non-essential or transiently useful functions to their host organism (Sherratt, 1982). Plasmids, from a number of organisms, have been isolated and engineered to become a valuable tool in the repertoire of the molecular biologist.

Although some plasmids have the ability to replicate and function in more than one organism, most plasmids are limited to one or a few closely related bacteria (Sherratt, 1982). The majority of plasmid vectors currently used in genetic transfer experiments are of this type. Since T. ferrooxidans has a relatively unique physiology there is no way of knowing which of the previously characterized plasmids from other bacteria would be able to replicate and be stably maintained in T. ferrooxidans strains.

An approach to this problem is to isolate and characterize plasmids from T. ferrooxidans. Several workers have reported the isolation of plasmids from T. ferrooxidans strains (Martin et al., 1983; Rawlings et al., 1983; Holmes et al., 1984). Such plasmids could form the basis of T. ferrooxidans vectors as they should be able to replicate when reintroduced back into T. ferrooxidans. Although the expression of promoters from a T. ferrooxidans plasmid in an in vitro E. coli DNA-directed cell-free system was shown, all attempts to identify a plasmid phenotype by heterologous expression in E. coli have been unsuccessful (Rawlings et al., 1984a).

Plasmids from T. ferrooxidans have been cloned in E. coli (Holmes et al., 1984; Rawlings and Woods, 1985). T. ferrooxidans plasmids, ligated to pBR325, were transformed into E. coli and the resistance of transformants to Ag, As^{III}, As^{V}, Cd, Co, Cr^{VI}, Cu^{II}, Hg, Li, Mo^{VI}, Ni, Sb,

Te, U^{VI}, and Zn was determined (Rawlings *et al.*, 1986). The plasmids did not affect the metal ion tolerance of the transformants, nor did they confer resistance to eight commonly used antibiotics. However, it was shown that at least one plasmid from *T. ferrooxidans* has a broad-host-range origin of replication (Rawlings *et al*, 1984b; Rawlings *et al.*, 1986) and mobilization functions (Rawlings and Woods, 1985).

1.6.2.1 Genetic markers for *T. ferrooxidans*. The transfer of plasmids into *T. ferrooxidans* requires a system for the selection of genetically transformed cells in an untransformed background. Mesophilic, heterotrophic bacteria, transformed with plasmid vectors, are easily identified by selection for a newly acquired genetic marker. This may be in the form of antibiotic resistance, resistance to heavy metals or the conversion of an auxotrophic mutant strain to prototrophy. In obligately autotrophic acidophiles, however, the growth conditions and physiology of the organism renders these techniques of marker selection unsuitable.

The effect of low pH, high iron concentration, and other growth requirements of *T. ferrooxidans* need to be considered in the search for suitable potential markers. Although *T. ferrooxidans* is inherently resistant to elevated levels of some metal ions, other metal ions are highly toxic to this organism (Lundgren and Silver, 1980; Sugio *et al.*, 1985). Genes conferring resistance to toxic metal ions have

the potential to serve as genetic markers and, at the same time, to confer an industrially significant characteristic to the bacterium. Genes that confer resistance to metals such as mercury (Hg) and arsenic (As), known to be toxic to T. ferrooxidans, have been isolated from other organisms (Summers and Silver, 1978).

The high concentrations of metal ions and the low pH of T. ferrooxidans growth media renders many antibiotics unstable and unsuitable for use against T. ferrooxidans. In addition, the slow growth rate of T. ferrooxidans on solid media requires that the antibiotic remain active over an extended period. Rawlings et al. (1983) reported that, of a range of antibiotics tested in iron- (pH 2,00) and sulphur-based (tetrathionate) (pH 4,00) media, some antibiotics exerted an inhibitory effect on T. ferrooxidans only in the sulphur-based, higher pH medium. Whereas cells were resistant to 200 μg/ml of tetracycline (Tc) in an iron-based medium, these organisms were sensitive to 10 μg/ml of the same antibiotic in tetrathionate medium (pH 4.00). The findings of Davidson and Summers (1983) that chloramphenicol (Cm), kanamycin (Km), streptomycin (Sm) and Tc remain stable at pH 2,00 over a 4 d period supports the observation by Rawlings et al. (1983) that the antibiotic instability was due to iron in the medium. Use of these antibiotics in sulphur-based media may therefore be possible. However, it was found that T. ferrooxidans was inherently resistant to Km, nalidixic acid (Nal) and Sm (Rawlings et al., 1983; unpublished results).

Auxotrophic amino acid or vitamin requirements can also serve as useful markers for the development of a genetic system. Although the effects of mutagens such as ultraviolet (UV) light and N-methyl, N-nitro, N-nitrosoguanidine (NTG) on T. ferrooxidans strains have been investigated (Groudeva and Groudev, 1980), no auxotrophic mutant enrichment techniques for T. ferrooxidans were reported. Auxotrophic mutants would facilitate the development of a gene transfer system using homologous DNA in marker rescue experiments. This approach has proved successful in optimizing a gene transfer system in Thiobacillus thioparus (Yankofsky et al., 1983).

The isolation of NTG-induced mutants of T. ferrooxidans that lack rusticyanin, a protein involved in the electron transport chain for the oxidation of iron (Cox et al., 1986), indicates that conventional mutagenesis techniques could be used for inducing mutations in T. ferrooxidans. However, a method for the selection of auxotrophs is lacking.

1.6.2.2 Construction of T. ferrooxidans vectors. It is desirable to use a vector that has the ability to shuttle between the industrial host and well characterized strains such as E. coli, Bacillus subtilis or Pseudomonas sp. A single broad-host-range origin of replication or at least two specific origins of replication are therefore required. The use of homologous genetic material would favour the

replication and functioning of a plasmid vector in T. ferrooxidans.

The T. ferrooxidans plasmid, pTF-FC2, cloned into pBR325 (Rawlings and Woods, 1985), is associated with selectable Cm^r and Tc^r markers from pBR325 and contains a T. ferrooxidans-derived broad-host-range origin of replication and mobilization functions. The plasmid, therefore, forms the basis for a potentially useful shuttle vector for genetic manipulation studies in T. ferrooxidans.

1.6.3 Assessment of the current state of T. ferrooxidans genetics

Progress towards the understanding of the molecular biology of T. ferrooxidans has been made. Although no phenotype associated with T. ferrooxidans plasmids has been conclusively identified, the expression of T. ferrooxidans plasmid DNA replication and mobilization functions in heterotrophic bacteria has been demonstrated. The T. ferrooxidans glnA gene product has been shown to function in E. coli. Some of the control elements associated with the glnA gene of heterotrophs are present in the promoter region of the glnA gene of T. ferrooxidans, and one appears to be missing. The amino acid sequences deduced from the nifHDK nucleotide sequence were shown to be highly homologous to analogous proteins from several nitrogen-fixing bacteria. Conservation of sequences was greatest between T. ferrooxidans and the bradyrhizobia. In regard to

gene expression and control, all the elements required for the expression and control of the nifHDK genes in other nitrogen-fixing bacteria are readily identifiable in the nucleotide sequence of the promoter region in T. ferrooxidans.

The findings described above indicate that at least some of the signals for gene expression in T. ferrooxidans are shared with heterotrophic bacteria. However, characterization and sequencing of a wider range of T. ferrooxidans genes is necessary to give an insight into the patterns of preferred codon usage and the nature of promoter sequences in this organism. Of particular interest would be the study of genes not functionally related to the anabolic nitrogen-assimilation pathway. The nitrogen-metabolism genes and their regulatory sequences are strongly conserved in a wide range of organisms. Therefore, analysis of genes within a single anabolic group of functions may not provide an accurate representation of general gene structure, expression and regulation in the rest of the organism. A study of genes from other pathways would supplement currently available information to provide a greater understanding of the genetics of T. ferrooxidans. This information would add to current data to enable the more accurate identification of the most compatible donors with regard to DNA for use in T. ferrooxidans.

1.7 The present study

This dissertation is a report on:
(i) attempts to transfer recombinant plasmid DNA to T. ferrooxidans,
(ii) the construction of genomic libraries for use in the genetic analysis of T. ferrooxidans, including attempts to isolate and characterize a homologous marker for use in T. ferrooxidans,
(iii) the isolation and characterization of a gene from T. ferrooxidans, unrelated to the nitrogen-metabolism genes previously characterized
(iv) an investigation of the presence of a chromosomal recombination system in T. ferrooxidans.

Attempts at the conjugational transfer of plasmids to T. ferrooxidans are described in Chapter 2. Both the direct transfer from E. coli to T. ferrooxidans and a multistage transfer system using non-iron-oxidizing thiobacilli as intermediates, were investigated.

Chapter 3 is a record of the construction of three separate libraries of the T. ferrooxidans genome. The first library consisted of small inserts (3-12 kb) cloned into a positive selection vector. This library was essentially for the isolation of a recA-like gene from a wild-type T. ferrooxidans strain.

In an effort to isolate a reliable homologous marker for use in T. ferrooxidans a rifampicin-resistant (Rif^r) mutant of the organism was isolated. The construction of a small-insert plasmid library, as well as a large-insert (35-55 kb) cosmid library, using chromosomal DNA from the T. ferrooxidans Rif^r mutant, is described in Chapter 3.

The isolation and characterization of a recA-like gene from T. ferrooxidans is described in Chapter 4. The RecA protein is an essential component of DNA repair and homologous genetic recombination mechanisms in E. coli. If a functional recA gene was found to be present in T. ferrooxidans, it would indicate that T. ferrooxidans is capable of homologous genetic recombination and the genetic processes associated with it. The following notations will be adhered to in this dissertation: recA for the recA gene, and RecA for the recA gene product, the RecA protein.

In Chapter 5, the nucleotide sequence of the T. ferrooxidans recA gene and the amino acid sequence of the deduced protein is reported. Expression of the cloned recA gene in E. coli was studied and the deduced amino acid sequence of the T. ferrooxidans recA gene was compared with those of E. coli and P. aeruginosa.

CHAPTER 2

STUDIES ON THE CONJUGAL TRANSFER OF DNA TO *T. ferrooxidans*

CHAPTER 2

STUDIES ON THE CONJUGAL TRANSFER OF DNA TO T. ferrooxidans

Summary. Conjugation was attempted as a means for plasmid DNA transfer to T. ferrooxidans. Recombinant T. ferrooxidans plasmids, pDER401 and pDER405, were shown to code for mobilization and replication functions in E. coli and T. novellus strains. The plasmids were mobilizable at high frequency by the IncP plasmid, R68.45. Attempts to transfer the T. ferrooxidans recombinant plasmids directly from E. coli to T. ferrooxidans were unsuccessful. In multi-stage mating experiments, plasmid DNA was transferred from E. coli to T. novellus, and from T. novellus to T. intermedius. However, in subsequent matings, plasmid transfer from these thiobacilli to T. ferrooxidans could not be shown.

2.1 INTRODUCTION

To enhance the productivity of bacteria with recombinant-DNA techniques, it is essential that a method be developed for introducing desirable genetic information into these organisms. The three commonly used methods for achieving this are transduction, transformation and conjugation.

2.1.1 Transduction

Transduction involves the transfer of bacterial DNA from a donor cell to a recipient by a bacteriophage capsid (Hayes, 1968). Specific bacteriophages have been engineered and are currently used as vectors to transfer in vitro-constructed recombinant DNA molecules into their host bacteria (Hohn,

1979). Because no virus specific for T. ferrooxidans has been reported to date, transduction as a means for delivering DNA is not available for use in this organism.

2.1.2 Transformation

Transformation is a process whereby cells assimilate naked DNA from their surroundings and functionally integrate such exogenous DNA into their genetic material (Smith et al., 1981; Saunders et al., 1984). Transformation is applicable only to a restricted range of bacteria and the physiochemical conditions required for such DNA transfers may vary considerably from organism to organism.

Transformation systems may be divided into two categories, natural and artificial (Saunders et al., 1984). Naturally-transformable bacteria such as Streptococcus pneumoniae, B. subtilis, Haemophilus sp. and Neisseria gonorrhoeae have specialized means of assimilating DNA (Smith et al., 1981; Stewart and Carlson, 1986). The mechanism for DNA uptake and integration in these organisms is a genetically-encoded trait. Genetically-determined transformation mechanisms are usually selective for the uptake of specific topological forms of DNA. In some cases, recognition by the cell of a particular sequence on the transforming DNA is necessary (Sisco and Smith, 1979; Saunders et al., 1984; Stewart and Carlson, 1986).

Although a well developed, genetically-determined DNA uptake and integration mechanism does exist in some organisms , most bacterial species are not naturally competent. These organisms must be rendered permeable to DNA by artificial means. Mandel and Higa (1970) found that phage DNA, in the presence of Ca^{2+}, could transform E. coli. Cohen et al. (1972) subsequently showed that under these conditions E. coli cells became effective recipients for plasmid DNA. Artificial transformation is not a genetically-encoded mechanism. Uptake by the cell does not depend on the topological form of the invading DNA nor on the recognition of specific sequences. However, the efficiency of successful transformation by different topological forms may vary (Saunders et al., 1984). This may be due to the susceptibility of the transforming DNA form to the host cell's restriction mechanisms. In addition, establishment of the DNA in the cell may be dependent on whether it is a replicon.

Other techniques have been used with cells that prove refractory to natural and divalent ion-induced transformation. Freeze-thaw procedures involve treatment of the target cells with cycles of freezing (-70^0C or -196^0C) and thawing (at either 37^0 or 42^0C) in the presence of DNA (Saunders et al., 1984). Another method for inducing cells to take up DNA makes use of protoplasts and spheroplasts (Hopwood, 1981; Saunders et al., 1984). Spheroplasts and protoplasts may be induced to absorb DNA in the presence of divalent cations and polyethylene glycol (PEG). An

additional method for the transformation of cells involves the use of artificial lipid vesicles, termed liposomes, as vehicles for the delivery of DNA into cells (Fraley et al., 1979; Nicolau et al., 1983).

A relatively new methodology for the transfer of DNA into bacteria is electroporation (Calvin and Hanawalt, 1988). The technique uses controlled high voltage electric impulses discharged through a suspension of cells. The electric impulses induce transient pores in the cell membrane through which DNA, in the medium, enters the cell. Electroporation has found application in the transformation and transfection of many types of eukaryotic cells (Potter et al., 1984; Shillito et al., 1985; Knutsen and Yee, 1987). Prokaryotes successfully transformed by electroporation include Lactobacillus casei (Chassy and Flickinger, 1987), Streptomyces lividans (MacNeil, 1987), Campylobacter jejuni (Miller et al., 1988), and E. coli (Calvin and Hanawalt, 1988)

2.1.2.1 Transformation of thiobacilli. No plasmid or chromosomal DNA transformation systems have been developed in T. ferrooxidans. Rudimentary transformation systems have been reported for non-iron-oxidizing thiobacilli. Yankofsky et al. (1983) reported the transformation of auxotrophic strains of T. thioparus cells with chromosomal DNA. These workers showed that T. thioparus could be successfully transformed only with homologous DNA. Optimum competence in T. thioparus was exhibited during the early logarithmic

phase of growth and was enhanced in the presence of an energy substrate. This is in contrast with transformation mechanisms of other naturally-transformable Gram-negative species in which competence is induced through nutrient depletion or nitrogen starvation (Smith et al., 1981; Stewart and Carlson, 1986).

Attempts to transform whole cells of various T. ferrooxidans strains with recombinant T. ferrooxidans plasmids, using established techniques, have been unsuccessful (M.E.C. Barros, Ph.D Thesis, University of Cape Town, 1985). Plasmid pDER401, used in these studies, is a recombinant consisting of a T. ferrooxidans plasmid, pTF-FC2, ligated to a fragment of pBR325 (Rawlings and Woods, 1985). Barros, using radiolabelled pDER401, showed that 11% of added DNA was irreversibly bound by cells, even though no transformants could be detected on selective media subsequently. Attempts to transform spheroplasts of T. ferrooxidans with plasmids were also unsuccessful, despite a high frequency of spheroplasting (of greater than 90%), and a regeneration frequency of about 75%.

Several possible explanations for the lack of transformation were given. One possibility was that once inside the cell the DNA was unable to replicate. A lack of expression of marker genes was also suggested. Barros further suggested that after entry into T. ferrooxidans, the heterologous pBR325 DNA fragment in the recombinant plasmid may have been

recognized as foreign and subjected to degradation by the cell's restriction system.

The present author suggests that the homologous fragment of T. ferrooxidans plasmid DNA on the recombinant plasmid, pDER401, is also likely to be recognized as foreign once inside T. ferrooxidans. It is likely that the recombinant plasmid, which had been propagated in E. coli, would have a modification pattern compatible with its existence in E. coli. Methylases place methyl groups only on appropriate bases within the recognition sites of their restriction endonucleases rather than elsewhere on the DNA within an organism (Watson et al., 1987). Thus, DNA propagated in an organism is protected only from cutting by its own restriction enzymes, and not from cutting by enzymes that bind to different recognition sites. The protective modification that may have occurred in E. coli would, therefore, not protect even the homologous plasmid DNA from degradation in T. ferrooxidans.

2.1.3 Conjugation

Conjugation is a mechanism of DNA transfer from one cell to another that requires cell to cell contact (Willetts, 1984). DNA moves from the donor to the recipient cell by replicative transfer. Since conjugation is essentially a replication process, it allows plasmids to replicate more frequently than chromosomal genes.

Conjugal transfer is an intricate genetic process invariably controlled by genes located on plasmids (Willetts and Wilkins, 1984). In the conjugative plasmid F, this is equivalent to 33 kb and involves 25 or more genes (Low and Porter, 1978; Willetts, 1984).

Plasmids are classified on the basis of mating type and replication origin, with a notable correlation between the incompatibility group using either criterion (Stanisch, 1984). This relationship probably reflects coevolution of replication and conjugation systems since these function independently of each other.

The conjugative process occurs within Gram-positive and Gram-negative bacteria and can be divided into two stages. The first is the recognition of recipient cells by donor cells, leading to mating-pair formation, and the second is the physical transfer of plasmid DNA (Low and Porter, 1978). Plasmids specify a surface or entry exclusion system that prevents the unproductive transfer of the plasmid to a cell that already possesses a copy (Willetts, 1984).

In Gram-negative bacteria, recognition of recipient cells by donors and the formation of mating pairs depends on the synthesis of pili (Ou and Anderson, 1970; Willetts, 1984). In addition, pili, encoded by the conjugative plasmids, provide the site for adsorption of certain bacteriophages which are plasmid- or pilus-specific (Willetts, 1984). Since pili and the specific phages they adsorb vary between

complementary DNA synthesis in the donor cell may aid linear ssDNA movement to the recipient cell.

Circularization of the transferred plasmid DNA in the recipient cell is thought to result from the covalent link of the 5′ terminus at oriT to a membrane protein which recognizes the 3′ terminus once DNA transfer is complete (Willetts and Wilkins, 1984). Once in this state the plasmid can replicate in the recipient and become part of the hereditary information of that cell. Some small, naturally occurring, non-conjugative plasmids possess an oriT and mobilization (mob) genes that enable them to be transferred by the conjugation system of a coexisting conjugative or self-transmissible plasmid (Willetts, 1984).

An advantage of the conjugation system for plasmid transfer between cells is that it circumvents the double-stranded DNA (dsDNA)-dependent restriction system of the new host organism. Donor DNA is transferred to the recipient in the single-stranded form. The transferred strand is therefore not susceptible to enzymes that recognize sequences on dsDNA (Watson et al., 1987). The single strand may be modified by methylation within the recipient cell, or alternatively, modification of the newly-synthesized complementary strand may occur.

2.1.3.1 Conjugation studies in the thiobacilli. Using conjugation systems, Davidson and Summers (1983) investigated the transfer of the broad-host-range plasmids, S-a (IncW, and which is similar or identical to pSa), RP4 (IncP), R300b (IncQ), and their derivatives between E. coli and non-iron-oxidizing thiobacilli. These workers demonstrated conjugative plasmid transfer from E. coli to Thiobacillus novellus at a low frequency. Plasmids were not transferred from E. coli to any of the other thiobacilli tested. When T. novellus was used as donor, plasmid transfer to Thiobacillus intermedius, Thiobacillus perometabolis, Thiobacillus neapolitanus and Thiobacillus acidophilus was observed.

Similarly, Kulpa et al. (1983) showed transfer of the broad-host-range plasmid, RP1 (which is similar or identical to RP4), from E. coli to T. neapolitanus at frequencies comparable to those of other heterogeneric conjugation systems. RP1 was subsequently transferred from T. neapolitanus to E. coli, showing that the transfer functions of the plasmid were expressed in T. neapolitanus. Antibiotic-resistance marker genes on RP1 were also expressed in T. neapolitanus.

Rawlings and Woods (1985) investigated the conjugative properties of T. ferrooxidans plasmids which were ligated to pBR325. These workers showed that recombinants containing the T. ferrooxidans plasmid, pTF-FC2, were mobilized between E. coli strains. In E. coli, the recombinant pTF-FC2 -

pBR325 plasmid was mobilized at a high frequency by the IncP plasmid, RP4, and at a low frequency by the IncN plasmid, R46, but not by the IncW plasmid, pSa. The mobilization region on pTF-FC2 was identified.

2.1.4 The present study

The object of the present investigation was to transfer the pTF-FC2 - pBR325 recombinant plasmids, pDER401 (Rawlings and Woods, 1985) and pDER405, into T. ferrooxidans by conjugation. Plasmids pDER401 and pDER405 are identical except for a deletion in pDER405 that has removed the pBR325 origin of replication (D.E.Rawlings, personal communication; refer to Appendix C for plasmid maps). The deletion plasmid is still able to replicate in E. coli from an origin of replication on the T. ferrooxidans plasmid. Both plasmids carry an origin of replication from the T. ferrooxidans plasmid, as well as the Tc^r and Cm^r genes of pBR325.

T. ferrooxidans FD1 was used as a potential recipient of plasmid DNA for the following reasons.

(i) This strain was reported to be mixotrophic and is able to utilize glucose at a low level in the presence of iron (Barros et al., 1984).

(ii) It grows well on agar-containing solid media.

(iii) Direct conjugation between T. ferrooxidans and heterotrophic bacteria requires a composite medium that will support the growth of donor and recipient organisms. However, T. ferrooxidans strains are normally inhibited by

organic compounds (Tuovinen and Kelly, 1973). *T. ferrooxidans* FD1, which is less affected by organic compounds, may be more tolerant of composite mating media.

An important requirement for the conjugative transfer of DNA between bacteria is the optimization of a suitable medium on which both donor and recipient cells are able to grow well enough to mate. Curtiss (1969) reported that starvation of donor cells causes a progressive loss of donor pili which are required for initial mating-pair formation. *T. ferrooxidans* is an acidophilic autotroph and has very different growth conditions and requirements from the heterotrophic, neutral pH-requiring bacteria in which genetic engineering work is carried out. It may therefore not be possible to mate bacteria such as *E. coli* and *T. ferrooxidans* directly. An approach to solving this problem is to transfer DNA from heterotrophic bacteria to *T. ferrooxidans* in two or three stages using non-iron-oxidizing thiobacilli as transit hosts. Some non-iron-oxidizing thiobacilli grow at neutral or low pH, either in the presence of organic matter or autotrophically (Kuenen and Tuovinen, 1981).

A schematic representation of the conjugation strategy used in this study is given in Fig. 2.1. In the first stage of the multistage mating exercise, mating between *E. coli* and *T. novellus* would be carried out on an organic medium at neutral pH. In the second stage conjugation between *T. novellus* and another facultative "intermediate"

Thiobacillus sp. would be carried out on an organic medium at near neutral pH. The third stage of the multistage mating exercise would be between the facultative Thiobacillus sp. and T. ferrooxidans, carried out at low pH and in the absence of organic matter. Apart from the multistage mating study, direct matings between either E. coli or T. novellus donors and T. ferrooxidans were also attempted on a range of composite media.

The present investigation involved the use of the large self-transmissible IncP plasmid, R68.45, in conjunction with the recombinant T. ferrooxidans plasmids, pDER401 and pDER405, in biparental and triparental matings. As described earlier, the self-transmissible plasmid provides the mechanism necessary for the activation of transfer functions on the recombinant plasmids. For a plasmid such as pDER401 or pDER405 to be useful in the multistage mating process, it is essential that it has the ability to replicate and that its mobilization functions are expressed in T. novellus and in the intermediate Thiobacillus sp. hosts.

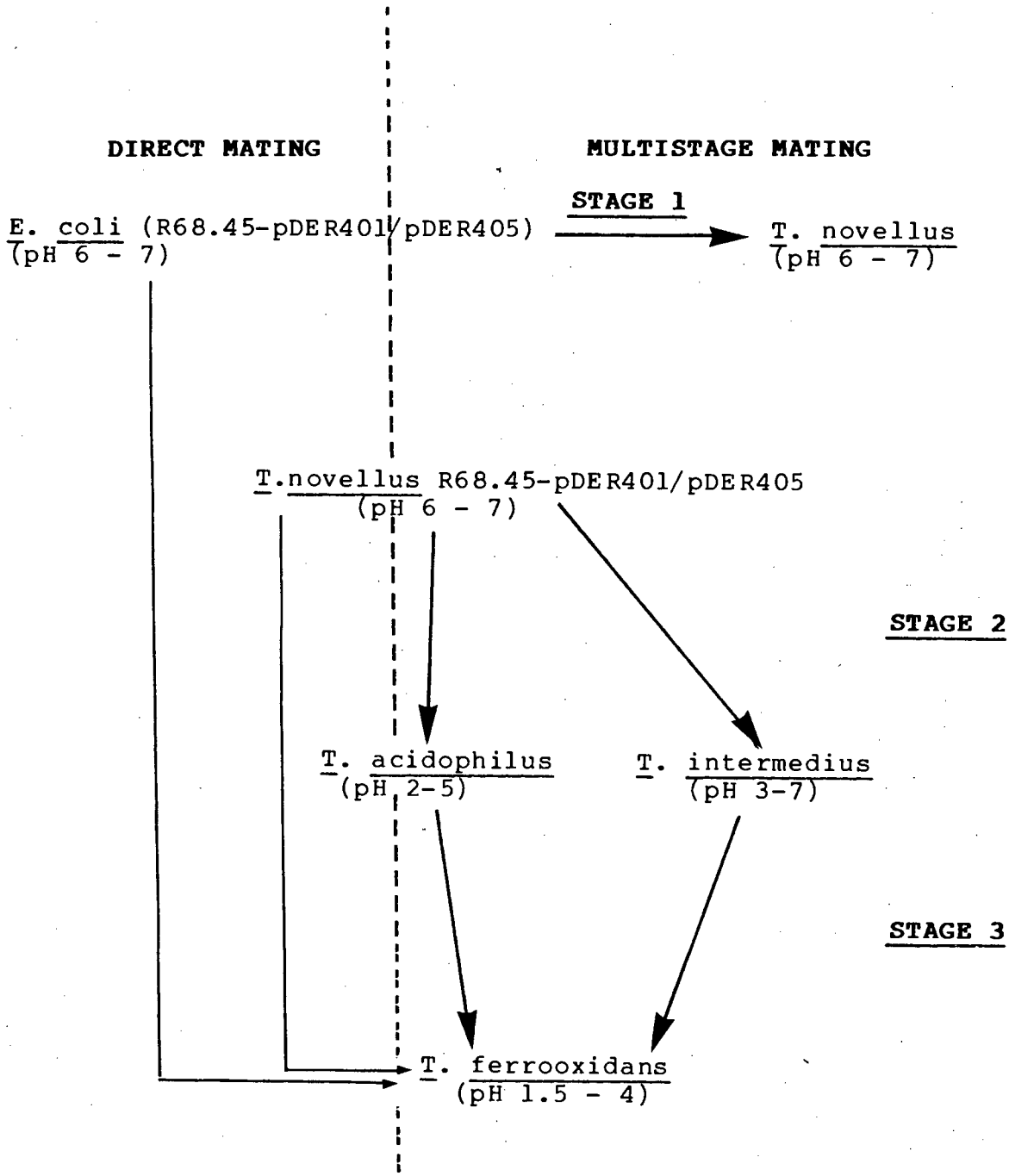

Fig. 2.1 Schematic illustration of the mating strategy adopted in this study. The pH range for the growth of each organism is indicated (Kuenen and Tuovinen, 1981).

2.2 MATERIALS AND METHODS

2.2.1. Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 2.1

2.2.2 Media

Details of media used are provided in Appendix B. E. coli was grown in Luria-Bertani (LB) medium, while T. novellus was grown in either TMB or TMYE (Davidson and Summers, 1983). T. intermedius and T. acidophilus were grown heterotrophically in either TMB or 9KD medium (Davidson and Summers, 1983). T. ferrooxidans was routinely grown in ferrous iron (9K) medium (Silverman and Lundgren, 1959), and platings were on tetrathionate agar (TA) medium (Tuovinen and Kelly, 1974). Antibiotics were added to the media at the following concentrations: Ap, 100 μg/ml; Cm, 20 μg/ml; Km, 25 μg/ml; Nal, 25 μg/ml; Tc, 20 μg/ml.

2.2.3 Conjugation

2.2.3.1 Technique. Cells were harvested from exponentially growing cultures by centrifugation, washed twice and resuspended in Ringer's solution (Appendix B) at approximately 10^8 cells/ml. Mating strains were mixed at a donor to recipient ratio of 1:1 for biparental crosses and 1:1:1 for triparental crosses. Mating mixtures were

Table 2.1 List of bacteria, plasmids and their relevant phenotypes and genotypes

Strain or plasmid	relevant phenotype or genotype	Source or reference
E. coli strains		
HB101	rpsL leu pro thi hsd hsm lacY recA	Maniatis et al. (1982)
ED8654	Nal^s	N. Willetts[1]
CSH56	Nal^r	Cold Spring Harbor Laboratory[2]
Thiobacillus strains		
Tn100 (T. novellus)	Nal^r	ATCC 8093
TnMD101 (T. novellus)	Nal^r Sm^r/Km^r bio^-	M. Davidson[3]
Ti200 (T. intermedius)	wt	ATCC 15466
Ta300 (T. acidophilus)	wt	ATCC 27807
TF FD1 (T. ferrooxidans)	wt	Gencor[4]
Plasmids		
pBR325	Cm^r Tc^r	Bolivar (1977)
R68.45	Ap^r Km^r Tc^r IncP Tra^+	P. Phibbs[5]
pDER401	Cm^r Tc^r	Rawlings et al. (1984b)
pDER405	Cm^r Tc^r	D.E.Rawlings[6]

[1] N. Willetts: Biotechnology Australia Pty. Ltd., Roseville, New South Wales, Australia
[2] Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, U.S.A.
[3] M. Davidson: University of Georgia, Athens, Georgia, U.S.A.
[4] Gencor : Gencor Group Laboratories, Johannesburg, South Africa
[5] P. Phibbs: Commonwealth University, Richmond, Virginia, U.S.A.
[6] D.E. Rawlings: University of Cape Town, Cape Town, South Africa

filtered onto nitrocellulose filters (pore size, 0,45 μm; diameter, 13 mm) (Millipore Corp., Bedford, Massachusetts, U.S.A.), held in a Swinny adaptor. Filters were placed on the appropriate mating media and incubated for the required time. Filters were transferred to standard containers with 2 ml sterile Ringer's solution (Appendix B) and vortexed. The cell suspension was diluted and plated onto specific selective media for enumeration of transconjugants, donors and recipients. Where transconjugants were expected at a low frequency, the cell suspension was plated directly onto selective media.

2.2.3.2. Multistage mating. Mating conditions, organisms and media used are indicated in Tables 2.2, 2.3, 2.4, 2.5 and 2.6. The strategy of the conjugation exercise was as follows: E. coli donor cells carrying R68.45 and either pDER401 or pDER405 were mated with recipient E. coli cells on LA medium to determine the transconjugation frequency of the self-transmissible and recombinant plasmids. Matings between E. coli donors and T. novellus recipients were attempted in the first stage of the proposed multistage mating strategy. In the second stage of the exercise, transfer of R68.45 and either pDER401 or pDER405 from T. novellus to both T. intermedius and T. acidophilus was attempted. To determine their potential to transfer newly acquired plasmids, transconjugants were mated with differently marked T. novellus and E. coli recipient strains. Only if plasmids were shown to be transferred out

of the non-iron-oxidizing thiobacilli, were these organisms used in the third-stage matings with T. ferrooxidans.

2.2.3.3 Evaluation of media for direct matings. To determine the effect of the medium composition on the ability of E. coli to mate directly with T. ferrooxidans, E. coli strains were mated on composite media containing TA medium and LA medium in different ratios. The efficiency of plasmid transfer between two E. coli strains on these media would indicate the effect of the medium composition on the mating performance of E. coli donors. Similarly, the effect of mating medium composition on the ability of T. novellus to act as a donor in direct matings with T. ferrooxidans was assessed. Donor and recipient T. novellus strains were mated on a range of composite media, containing different ratios of TMB and TA medium.

2.2.4 Phenotype verification

Phenotypes of putative transconjugant colonies were examined by subculturing on selective media containing more than one antibiotic, where possible. A number of individual transconjugants from each new mating were selected and the presence of the plasmid confirmed using the miniprep method of plasmid extraction (Appendix A.1.1) and agarose gel electrophoresis (Appendix A.3). Putative T. ferrooxidans transconjugants were investigated for plasmids using Southern hybridization (Appendix A.7). To confirm the presence of either pDER401 or pDER405, pBR325 was

radiolabelled (Appendix A.7.1) and used as a probe (Appendix A.7.2). Since the T. ferrooxidans recipient could conceivably contain a pTF-FC2-like resident plasmid, probing with either pDER401 or pDER405 directly could provide ambiguous results. Homology between the pBR325 probe and the pBR325 fragment on pDER401 or pDER405 would be sufficient to detect the presence of either recombinant plasmid in T. ferrooxidans.

2.3 RESULTS

2.3.1 Mobilization of pDER401 and pDER405 between E. coli strains

Self mobilization of the recombinant T. ferrooxidans plasmids, pDER401 and pDER405, between E. coli strains was investigated. Neither plasmid was able to transfer itself from a Nal-sensitive (Nal^S) strain to a Nal^r strain (Table 2.2; matings 1 and 2).

Plasmid R68.45 did not mobilize pBR325 (Table 2.2; mating 5), but was able to mobilize pDER401 and pDER405 between E. coli strains at high frequencies (Table 2.2; matings 6 and 7). These results indicated that the pTF-FC2 region on plasmids pDER401 and pDER405 was involved in mobilization.

Table 2.2 Conjugal transfer of non-conjugative plasmids between strains of E. coli. Donor E. coli ED8654 was mated[1] with recipient E. coli CSH56 (Nal^r)

Mating number	Plasmid/s in donor	Transconjugation frequency of non-conjugative plasmids
1	pDER401	$< 2.3 \times 10^{-8}$
2	pDER405	$< 2.3 \times 10^{-8}$
3	pBR325	$< 2.3 \times 10^{-8}$
4	R68.45	$< 2.9 \times 10^{-8}$
5	R68.45-pBR325	$< 2.5 \times 10^{-8}$
6	R68.45-pDER401	4.3×10^{-1}
7	R68.45-pDER405	3.1×10^{-1}

[1] Matings were carried out on LA plates for 7 h, and selection for transconjugants carrying the non-conjugative plasmids (i.e. pBR325, pDER401, or pDER405) was on LA plates containing Nal and Cm.

These experiments confirmed earlier findings in this laboratory (Rawlings and Woods, 1985).

2.3.2 Transfer of plasmids from E. coli to Thiobacillus sp.

The findings of the multistage and direct mating study, described hereafter, are schematically summarized in Fig. 2.2.

2.3.2.1 STAGE 1: Transfer of plasmids pDER401 and pDER405 from E. coli to T. novellus. A triparental mating between E. coli ED8654, containing pDER401 (Cm^r, Tc^r), E. coli HB101 containing the conjugative plasmid, R68.45 (Ap^r ,Km^r, Tc^r), and T. novellus was carried out on LA medium. As E. coli is unable to grow on the T. novellus TMB medium, and because Ap^r is not expressed in T. novellus (Davidson and Summers, 1983), post-mating mixtures were plated onto TMB plates containing Km and Cm. Tc was not used as a selective agent, since the Tc^r marker was present on both pDER401 and R68.45. A Tc^r phenotype would therefore not be indicative of the presence of individual plasmids within the cells. T. novellus colonies, resistant to both Km and Cm, were isolated at a frequency of 4.5×10^{-6} transconjugants per recipient (Table 2.3, mating 8). Similar results were obtained using pDER405 in place of pDER401 (Table 2.3, mating 9). Since pDER405 has the origin of replication of pBR325 deleted, this demonstrated the ability of the T. ferrooxidans plasmid, pTF-FC2, to

replicate in T. novellus. Both plasmids, pDER401 and pDER405, could be isolated from T. novellus transconjugants.

2.3.2.2 Mobilization of the recombinant plasmids between T. novellus strains. In a biparental mating between T. novellus (R68.45) (Ap^r, Km^r, Tc^r) and T. novellus MD101 (Sm^r), colonies capable of growth on TMYE containing Sm and Tc were isolated at a frequency of 1.4×10^{-3} transconjugants per recipient (Table 2.3, mating 10). In a triparental mating between T. novellus (R68.45), T. novellus (pDER401) and T. novellus MD101, colonies able to grow on TMYE, containing Km, Cm and Sm, were isolated at a frequency of 8.7×10^{-6} transconjugants per recipient (Table 2.3, mating 11). Reisolation of plasmids from these colonies confirmed the presence of pDER401. Similar results using pDER405 in place of pDER401 were obtained (Table 2.3, mating 12). These results demonstrated the expression in T. novellus of mobilization functions located on the T. ferrooxidans plasmid, pTF-FC2. Plasmids pDER401 and pDER405, containing the mobilization region, were able to be conjugated from one T. novellus strain to another. Biparental matings involving a T. novellus donor strain, containing R68.45 and either pDER401 or pDER405, mated with a recipient T. novellus strain, were shown to be more efficient than triparental matings, where each plasmid was present in a different donor strain (Table 2.3, matings 11 and 13, matings 12 and 14).

TABLE 2.3 SUMMARIZED CONDITIONS AND RESULTS OF STAGE 1 MATINGS BETWEEN HETEROTROPHIC ORGANISMS

MATING NUMBER	DONOR/S [x]	RECIPIENT [x]	MATING MEDIUM	MATING PERIOD	POST-MATING SELECTION MEDIUM	TRANSCONJUGATION FREQUENCY
8	Ec ED8654 (pDER401) Ec HB101 (R68.45)	Tn 8093	LA	24 H	TMB Cm Km	4.5×10^{-6}
9	Ec ED8654 (pDER405) Ec HB101 (R68.45)	Tn 8093	LA	24 H	TMB Cm Km	4.1×10^{-6}
10	Tn 8093 (R68.45)	Tn MD101	TMYE	40 H	TMYE Tc Sm	1.4×10^{-3}
11	Tn 8093 (pDER401) Tn 8093 (R68.45)	Tn MD101	TMYE	40 H	TMYE Cm Km Sm	8.7×10^{-6}
12	Tn 8093 (pDER405) Tn 8093 (R68.45)	Tn MD101	TMYE	40 H	TMYE Cm Km Sm	8.1×10^{-6}
13	Tn 8093 (pDER401 R68.45)	Tn MD101	TMYE	40 H	TMYE Cm Km Sm	6.5×10^{-5}
14	Tn 8093 (pDER405 R68.45)	Tn MD101	TMYE	40 H	TMYE Cm Km Sm	4.4×10^{-5}
15	Tn 8093 (pDER401 R68.45	Ec CSH56	LA	24 H	LA Ap Cm Nal	2.1×10^{-7}
16	Tn 8093 (pDER405 R68.45	Ec CSH56	LA	24 H	LA Ap Cm Nal	0.8×10^{-7}

[x] GENERIC CODES USED:
Ec = E. COLI
Tn = T. NOVELLUS

The transconjugation frequency of the self-transmissible and mobilizable plasmids was higher between E. coli strains than for intergeneric transfers, or transfer between T. novellus strains. However, once transferred to T. novellus, R68.45, pDER401 and pDER405 were stably maintained under antibiotic marker selection. All three plasmids were transferred between T. novellus strains at a relatively high frequency. However, transfer from T. novellus to E. coli was less efficient (Table 2.3, matings 15 and 16).

2.3.2.3 Transfer of recombinant plasmids from E. coli to other thiobacilli. Attempts to conjugate either R68.45, pDER401 or pDER405 from E. coli donors directly to T. intermedius and T. acidophilus proved unsuccessful.

2.3.3. STAGE 2: Transfer of plasmids from T. novellus to "intermediate" thiobacilli. Matings between T. novellus and T. acidophilus were unsuccessful (Table 2.4, matings 17 and 18). However, the low frequency conjugative transfer of R68.45 and pDER401 from T. novellus to T. intermedius was demonstrated (Table 2.4, mating 19). Plasmid pDER405 was not transferred in similar crosses (Table 2.4, mating 20). These last findings were not pursued.

In order to establish whether T. intermedius might serve as a suitable donor, matings between T. intermedius (R68.45-pDER401) and E. coli, and between T. intermedius (R68.45-pDER401) and T. novellus were carried out. The self-transmissible plasmid was transferred at a low frequency to

T. novellus (Table 2.4, mating 21, transconjugation frequency (a)), but not to E. coli. Plasmid pDER401 was not transferred to either recipient (Table 2.4, mating 21, transconjugation frequency (b)). Although the presence of pDER401 was detected in T. intermedius recipients after conjugation with T. novellus (R68.45-pDER401), the plasmid was lost during repeated subculture. However, plasmid R68.45 was maintained in this organism in the presence of Tc.

2.3.4 STAGE 3: Transfer of plasmids from T. intermedius to T. ferrooxidans. Conjugation between T. intermedius (R68.45-pDER401) and T. ferrooxidans was carried out on TA medium (Table 2.4, mating 22) since both organisms grew on this medium. Since T. ferrooxidans was insensitive to Tc in 9K iron medium, selection for T. ferrooxidans transconjugants could not be carried out on 9K plates containing Tc. TA medium containing Tc was used for plating of diluted conjugation mixtures, followed by a replica-plating step on 9K plates. In this way T. ferrooxidans colonies could be distinguished from among the donor T. intermedius (R68.45-pDER401) Tc^r background colonies. T. ferrooxidans colonies were expected to oxidize ferrous iron in the 9K solid medium, and these would then be correlated to their position on the TAM plate containing Tc. However, no iron-oxidizing colonies were isolated from the TAM plates containing Tc.

TABLE 2.4 SUMMARIZED CONDITIONS AND RESULTS OF STAGE 2 MATINGS BETWEEN HETEROTROPHIC DONORS AND FACULTATIVELY AUTOTROPHIC RECIPIENTS; STAGE 3 MATINGS WITH T. FERROOXIDANS; AND DIRECT MATINGS BETWEEN T. FERROOXIDANS AND EITHER E. COLI OR T. NOVELLUS.

MATING NUMBER	DONOR/S[X]	RECIPIENTS[X]	MATING MEDIUM	MATING PERIOD	POST-MATING SELECTION MEDIUM	TRANSCONJUGATION FREQUENCY
17	TN MD101 (pDER401 R68,45)	TA 300	TMB	40 H	9KD Ap Cm	$< 3.2 \times 10^{-9}$
18	TN MD101 (pDER405 R68,45)	TA 300	TMB	40 H	9KD Ap Cm	$< 3.2 \times 10^{-9}$
19	TN MD101 (pDER401 R68,45)	TI 200	TMB	40 H	9KD Ap Cm	6.3×10^{-8}
20	TN MD101 (pDER405 R68,45)	TI 200	TMB	40 H	9KD Ap Cm	$< 3.5 \times 10^{-9}$
21	TI 200 (pDER401 R68,45)	TN MD101	TMB	40 H	TMYE Km Sm AND TMYE Ap Cm Sm	(A) 1.56×10^{-8} (B) $< 3.5 \times 10^{-9}$
22	TI 200 (R6845 (pDER401)	TF FD1	TA	8 D	9K/ TAM (Tc)	$< 5.5 \times 10^{-9}$
23	EC (pDER401 R68,45)	TF FD1	x% LA[Y]: x% TAM	8 D	TAM Ap Cm Tc	$< 5.5 \times 10^{-9}$
24	EC (pDER405 R68,45)	TF FD1	x% LA: x% TAM	5 D	TAM Tc	$< 5.5 \times 10^{-9}$
25	-----	TF FD1	TAM		TAM Tc	2.4×10^{-9}
26	TN MD101 (pDER401 R68,45)	TF FD1	x%TMB[Z]: x%TAM	5 D	TAM Tc	2.4×10^{-9}
27	TN MD101 (pDER405 R68,45)	TF FD1	x%TMB: x%TAM	5 D	TAM Tc	2.5×10^{-9}

[X] GENERIC CODES USED:
EC = E. COLI
TN = T. NOVELLUS
TA = T. ACIDOPHILUS
TI = T. INTERMEDIUS
TF FD1 = T. FERROOXIDANS

[Y] INDICATES MATING ON A RANGE OF LA:TA COMPOSITE MEDIA AND THE TRANSCONJUGANTION FREQUENCY IS THE AVERAGE FOR THE ENTIRE RANGE OF MEDIA

[Z] INDICATES MATING ON A RANGE OF TMB:TA COMPOSITE MEDIA. THE TRANSCONJUGATION FREQUENCY INDICATED IS AS FOR [Y]

In the control, T. ferrooxidans was shown to grow on 9K plates after growth on TAM plates. Known T. ferrooxidans colonies on TAM plates were subcultured onto 9K plates. After 5 d, iron oxidation resulted in a zone of brown ferric iron deposit around the colonies.

2.3.5 Direct matings between heterotrophs and T. ferrooxidans.

2.3.5.1 Evaluation of media for direct mating of E. coli with T. ferrooxidans. The effect of mating medium composition on the efficiency of plasmid conjugation between E. coli strains is shown in Table 2.5.

Although E. coli cells were able to grow on all media except the totally inorganic 100% TA medium (pH 4,00), the mating efficiency decreased markedly as the medium was changed towards that more favourable to T. ferrooxidans. Although T. ferrooxidans cells remained viable on all the media investigated, they were unable to grow on any of the mating media except 100% TA medium. There was an approximately 10-fold reduction in growth of E. coli on 100% LA medium compared with 10% LA:90% TA media, but a 9 X 10^3-fold decrease in conjugation efficiency. No marker transfer was evident between E. coli strains that were mated on 100% TA medium. Whether it was the low pH or the lack of suitable nutrients that was responsible for the drop in mating efficiency was not determined.

Table 2.5 Effect of medium composition on the mating frequency between E. coli strains. Donor E. coli ED8654 (R68.45-pDER401) was mated with E. coli CSH56 (Nal^r)[1].

Mating medium %LA : %TAM	pH	Mating frequency: Transconjugants per recipient
100 : 50	6.9	7.6×10^{-1}
50 : 50	4.4	5.4×10^{-1}
40 : 60	4.3	4.8×10^{-2}
30 : 70	4.2	5.1×10^{-3}
20 : 80	4.1	6.3×10^{-4}
10 : 90	4.1	8.3×10^{-6}
0 : 100	4.0	$<3.2 \times 10^{-9}$

[1] mating was for 7 h and transconjugants were selected on LA plates containing Ap and Cm

2.3.5.2 Direct matings with E. coli. Attempts to mate E. coli (R68.45-pDER401), or E. coli (R68.45-pDER405) with T. ferrooxidans on the range of media described in section 2.3.5.1, produced no Tc^r colonies on selective media (Table 2.4, matings 23 and 24). However Tc^r colonies appeared at a frequency of 2.4×10^{-9} on plates spread with unconjugated T. ferrooxidans cells (Table 2.4, mating 25).

2.3.5.3 Evaluation of media for the direct mating of T. novellus with T. ferrooxidans. Mobilization of pDER401 between T. novellus strains on a range of composite TMB:TA media, containing different ratios of TMB and TA media is shown in Table 2.6. The frequency of mobilization decreased as the proportion of TA medium increased. There was an approximately 15-fold reduction in growth of T. novellus on TMB medium compared with 10% TMB:90%TAM medium. No plasmid transfer was detected on either 20%TMB:80%TAM, 10%TMB:90%TAM or 100% TA medium.

Table 2.6 Effect of medium composition on the mating frequency between T. novellus strains. Donor T. novellus (R68.45-pDER401) was mated with T. novellus MD101 (Sm^r)[1].

Mating medium %TMB : %TAM	pH	Mating frequency: transconjugants per recipient
100 : 0	6.7	6.1×10^{-5}
50 : 50	4.7	5.2×10^{-6}
40 : 60	4.4	2.1×10^{-6}
30 : 70	4.3	7.6×10^{-7}
20 : 80	4.3	$<2.3 \times 10^{-9}$
10 : 90	4.1	$<2.3 \times 10^{-9}$
0 : 100	4.0	$<2.3 \times 10^{-9}$

[1] Matings were for 24 h and transconjugants were selected on TMYE containing Km, Cm and Sm.

2.3.5.4 Direct matings with T. novellus. Matings between T. novellus and T. ferrooxidans on a range of composite TMB:TA media produced T. ferrooxidans Tc^r colonies at a low frequency ($2.4\text{-}2.5 \times 10^{-9}$) on selective plates (Table 2.4, matings 26 and 27). However, Tc^r colonies also developed at a similar frequency on selective plates spread with equivalent quantities of T. ferrooxidans cells which were not exposed to potential donor cells (Table 2.4, mating 25). Subculturing T. ferrooxidans Tc^r colonies on TA medium, containing other antibiotics, to confirm the presence of either the conjugative plasmid R68.45 (Ap), and either pDER401 (Cm) or pDER405 (Cm), showed no growth. Only about 30% of Tc^r colonies from both mated and unmated platings of T. ferrooxidans were stably propagated on TA medium containing Tc. However, DNA preparations of the T. ferrooxidans Tc^r cells, probed separately with radiolabelled pBR325 and R68.45, failed to show the presence of either R68.45 or pDER401.

Plasmids mated	markers
R68.45	Ap^r Km^r Tc^r
pDER401	Cm^r Tc^r
pDER405	Cm^r Tc^r

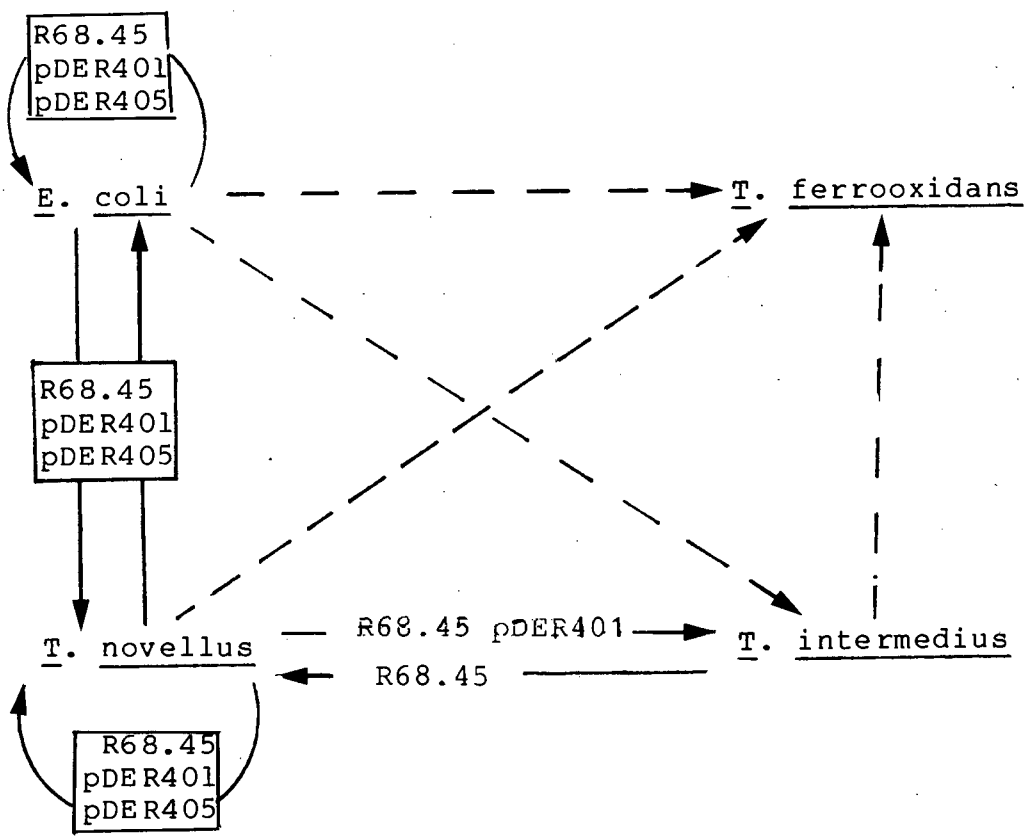

Figure 2.2 Schematic illustration of the results of conjugation experiments. Arrows indicate directions of matings. Complete lines indicate successful plasmid transfer. Interrupted lines indicate unsuccessful matings.

2.4 DISCUSSION

The recombinant plasmid, pDER401, had previously been shown to replicate in laboratory genetic strains such as E. coli and P. aeruginosa (Rawlings et al., 1986). The experiments reported here have demonstrated that this plasmid, and its derivative, pDER405, are also capable of replication in T. novellus. Because the parental plasmid, pTF-FC2, was originally isolated from T. ferrooxidans and shown to have a functional broad-host-range origin of replication, plasmids pDER401 and pDER405 were expected to replicate in T. ferrooxidans as well.

Plasmids pDER401 and pDER405 were mobilized at high frequencies between E. coli strains by the IncP plasmid, R68.45. Furthermore, mobilization functions located on the T. ferrooxidans portion of the recombinant plasmids were expressed in T. novellus since R68.45 was able to mobilize the vectors between T. novellus strains. The mobilization of pDER401 and its derivative, pDER425 (which carries As^{III} and As^{V} resistance genes from plasmid R46), between strains of E. coli, and from E. coli to T. novellus, has been reported by the present author and coworkers (Rawlings et al., 1986).

It is interesting to compare the expression of foreign genes in T. novellus, as observed in the present study, with that reported by Davidson and Summers (1983). These workers

reported that the Cm^r gene of plasmid S-a and the Ap^r gene of RP4 were not expressed in T. novellus, although the Tc^r determinant was expressed. Results obtained in the present study confirmed their findings with respect to the Tc^r and Ap^r genes. However, the Cm^r determinant on pBR325, used in the construction of pDER401 and pDER405, was expressed in T. novellus. This discrepancy of (S-a and pBR325) Cm^r-gene expression in T. novellus may be due to the nature of the Cm^r genes on the two plasmids. The respective genes are not identical and have different restriction enzyme sites (Rawlings et al., 1986).

Although pDER401 was shown to be transferred from T. novellus to T. intermedius, it was not stably maintained in this organism. However, R68.45 was retained and could be retransferred from T. intermedius to T. novellus at a low frequency. These findings corroborate those of Davidson and Summers (1983), and of Kulpa et al. (1983) that broad-host-range plasmids may be mobilized between E. coli and non-iron-oxidizing Thiobacillus sp. and among the non-iron-oxidizing thiobacilli.

No plasmid exchange between T. intermedius and T. ferrooxidans was detected. The loss of pDER401 from T. intermedius on subculturing indicated that pDER401 was not stably maintained in this organism. However, in spite of R68.45 being maintained in T. intermedius, transfer of either the Tc^r or Ap^r markers of R68.45 from this organism to T. ferrooxidans was not detected. Attempts to transfer

plasmids directly from either E. coli or T. novellus donors to T. ferrooxidans, on a range of mating media, were also unsuccessful.

The primary method of detecting plasmid transfer in this study was through the expression of antibiotic markers. Although markers on pDER401 and pDER405 (and R68.45) were expressed in E. coli, T. novellus and T. intermedius, it is not known whether these markers would be expressed in T. ferrooxidans. An explanation for the failure to isolate T. ferrooxidans transconjugants, using antibiotic selection, could be that the functional mechanisms of Tc^r and Cm^r, as encoded by pBR325 in E. coli, are not compatible with the physiology of T. ferrooxidans.

Tc was found to be stable in TA medium (pH 4,00) over an extended period of 12-14 d. This finding supports that of Davidson and Summers (1983) who showed that Tc was stable at pH 2,00. In spite of this stability, the use of Tc as a selective agent is not entirely satisfactory. The spontaneous mutagenesis of T. ferrooxidans to Tc^r was a major drawback. The frequency of Tc^r colonies appearing on plates in the present study was typical of a bacterial population undergoing spontaneous mutagenesis (i.e. approximately 10^{-8}-10^{-9}). Use of Tc is therefore limited to DNA transfer techniques that produce transformants or transconjugants at a level of at least 10-fold higher than the spontaneous mutation background. Plasmid transfer to T. ferrooxidans requires a more reliable selective system

for detection of this process. The use of the pBR325-derived Cm^r determinant has not been pursued exhaustively, and was used only in confirmatory studies, i.e. to determine whether putative Tc^r transconjugant colonies were also Cm^r. Mechanisms of resistance to Tc and Cm encoded on the recombinant T. ferrooxidans plasmids are different and Cm^r as a marker should be exploited further. Whether the mechanism of Cm^r will be compatible with the physiology of T. ferrooxidans is not known.

Although the plasmids, pDER401 and pDER405, were expected to replicate in T. ferrooxidans, it is possible that the natural plasmids in the T. ferrooxidans recipient may be incompatible with pDER401 and pDER405. A plasmid-cured T. ferrooxidans strain may prove useful as a recipient in DNA transfer studies.

Furthermore, since three out of four plasmids isolated from a single strain of T. ferrooxidans have been shown to be mobilizable (Rawlings and Woods, 1985), it is likely that T. ferrooxidans is capable of mating under the right conditions. Large cryptic plasmids are present within T. ferrooxidans, including the strain from which pTF-FC2 was isolated (D.E. Rawlings, personal communication). It is possible that these plasmids are capable of mobilization of the smaller plasmids in T. ferrooxidans. These large cryptic plasmids have already been isolated and are currently being investigated for conjugative transfer properties.

Further studies on DNA transfer to T. ferrooxidans would be aided by the identification of a suitable homologous marker. The use of auxotrophic complementation in T. ferrooxidans, as used for the genetic analysis of heterotrophic organisms and more recently in T. thioparus (Yankofsky et al., 1983), would then be possible. However, defined auxotrophic mutants of T. ferrooxidans are not available. Once a strategy for isolating such mutants of T. ferrooxidans has been established, it may be possible to use this as a selective mechanism. T. ferrooxidans raises unique problems which need to be addressed in attempting to study this organism at a genetic level. These include techniques for mutant enrichment, and a determination of whether T. ferrooxidans has uptake systems for presynthesized metabolites.

An alternative strategy towards the identification of a potential marker involves the induced mutagenesis of an antibiotic-sensitive T. ferrooxidans strain to antibiotic resistance. Once this has been achieved, cloning of the chromosomal gene responsible for conferring such a resistance into the available T. ferrooxidans plasmids (Rawlings and Woods, 1985), may be possible. Wild-type antibiotic-sensitive T. ferrooxidans strains could then serve as recipients for such a wholly homologous plasmid. Attempts to identify a potential homologous marker for T. ferrooxidans will be described in the next chapter.

CHAPTER 3

CONSTRUCTION OF T. ferrooxidans GENOMIC LIBRARIES

Chapter 3

CONSTRUCTION OF T. ferrooxidans GENOMIC LIBRARIES

Summary. A genomic library of T. ferrooxidans ATCC 33020 was constructed in the plasmid vector, pEcoR251, for the purpose of cloning a recA-like gene from this organism. The library consisted of approximately 1.78×10^4 clones carrying chromosomal DNA fragments of about 3-12 kb. The library was successfully screened for functional complementation of E. coli auxotrophic mutants. Clones that conferred resistance to MMS, a DNA-damaging agent, were isolated in an E. coli recA$^-$ mutant. In an attempt to clone a homologous marker, T. ferrooxidans ATCC 33020 was mutated to Rif^r and DNA from the mutant strain was used in the construction of plasmid- and cosmid-based libraries. The plasmid library contained approximately 1.35×10^3 clones with inserts of about 1-13 kb. The cosmid library consisted of approximately 8.2×10^3 colonies, 4.0×10^4 in vitro packaged cosmids, and an amplified in vivo-packaged cosmid lysate containing approximately 1.82×10^{11} infectious particles, carrying inserts of about 35-55 kb. Complementation of E. coli auxotrophic mutants was observed with the plasmid and cosmid library of the T. ferrooxidans Rif^r strain. Screening both libraries for a Rif^r marker was unsuccessful.

3.1 INTRODUCTION

Studies on the molecular biology of prokaryotes and eukaryotes have been aided by the construction of genomic libraries. The current method of choice for constructing libraries is to clone into a vector, DNA fragments obtained by partial digestion of high molecular weight DNA. Partial digestion results in random scission of the parent DNA, which is necessary to ensure that the desired gene system

remains intact in at least a portion of the cleaved DNA fragments. The digested DNA, ligated to the linearized vector, is then transferred to a host strain. These techniques enable the propagation of specific DNA fragments (clones) in a foreign host.

The choice of vector used to construct a genomic library depends on the organism from which the DNA is to be isolated, and the expected size of the fragment of interest. Other factors include the method of screening to be used, and the application for which the DNA is to be used. Specific vectors have been developed for a wide range of organisms, including Pseudomonas (Bagdasarian and Timmis, 1982; Olsen et al., 1982; Sakaguchi, 1982), Bacillus sp. (Ehrlich et al., 1982), Streptomyces sp. (Kieser et al., 1982), yeast (Beggs, 1978; Hinnen et al., 1978) and various plant (Howell, 1982) and mammalian systems (Muzyczka, 1980). A comprehensive catalogue of vectors, their characteristics and potential uses has been published (Pouwels et al., 1985).

The most extensively studied vectors are those that function in E. coli. These include plasmids for cloning relatively small DNA fragments (of up to 12 kb); various bacteriophage derivatives (Williams and Blatner, 1979), and cosmids (Collins and Bruning, 1978; Hohn, 1979; Hohn and Collins, 1980; Ish-Horowicz and Burke, 1981; Little and Cross, 1985) which are used to clone relatively large DNA fragments (20-40 kb).

The number of recombinants, **N**, required to give a specific probability of having any DNA sequence represented in a genomic library can be calculated by applying the formula:

$$N = \frac{\ln (1 - P)}{\ln (1 - f)} ,$$

where **f** is the fraction of the total genome that each insert represents and **P** is the required probability (Clarke and Carbon, 1976).

Plasmids are frequently the cloning vector of choice in prokaryote genomic library construction, as inserts of up to 10 kb are sufficiently large to cover the entire prokaryotic genome in approximately 5 000 to 10 000 clones. Plasmid cloning vectors are available that carry a wide variety of features that aid in plasmid selection and cloned gene expression (Pouwels et al., 1985). Examples of these vectors are, the general purpose cloning vectors such as the pBR range of plasmids (pBR322-pBR328) (Bolivar et al., 1977; Bolivar, 1978), and plasmids containing bacteriophage and other promoters that control the expression of cloned genes (De Boer et al., 1983; Botterman and Zabeau, 1985). Some expression vectors are designed specifically for elevated level expression of cloned genes for product harvesting (Remaut et al., 1983).

Although plasmids remain the most widely used vectors, they have some disadvantages when used in the construction of genomic libraries, particularly relating to the cloning of large DNA fragments (> 15 kb). For example, (i) larger fragments often tend to be structurally unstable and are subject to rearrangement. The replication rates of plasmids slow down with an increase in size, with those that have lost sizeable chunks of insert DNA usually predominating (Watson et al., 1987). (ii) Larger plasmids (> 15 kb) have much reduced transformation frequencies (Saunders et al., 1984).

The disadvantages of plasmid vectors are emphasized when used in the construction of eukaryotic genomic libraries. Due to the size of the eukaryotic genome, a large number of clones would be required to obtain a high probability (99%) of any given gene being represented.

Bacteriophage λ substitution vectors and cosmids were developed to enable the cloning of larger DNA fragments. Cloned fragments of up to 23 kb for λ substitution derivatives (Maniatis et al., 1982) and 40 kb for cosmids (Bruning and Collins, 1978; Hohn, 1979;) may be isolated. In vitro assembled recombinant cosmids are favoured by some workers because of the large size of the insert and because selection of a particular gene involves screening of a smaller number of recombinant clones.

Recombinant cosmid DNA or λ DNA is conveniently packaged *in vitro* by adding a combination of two cell lysates, each of which is defective in a different step of λ morphogenesis (Hohn, 1979). Empty phage precursor particles accumulate after induction in bacteria carrying a prophage, mutant in gene *D*. The missing D protein may be complemented efficiently by an induced λ lysogen which is defective in another essential structural component, such as the product of the *E* gene. In the absence of this component, which effects the main capsid protein, all the other head proteins are present in soluble form. In the presence of putrescine, ATP and spermidine, DNA is packaged and a mature viable phage is assembled from components of both lysates, resulting in a DNase-resistant infectious particle, much like any *in vivo* produced phage (Hohn, 1979).

Normally, concatemeric λ DNA is the substrate for packaging into assembled phage heads (Hohn, 1979). The tandem polymer is packaged *in vivo* and *in vitro* producing cohesive ends. Because cosmid vector molecules are small and circular, they cannot be packaged. However, open forms of the vector ligated to suitable sized fragments of insert DNA resemble concatemeric DNA. The *cos* sites at each end of the cosmid facilitate the packaging of recombinant DNA in a λ coat. An important requirement for packaging is the amount of DNA between successive *cos* sites. Only *cos* sites 38-52 kb apart are subjected to packaging-dependent cleavage, yielding infectious particles (λ wildtype DNA is 49 kb in size). The efficiency of obtaining infectious particles with shorter or

longer DNA decreases markedly outside of this range (Hohn, 1979).

Libraries constructed in bacteriophage λ vectors are stored and propagated in the form of recombinant bacteriophage particles (Maniatis et al., 1982). In cosmids, however, these bacteriophage particles merely serve as vehicles whereby the recombinant DNA molecules are efficiently introduced into the bacteria where they are propagated as large plasmids (Hohn, 1979). Libraries constructed in cosmids are therefore maintained within populations of transformed bacteria.

Once a cosmid bank is constructed, DNA is isolated from recombinant clones as large plasmids. Because E. coli is poorly transformed with large plasmids, subsequent screening of the recombinant cosmid library is inefficient. However, recombinant cosmids carrying cos sites may be packaged in vivo in special strains of E. coli such as χ-2819 which is a λ lysogenic strain of E. coli K12 (R. Curtiss III, personal communication). Packaging of recombinant cosmid molecules in vivo results in the amplification of an original in vitro-packaged cosmid sample. The transfer of recombinant cosmids for screening of genomic libraries in E. coli strains is by simple transduction with the packaged lysate. This technique also allows the storage of cosmid libraries as infectious packaged lysates.

Regardless of whether plasmid- or cosmid-based vector systems are used, selection of the recombinant DNA molecules from a background of reassembled parental vectors determines the efficiency of the experiment. Efficient cloning strategies are those which reduce the parental vector background. One way in which this is achieved is the removal of 5' phosphates from the vector molecule. Since bacteriophage T4 ligase (T4 ligase) is unable to ligate DNA fragments lacking a 5' phosphate group (Weiss et al., 1968), dephosphorylation of the vector substantially reduces the parent vector background. In the present study, dephosphorylation of the cosmid vector, pHC79 (Hohn and Collins, 1980), was essential to prevent concatenation and packaging of the linearized vector.

Selection of recombinants at the transformation stage, using vectors such as pBR322 (Bolivar et al., 1977) and the cosmid pHC79 (Hohn and Collins, 1980), involves the insertional inactivation of one of two antibiotic-resistance markers on the vector. Each putative recombinant colony has to be screened for sensitivity towards the specific antibiotic; a laborious task when screening the number of colonies that constitute a library.

A method of positive selection for pBR322 recombinants that enables direct selection of Tc-sensitivity was developed by Bochner et al. (1980) and improved on by Kiel et al. (1987). Positive selection vectors are easier to use and more efficient than other types of vectors especially in the

construction of genomic libraries (Kuhn et al., 1986). These vectors typically rely upon the derepression of an antibiotic-resistance function (Roberts et al., 1980; Nilsson et al., 1983; Nikolnikov et al., 1984), inactivation of a dominant function conferring cell sensitivity to an antibiotic or metabolite (Dean, 1981; Hennecke et al., 1982; Ahmed, 1984; Burns and Beacham, 1984), removal of a lethal DNA sequence (Hagan and Warren, 1982) or inactivation of a lethal gene (Schumann, 1979; O' Connor and Humphreys, 1982; Cheng and Modrich, 1983; Vernet et al., 1985; Kuhn et al., 1986).

The plasmid, pEcoR251 (M. Zabeau, Plant Genetic Systems, Ghent, Belgium; Appendix C), used in the present study to obtain a genomic library of T. ferrooxidans, is of this last type. Essentially, it consists of the E. coli EcoR1 endonuclease gene under the control of the λ rightward promoter (P_r), the Ap^r gene and the origin of replication from pBR322. The EcoR1 gene product, when expressed at high levels by the λ P_r promoter is lethal unless insertionally inactivated, or regulated by a resident λ prophage. Effective temperature sensitive regulation can be achieved with plasmid pcI857 (Km^r), a colE1 compatible plasmid carrying the λ cI857 allele which codes for a temperature-sensitive repressor protein (Remaut et al., 1983). The EcoR1 gene has single restriction sites for HindIII, BglII and SstI endonucleases. The BglII endonuclease site can be used to clone fragments generated by BglII, BamH1, MboI, or Sau3A restriction endonucleases.

3.1.1 The present study

A genomic library in pEcoR251 was constructed for the analysis of loci which could be cloned on 4-10 kb inserts. Of particular interest in the construction of this library was the isolation of a *recA*-like gene from *T. ferrooxidans*.

RecA protein is nonessential for viability in *E. coli* (McEntee, 1977). However, mutations in the *recA* gene severely disable cells with respect to genetic recombination, DNA repair, and mutability by chemical agents (Walker, 1984). *E. coli recA*$^-$ strains are sensitive to low levels of DNA damaging agents, e.g. UV light, methyl methane sulfonate (MMS) and 4-nitroquinoline-1-oxide (NQO). Isolation of a *T. ferrooxidans recA*-like gene was attempted by interspecific complementation in *E. coli* HB101 (*recA*$^-$).

In an attempt to isolate a homologous Rif^r marker, a plasmid- and a cosmid-based library of a *T. ferrooxidans* Rif^r strain were constructed. The use of Tc as a selective agent for *T. ferrooxidans* is considered unsuitable for the reasons given in Chapter 2. In addition, antibiotics generally used as selective agents, e.g. Ap, Km, Sm, and Nal, have been shown to produce minimal or no inhibitory effect on *T. ferrooxidans* under normal growth conditions in iron- and sulphur-based media (Rawlings *et al.*, 1983; R.S Ramesar, unpublished results).

The most suitable antibiotic against T. ferrooxidans, in both sulphur- and iron-based media, that has thus far been identified is Rif (Rawlings et al., 1983). Rif is a derivative of the rifamycin group of antibiotics known to be potent inhibitors of DNA-dependent RNA polymerase (hereafter referred to as RNA polymerase) (Lester, 1972). Rif prevents initiation of the RNA chain but not subsequent elongation. RNA polymerase has a complex subunit structure and contains five different polypeptide chains: beta prime (β'), beta (β), alpha (α) and sigma (σ) (Scaife, 1976; Zillig et al., 1976). Mutations that render a cell resistant to Rif have so far been shown to map only in the rpoB gene, which codes for the β subunit of RNA polymerase (Scaife, 1976; Watson et al., 1987). The β subunit has a M_r of 151 000 and rpoB is one of the largest genes in E. coli (Scaife, 1976).

RNA polymerase has been found in all bacterial species where it has been sought (Chamberlin, 1976). RNA polymerases from different species appear to be closely related in subunit structure (Burgess, 1976). The subunits for RNA polymerase from Gram-negative bacteria resemble those of the E. coli enzyme, although M_r values obtained by gel electrophoresis for α and σ subunits may vary slightly (Burgess, 1976). Herzfeld and Zillig (1971) reported a similar subunit structure for RNA polymerase in the blue-green alga, Anacystis nidulans. RNA polymerase isolated from Bacillus sp. is analogous to that of E. coli except for the σ subunit, which is of lower M_r (Chamberlin, 1976). The interchangability of RNA polymerase subunits in vitro

between E. coli and the phylogenetically different, Gram-positive bacterium, Micrococcus luteus, has been reported to produce enzymatically active hybrid RNA polymerases (Lill et al., 1975).

The DNA encoding the Rif-insensitive β subunit of RNA polymerase in E. coli has already been cloned and shown to transform Rif^s strains of E. coli to Rif^r (Kirschbaum and Konrad, 1973). In these partial diploids the Rif-insensitive β subunit displaces the native β subunit during RNA polymerase assembly.

The strategy in the present study was to construct a T. ferrooxidans Rif^r mutant, and then attempt to clone the Rif^r-conferring gene. The first step involved the mutagenesis of T. ferrooxidans to Rif^r. Once obtained, the mutant would be the source of DNA for the construction of plasmid- and cosmid-based libraries.

Construction of a cosmid library from the T. ferrooxidans Rif^r strain was attempted to ensure that the long stretches of DNA coding for the β subunit of RNA polymerase, and its control sequences were cloned. It was considered that, once isolated, the cloned Rif^r trait would have a greater chance of functioning as a marker in T. ferrooxidans, than the heterologous E. coli-based antibiotic-resistance markers, presently on the pDER series of plasmids (Rawlings and Woods, 1985; Chapter 2).

3.2 MATERIALS AND METHODS

3.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Table 3.1

3.2.2 Media and reagents

Constituents of media, buffers, and reagents are listed in Appendix B. Details of methods, where not in the text, are given in Appendix A.

3.2.3 Mutagenesis of T. ferrooxidans to produce a Rif^r strain.

T. ferrooxidans ATCC 33020, grown to late logarithmic phase in 9K medium (Appendix B), was harvested at 8 000 rpm for 10 min in a Beckman JA14 rotor, washed twice in dilute H_2SO_4 (pH 2,00), twice in distilled water, and resuspended in Ringer's solution (pH 4,00). Approximately 10^9 cells were treated in one of four ways:

(i) Plated directly onto the surface of TAM plates (Appendix B), supplemented with Rif (50 μg/ml).

(ii) Treated with NTG at concentrations of 20, 50, 100, 250 and 500 μg/ml. The cells were exposed to the mutagen for 30 min, washed four times in fresh Ringer's solution, resuspended in distilled water and plated onto TAM plates

Table 3.1 Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
E coli BHB2688	N205 *recA*(λ *imm434 cIts b2 red3 Eam4 Sam7*) /λ	Hohn (1979)
E coli BHB2690	N205 *recA* (λ *imm434 cIts b2 red3 Dam15 Sam7*)/λ	Hohn (1979)
E coli BHB2600	803 *supE*$^+$ *supF*$^+$ $r_k^- m_k^+$ *met*	Hohn (1979)
E coli HB101	*rpsL leu pro thi hsd hsm lacY recA*	Maniatis *et al*. (1982)
E coli ~2819	F$^-$ *lacY1 glnV44 galK2 galT22* λ(*c1857 b2 redBβ3 Sam7*) *recA56* Δ*thyA57 metB1 hsdR2*	R. Curtiss III[1]
E. coli YMC11	*glnA ntrB ntrC*	Backman *et al*. (1981)
E. coli K514	*thr1 leu6 thi1 SupE44 lacY1 tonA21* r^-_k m^+_k	Wood, 1966
E. coli K514λ	λlysogen of *E.coli* K514	Zabeau and Stanley (1983)
E. coli LK111	*E.coli* K514 derivative *lacZ*Δ*M15 lacY*$^+$ *recA*	Zabeau and Stanley (1982)
T. ferrooxidans	wild-type	ATCC33020
T. ferrooxidans	Rif^r	This study
Plasmids and cosmids		
pEcoR251	Ap^r *EcoR1*	Zabeau, M[2]
pDER401	Tc^r Cm^r	Rawlings *et al*. (1984b)
pHC79	Tc^r Ap^r *cos*	Hohn and Collins (1980)
pBR322	Ap^r Tc^r	Bolivar *et al*. (1977)

[1] R. Curtiss III via S. Silver: University of Illinois College of Medicine at Chicago, Chicago, Illinois, U.S.A.
[2] M. Zabeau: Plant Genetic Systems, Ghent, Belgium

containing Rif (50 μg/ml).

(iii) Irradiated with UV light at 35 J/m^2, which produced 0,001% survivors. The irradiated suspension was plated onto TAM plates containing Rif (50 μg/ml).

(iv) Diluted and plated onto TAM plates as a control for the calculation of the frequency of Rif^r mutants.

T. ferrooxidans Rif^r colonies were, (i) inoculated into aqueous 9K medium containing Rif (50 μg/ml), and (ii) subcultured onto TAM plates containing Rif (50 μg/ml). Confirmed T. ferrooxidans Rif^r cultures were propagated in aqueous 9K medium containing Rif (50 μg/ml).

3.2.4 Partial digestion and sucrose gradient fractionation of chromosomal DNA

3.2.4.1 T. ferrooxidans ATCC 33020 (wild-type). Total cellular DNA from T. ferrooxidans was isolated and purified as described in Appendix A.1.4. A sample of DNA was partially digested in a pilot study using a 2-fold dilution series of Sau3A endonuclease to ascertain the optimum DNA to enzyme ratio to produce 4-10 kb fragments (Maniatis et al., 1982). Using the optimum ratio, the digestion system was scaled up for the digestion of 300 μg of DNA, after which the mixture was extracted twice with TE-buffered phenol (Appendix B). DNA was precipitated by adding 0.1 volume of sodium acetate (3 M) and two volumes of 95% ethanol. The DNA was pelleted by centrifugation in a microfuge (12 000 rpm for 10 min), dried in a Speedyvac concentrator (Savant

Instruments, Inc., Hicksville, New York, U.S.A.), resuspended in 500 µl of TE buffer (Appendix B) and loaded onto a sucrose density gradient (10-40% (w/v) in 10 mM Tris-HCl, pH 8.00; 1 mM EDTA; 1 M NaCl) in a nitrocellulose tube and centrifuged at 26 000 rpm for 24 h at 20^{0}C in a Beckman SW28 rotor. The gradient was fractionated into microfuge tubes and samples of every third fraction were analyzed by agarose gel electrophoresis (Appendix A.3). Fractions containing fragments of approximately 4-10 kb were pooled, the DNA precipitated and resuspended in TE buffer.

3.2.4.2 *T. ferrooxidans* Rif^r strain. Chromosomal DNA from the *T. ferrooxidans* Rif^r mutant was isolated, purified and subjected to partial digestion as described for the wild-type strain. Two pilot partial digests were set up using *Sau*3A endonuclease; one to produce fragments in the 4-10 kb range and the other to produce fragments of 35-50 kb. The respective systems were scaled up for the digestion of 300 µg of DNA. Fragment separation according to size and subsequent retrieval of the required DNA fragments are described in section 3.2.4.1.

3.2.5 Construction of the plasmid libraries

3.2.5.1 Preparation of pEcoR251 vector DNA. The vector, pEcoR251, was isolated from the *E. coli* K514λ host using the maxiprep method (Appendix A.1.2). Purified plasmid was digested with *Bgl*II endonuclease (Appendix A.2),

extracted with phenol, precipitated with ethanol and redissolved in 50 μl of TE buffer.

3.2.5.2 Ligation of pEcoR251 and insert DNA. Size-fractionated DNA fragments (4-10 kb) of the wild-type and the Rif^r mutant strains were used in separate ligations with vector DNA. Ligations were carried out as described in Appendix A.4, with a vector to insert DNA ratio of 1:1 (at a concentration of 5 pmol/ml) to optimize closed circular recombinant molecule formation.

3.2.5.3 Transformation of ligation. Preparation of competent cells and transformation protocols are outlined in Appendices A.5 and A.6, respectively.

Competent E. coli K514λ cells were transformed with 2 μl of the ligated DNA suspension. Unrestricted plasmids, pBR322 and pEcoR251 (at 5 ng of each), transformed as above, served as controls to evaluate cell competence and the efficiency of the pEcoR251 counterselection mechanism. Transformation mixes were plated onto LA plates containing Ap (100 μg/ml).

After incubation for 1 h, pBR322-transformed cells showed a 1- to 2-fold increase in the number of colonies on selective plates, whereas the cells transformed with integral pEcoR251 showed a greater than 50-fold reduction in the number of transformants on selective plates.

3.2.5.4 Storage of recombinant plasmids. Using the maxiprep method, plasmid DNA was extracted from pools of approximately 500 Ap^r colonies. The DNA pellets were resuspended at 1 mg/ml in TE buffer, without CsCl purification, and stored at -70^0C.

3.2.6 Construction of the cosmid library

3.2.6.1 Preparation of the cosmid vector. The cosmid vector, pHC79 (Appendix C), was digested to completion with BamH1 endonuclease, extracted with phenol, precipitated with ethanol and redissolved in TE buffer.

Digested pHC79 was treated with calf intestinal phosphatase (CIP; Boehringer-Mannheim GmbH, Mannheim, West Germany) at 0.01 u CIP per 1 μg of DNA. The mixture was incubated at 37^0C for 30 min, extracted with phenol, precipitated with ethanol and redissolved in TE buffer.

3.2.6.2 Ligation of pHC79 and insert.
Dephosphorylated pHC79 was added to T. ferrooxidans Rif^r size-fractionated chromosomal DNA (35-50 kb) at a molar ratio of 10:1 (vector:target chromosomal DNA). The final DNA concentration in the mixture was 600 μg/ml in a volume of 15 μl. Ligation conditions are described in Appendix A.4.

3.2.6.3 Construction of the packaging system for cosmids. Recombinant cosmid DNA was packaged *in vitro*. Details of the construction of packaging mixes, assays for their efficiency, *in vivo* amplification of cosmids, generation of cosmids as phage lysates, and storage of packaging mixes and cosmid libraries, are detailed in Appendix A.8.

3.2.6.4 Recombinant cosmid packaging. Packaging mixes, in microfuge tubes, were transferred from storage to liquid nitrogen in a portable Thermos flask. The mixes were placed in an ice bath to thaw and assayed as follows:

5 μl ligated cosmid-chromosomal DNA mixture
1 μl 40 mM ATP

The still partially frozen contents of the tubes were centrifuged for 5 s (in a microfuge) and again placed on ice. The packaging-reaction mixture was mixed well using a capillary tube, immediately centrifuged for 5 s and incubated at 37^0C for 60 min. The reaction was terminated by dilution with 0,5 ml SM buffer (Appendix B), and a drop of chloroform. Debris was removed by centrifugation for 30 s. The unused ligation mix was stored at -70^0C.

3.2.6.5 Titration of packaged cosmids. A fresh colony of *E coli* HB101 was inoculated into LM medium (Appendix B), supplemented with 0,4% (w/v) maltose and grown to late

exponential phase. Cells were harvested by centrifugation and resuspended in $MgSO_4 \cdot 7H_2O$ (10mM) to an OD_{600} of 0,5.

A sample (50 μl) of the diluted packaged cosmids was added to 200 μl of the E. coli HB101 recipient cell suspension and adsorbed at 30^0C for 30 min. The suspension was diluted with 2 ml of LB medium and incubated for 1 h at 37^0C with shaking to allow expression of the Ap^r marker. Samples (50 μl) were plated onto LA plates containing Ap (100 μg/ml) and incubated overnight at 37^0C.

3.2.7 Library Characterization

3.2.7.1 Nature of inserts. A sample of clones from each library was analyzed to provide an indication of the nature of insert DNA in the parent libraries. Ten colonies of E. coli HB101 from primary platings of each library in pEcoR251 were grown in 5 ml of LB medium overnight at 37^0C. Plasmid DNA was isolated from the cultures using the miniprep method (Appendix A.1.1). A sample from each DNA preparation was restricted with PstI endonuclease and analyzed by agarose gel electrophoresis; the remaining plasmid DNA was used to retransform competent E. coli HB101 cells.

One hundred putative cosmid clones were subcultured onto LA plates containing Tc (25 μg/ml) to determine whether the Tc^r gene on the pHC79 vector was insertionally inactivated. To determine the size of the cosmids, 12 colonies were randomly

chosen and grown overnight at 37^{0}C in LB medium containing Ap (100 μg/ml). Plasmid DNA, isolated using the miniprep method, was restricted with Sal1 endonuclease and resolved by agarose gel electrophoresis.

3.2.7.2 Biological evidence of the quality of the genomic libraries. The pEcoR251-*T. ferrooxidans* ATCC 33020 wild-type genomic library will hereafter be referred to as the plasmid-wt library; the pEcoR251-*T. ferrooxidans* Rif^r strain DNA library as the plasmid-Rif^r library, and the pHC79-*T. ferrooxidans* Rif^r strain DNA library as the cosmid-Rif^r library.

Complementation of *E. coli* mutants provides additional evidence, at the functional level, for the quality of genomic libraries. Complementation studies were carried out using 2 μg of plasmid DNA from either the plasmid-wt, or the plasmid-Rif^r library to transform approximately 10^9 competent *E. coli* cells. A sample (20 μl) of the diluted *in vitro* packaged cosmids was used to transduce approximately 10^9 *E. coli* cells as described in section 3.2.6.5. Transformed and transduced cells were supplemented with 1 ml of LB medium and allowed to express at 37^{0}C for 1 h before plating onto selective media. Plates were incubated overnight at 37^{0}C. Sample clones were examined for the presence of recombinant plasmids. Recombinant plasmids were screened for their ability to retransform the respective auxotrophs to prototrophy.

(i) leucine and (ii) proline biosynthesis. E. coli HB101 is deficient in the genes for the production of the amino acids proline (Pro) and leucine (Leu). Transformants were selected directly on minimal medium (Appendix B) containing Ap (100 μg/ml) and either Pro or Leu (50 μg/ml).

(iii) glutamine synthetase (GS). E. coli YMC11 (gln$^-$) is a glnA deletion mutant. Growth on minimal medium containing $(NH_4)_2SO_4$ as sole source of nitrogen is indicative of functional complementation by a glnA gene. The transformants were selected on minimal medium containing $(NH_4)_2SO_4$ (20 mM), and Ap (100 μg/ml).

3.2.8 Resistance to MMS

The minimum inhibitory concentration of the DNA-damaging agent, MMS, for E. coli HB101 was determined to be 0,002% (v/v). Five μg of pooled plasmids from the plasmid-wt library were used to transform competent E. coli HB101 cells. After expression, cells were spread onto LA plates supplemented with Ap (100 μg/ml) and MMS at 0,01% (v/v). Putative Ap^r MMS^r colonies were checked for recombinant plasmids and for the ability of the plasmids to retransform E. coli HB101 to Ap^r and MMS^r.

E. coli HB101 was transformed with the plasmid-Rif^r library as described for the plasmid-wt library. Transduction of E. coli with packaged cosmids is described in section 3.2.6.5. Ap^r MMS^r transformants provided further evidence

of the biological quality of the plasmid- and cosmid-Rif^r libraries.

3.2.9 Screening for Rif^r

Five μg of plasmid DNA from each pool of the plasmid-Rif^r library were transformed into approximately 10^9 competent E. coli HB101 and E. coli LK111 cells. One ml of LB medium was added to each transformation mix and incubated at 37^0C for 2 h to allow for expression of cloned genes. Cells were plated onto LA plates containing Rif (25 μg/ml). An equivalent amount of cells of each strain, not exposed to transforming DNA, was spread on LA plates containing Rif (25 μg/ml) to provide an indication of the levels of spontaneous Rif^r mutations.

For screening of the cosmid-Rif^r library, transduction was carried out by adding 50 μl samples of the original diluted, in vitro-packaged cosmids and 5 μl samples of the amplified cosmid lysate (described in Appendix A.8.7), separately to about 10^9 cells of E. coli HB101 and E. coli LK111. After adsorption and expression at 37^0C for 2 h, the cell suspension was spread onto LA plates containing Rif (25 μg/ml), and incubated overnight at 37^0C. At the same time an equivalent quantity of an untransduced cell suspension, for each E. coli strain, was spread onto LA plates containing Rif (25 μg/ml) to ascertain the level of spontaneous mutations to Rif^r.

Colonies on selective plates were restreaked onto LA plates containing Rif (100 μg/ml) and Ap (100 μg/ml), and incubated overnight at 37^0C. Colonies were also inoculated into 5 ml LB medium containing Rif (50 μg/ml) and grown overnight at 37^0C. These cultures were examined for plasmids and the restriction pattern of the plasmid DNA was determined. E. coli HB101 and LK111 cells were retransformed with plasmid DNA from the Ap^r Rif^r cultures to determine whether the Rif^r determinant was present on any of the recombinant plasmids.

3.3 RESULTS

3.3.1 Construction of T. ferrooxidans Rif^r mutants.

UV irradiation or NTG treatment did not produce any T. ferrooxidans Rif^r mutants over the range of conditions used. However, untreated cells plated onto TAM plates containing Rif (50 μg/ml) produced three colonies from approximately 10^9 cells.

These colonies were able to oxidise iron (in aqueous 9K medium) within 5 d in the presence of Rif (50 μg/ml). Cultures inoculated with T. ferrooxidans ATCC 33020 wild-type colonies remained unoxidized for weeks or months, an indication of the effectiveness of Rif in 9K medium.

3.3.2 Construction of the cosmid library

3.3.2.1 Titration of packaged particles

(i) background packaging frequency. No background plaques were detectable when mock packaging reactions (no DNA added) were carried out.

(ii) λ DNA packaging. In vitro packaging of λ DNA resulted in 5.6 X 10^7 plaque forming units (pfu) per 1 μg of DNA, per packaging mix.

(iii) cosmid packaging. Approximately 8.2 X 10^3 Ap^r colony forming units (cfu) were produced by the transduction of E. coli HB101 with 100 μl of diluted, packaged cosmid (i.e. approximately 4.0 X 10^4 Ap^r cfu per packaging reaction per 1 μg of insert DNA).

3.3.2.2 Amplification, storage and viability of cosmids. Amplification of the cosmid library in E. coli χ2819 resulted in an in vivo packaged recombinant cosmid suspension (7 ml), containing approximately 2.6 X 10^{10} infectious particles per ml. Different forms of stored cosmids were assayed for viability. Transformation of pooled DNA, transduction of packaged cosmid lysate, and plating of originally transduced E. coli HB101 cells showed that these forms were viable after 6 months in storage.

3.3.3 Library Characterization

3.3.3.1 Nature of inserts

(i) Plasmid libraries. Transformation of E. coli HB101 with ligated pEcoR251-T. ferrooxidans ATCC 33020 (wild-type) DNA resulted in more than 1.78 X 10^4 colonies on LA medium containing Ap. Transformation of E. coli HB101 with the pEcoR251-T. ferrooxidans Rif^r DNA ligation mixture produced in excess of 1.35 X 10^4 colonies.

The integrity of the gene banks was assessed by isolation of plasmids from randomly selected clones. Fig. 3.1 shows an

agarose gel of recombinant plasmids isolated from 10 colonies from each of the two plasmid libraries, restricted with PstI endonuclease. The presence of a 1,8 kb vector fragment confirms the pEcoR251 origin of the recombinants. Inserts of various sizes were present in all the clones. In some cases the inserts contained internal PstI endonuclease sites. Sizes of insert fragments ranged between 3 - 12 kb, with an average of 7 kb for the plasmid-wt library, and between 1 - 13 kb, with an average of approximately 5 kb, for the plasmid-Rif^r library.

Using the T. ferrooxidans genome size of 2.8 X 10^6 bp (Yates and Holmes, 1987), 1 840 clones containing an average insert of 7 kb are required for a library with a 99% confidence of isolating a given gene (P = 0.99). An average insert size of 5 kb requires approximately 2 580 clones for a library of 99% confidence.

(ii) Cosmid library. The proportion of clones carrying DNA fragments, as indicated by insertional inactivation of the Tc^r gene on pHC79, was estimated at greater than 90%. The high efficiency of cosmid inserts was confirmed by restriction analysis of plasmid DNA from sample clones. The majority of inserts were in the size range 35-50 kb, as shown in Fig. 3.2. The smallest insert measured was approximately 31 kb and the largest was approximately 58 kb, with an average size of approximately 38 kb. For a library of 99% confidence, 337 colonies carrying recombinant cosmids, with an average insert size of 38 kb, are required.

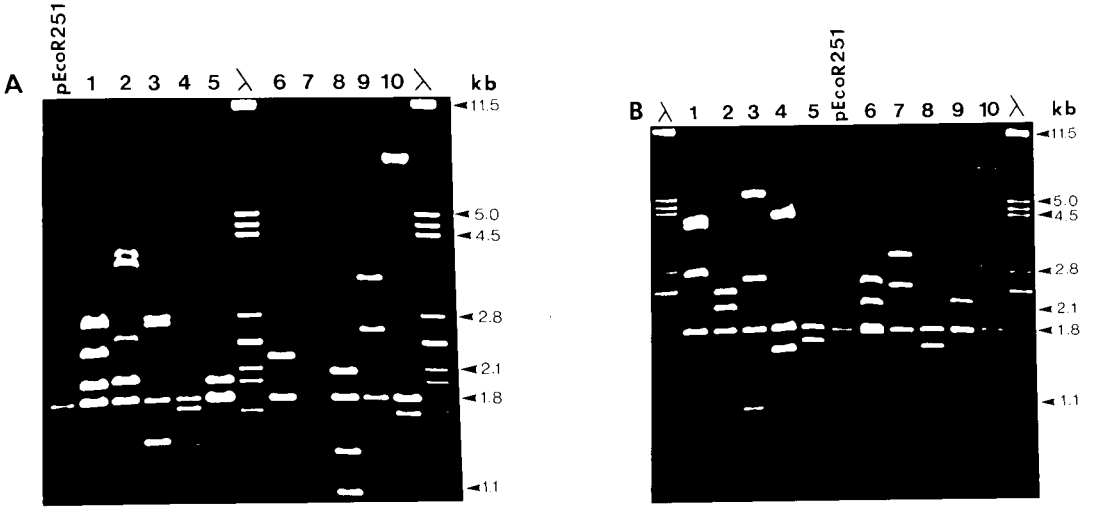

Fig 3.1 Agarose gel analysis of plasmids from a random selection of clones (1-10) from:
(A) the plasmid-wt genomic library, and
(B) the plasmid-Rif^r genomic library of *T. ferrooxidans*.

Plasmids from clones and the vector, pEcoR251, were digested with *Pst*I endonuclease to release an internal vector fragment of 1.8 kb*. The smaller vector fragment contained the *Bgl*II cloning site and increased in size according to the size of the insert (1-10 in each photograph). The inserts in (**A**) 1,2,3,4,8,9,10 and (**B**) 1,2,3,4,6,7,9 and 10, contained internal *Pst*I restriction endonuclease sites. Lanes carrying λ DNA, digested with *Pst*I endonuclease, are indicated, with the fragment sizes in kb alongside.

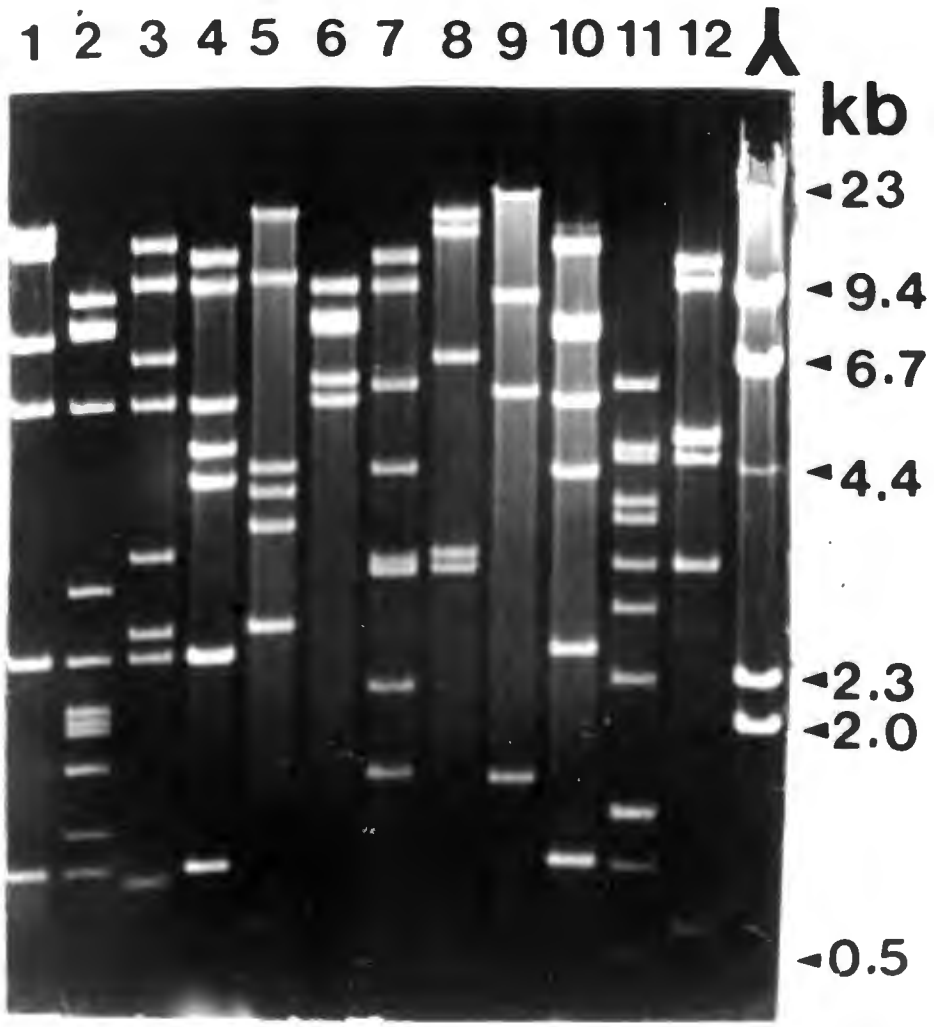

Fig. 3.2 Agarose gel analysis of plasmid DNA from 12 random clones (1-12) in the **cosmid-Rif^r genomic library** of *T. ferrooxidans* ATCC 33020. DNA was digested with *Sal*1 endonuclease. The lane carrying λ DNA digested with *Hin*dIII endonuclease is indicated, with the fragment sizes in kb alongside.

3.3.3.2 Biological evidence of bank quality. All three libraries contained recombinant clones that complemented auxotrophic E. coli mutants. The number of transformed clones on minimal medium for each of the three auxotrophs screened is listed in Table 3.2.

Table 3.2 Complementation of E. coli auxotrophic markers by T. ferrooxidans genomic libraries[1].

	LIBRARY		
	plasmid-wt	plasmid-Rif^r	cosmid-Rif^r
Leu$^+$ Ap^r	11	8	17
Pro$^+$ Ap^r	16	11	23
Gln$^+$ Ap^r	10	13	29

[1] Figures indicate the number of transformed prototrophic clones on minimal medium.

Retransformation of the appropriate E. coli auxotroph with plasmid DNA from these clones resulted in an equivalent number of colonies on both minimal medium plates and on LA plates containing Ap. This confirmed the ability of the plasmid clones to functionally complement the deficiencies in the respective E. coli auxotrophic strains.

3.3.4 Expression of MMS^r

E. coli HB101 recA$^-$ cells, transformed with DNA from a pool of the plasmid-wt library, produced 18 colonies on LA plates containing Ap and MMS. Three families of DNA fragments, as shown by restriction digests of plasmid DNA, were isolated

from the gene library indicating independent cloning events. Plasmid DNA from each of the three clones was capable of retransforming E. coli HB101 to MMS^r and Ap^r.

Transformation of E. coli HB101 with plasmid DNA from the plasmid-Rif^r library produced 11 colonies on selective plates containing Ap and MMS. These clones were not investigated further. E. coli HB101 transduced with packaged cosmids produced 27 colonies on the selective plates. One randomly chosen Ap^r MMS^r cosmid clone, pRSR500, was pursued further (Chapter 4, section 4.2.5).

3.3.5 Screening for Rif^r

E. coli HB101 or E. coli LK111 transformed with 5 μg of the plasmid-Rif^r library DNA, produced 55 ($\pm$8) colonies on LA plates containing Rif. Approximately 5% of the Rif^r clones were able to grow when subcultured onto LA plates containing Rif and Ap, suggesting the presence in these clones of recombinant plasmid DNA. Plasmid DNA isolated from these clones, however, was unable to retransform either E. coli strain to Rif^r, although Ap^r was transferred.

Transduction of E. coli HB101 with either the in vitro packaged cosmids or with the amplified cosmid lysate resulted in 45 ($\pm$12) colonies on LA plates containing Rif (25 μg/ml). Approximately 5% of the Rif^r colonies resulting from the in vitro packaged cosmid transduction, and 20% of Rif^r colonies resulting from cells transduced with the

in vivo-packaged cosmid lysate, were also Ap^r, suggesting the presence of a cosmid. Plasmid DNA from these clones retransformed *E. coli* HB101 and *E. coli* LK111 to Ap^r, but failed to retransform the Rif^r phenotype. The number of *E. coli* Rif^r colonies on selective plates indicated above represents the average of eight independent cosmid-Rif^r library transductions and 14 plasmid-Rif^r library transformations.

The similar number of Rif^r colonies resulting from using either the plasmid or the cosmid-Rif^r library suggested that the *E. coli* Rif^r colonies on selective plates were spontaneous mutants. This was confirmed by similar numbers of Rif^r colonies appearing on selective plates spread with *E. coli* cells that were not exposed to DNA.

3.4 DISCUSSION

The number of clones obtained for each of the libraries in the present study is several-fold higher than that required for a library of 99% confidence. In the case of the cosmid library there is approximately a 120-fold excess of clones in the primary *in vitro*-packaged suspension alone. The number of clones in each library, together with the range of fragments evident from restriction digests of recombinant DNA from random clones, is indicative of libraries of sound statistical confidence. The reliability of the libraries was corroborated by the isolation of prototrophs of auxotrophic strains of *E. coli* after either transduction with the packaged cosmid library, or transformation with either of the plasmid-based libraries.

The expression of *T. ferrooxidans* amino acid markers in *E. coli* indicated that some steps in anabolic pathways in both *E. coli* and *T. ferrooxidans* may be functionally analogous. This was previously shown to be the case for GS (Barros *et al.*, 1985).

The isolation of 18 MMS^r clones from the plasmid-wt library was a further indication of the functional integrity of the library. Characterization of these MMS^r clones is the subject of the next chapter. Functional integrity of the plasmid-Rif^r and cosmid-Rif^r libraries was also supported by the isolation of MMS^r clones.

Despite exhaustive screening in the present study, a gene that could confer Rif^r to *E. coli* cells was not isolated from the *T. ferrooxidans* Rif^r libraries. Because of the integrity of both the plasmid- and cosmid-Rif^r libraries, it is unlikely that the appropriate locus was not cloned. A cloned Rif^r marker may not be expressed in *E. coli* for several reasons.

Rif^r mutants of *E. coli* arise spontaneously at a frequency of approximately 10^{-8} (Kirschbaum and Konrad, 1973). The *rif*^{r} mutations are recessive to the wild-type *rif*^{s} allele since *rif*^{r}/*rif*^{s} partial heterodiploids fail to grow under Rif selection. However *rif*^{r} mutations which are dominant (*rif*^{d}) to the wild-type *rif*^{s} allele, occur spontaneously at a frequency of approximately 10^{-9} - 10^{-10} (Kirschbaum and Konrad, 1973). The Rif^r marker which had previously been cloned in *E. coli* was a *rif*^{d} mutation (Kirschbaum and Konrad, 1973). When present in a partial diploid state, as on a plasmid, in a sensitive strain, expression and function of the mutant protein was found to override the Rif^s native *rpoB* gene product. The nature of the *T. ferrooxidans* locus could not be determined with reference to whether it was a dominant or recessive allele.

Detection of a cloned Rif^r phenotype in *E. coli* also depends on the transcription of the modified *T. ferrooxidans rpoB* gene, its subsequent translation, and assembly into a functional RNA polymerase. Impedance at any one of these

levels would fail to produce a Rif^r phenotype. Even if the cloned T. ferrooxidans rpoB gene were transcribed at levels comparable to the native E. coli rpoB gene product, phenotypic detection requires that the T. ferrooxidans rpoB gene product is sufficiently similar to the E. coli β subunit to form an active Rif^r RNA polymerase holoenzyme.

The structural similarity of RNA polymerase in a wide range of organisms, and the finding that subunits of such diverse organisms as M. luteus and E. coli could form enzymatically active hybrid RNA polymerases in vitro, suggested that functional T. ferrooxidans-E. coli hybrid RNA polymerases could be assembled in E. coli. The results of Lill et al. (1975), however, indicated that a hybrid of E. coli RNA polymerase assembled with the β subunit of M. luteus, which is much larger than its E. coli counterpart, exhibited minimal enzymatic activity. These workers concluded that the β subunit of M. luteus cannot replace the β subunit in E. coli RNA polymerase under the conditions they used. Addition of an excess of M. luteus β subunits did not increase enzymatic activity. In contrast a hybrid M. luteus RNA polymerase, which carries the E. coli β subunit, in spite of its different net charge, was enzymatically active. This suggested that RNA polymerase subunits may be exchanged at least in one direction between bacterial RNA polymerases (Lill et al., 1975).

Lill et al. (1975) showed that although subunits of RNA polymerase from different organisms form catalytically

active hybrids *in vitro*, their rate of formation or assembly was significantly slower than the reconstitution of the *E. coli* enzyme from its subunits. These results suggested a strong affinity of *E. coli* RNA polymerase subunits for one another to the exclusion of the *M. luteus* subunits.

Unlike *M. luteus*, *E. coli* and *T. ferrooxidans* are both Gram-negative bacteria and the similarity between their respective RNA polymerase subunits was expected to be greater. Nevertheless, if the *in vitro* *M. luteus* and *E. coli* results are a reflection of RNA polymerase assembly within *E. coli*, the *E. coli* Rif^s β subunit may be incorporated to the exclusion of the *T. ferrooxidans* Rif^r β subunit. Further work would require *in vitro* reconstitution studies of the *T. ferrooxidans* β subunit with the α , β' and σ subunits of *E. coli* RNA polymerase to test the viability of such a hybrid assemblage, as described by Lill *et al.* (1975).

Most important in an attempt to isolate a homologous Rif^r marker for use in *T. ferrooxidans* is the identification of a *T. ferrooxidans* rif^d mutant. This requires a method, not yet available, to differentiate between dominant and recessive loci in *T. ferrooxidans*. In *E. coli*, a rif^d mutation occurs in about 1 - 10 % of rif^r mutations (Kirschbaum and Konrad, 1973). If this is also the case in *T. ferrooxidans*, isolation of the rif^d allele through the construction of a number of genomic libraries from individual *T. ferrooxidans* Rif^r clones, and their screening

in E. coli, would be required. However, the potential problems of heterologous gene expression, and assembly of the T. ferrooxidans β subunit into the E. coli RNA polymerase holoenzyme, make such an investigation impractical.

Because Rif is an effective antibiotic in iron- and sulphur-based media, and because no other homologous marker has been identified, it is necessary to consider the E. coli rif^d allele for use as a marker in T. ferrooxidans. A heterologous Rif^r marker falls short of the ideal of using a homologous marker for the development of a genetic system for T. ferrooxidans. However, the E. coli chromosomal Rif^r marker has some advantages over the Tc^r and Cm^r plasmid-derived markers on the pDER plasmids (Rawlings and Woods, 1985), described in Chapters 1 and 2.

The E. coli Rif^r marker is a chromosomal allele and presumably has an analogous Rif^s counterpart in T. ferrooxidans. Chromosomal genes from T. ferrooxidans, so far characterized, have shown a strong conservation of nucleotide and derived amino acid sequences with analogous genes from E. coli (Pretorius et al., 1986; Rawlings et al., 1987; refer to Chapter 5). In addition, RNA polymerase subunits have been shown to be structurally conserved in a wide variety of prokaryotes (Burgess et al., 1976). Since Rif^r is known to occur in T. ferrooxidans, the expression of the E. coli rif^d allele would not require the de novo

synthesis of a foreign resistance mechanism in *T. ferrooxidans*, as is the case with the Tc^r and Cm^r genes.

The results of Lill *et el.* (1975), discussed earlier, indicated that assembly of a hybrid RNA polymerase from subunits of the Gram-positive *M. luteus* and the Gram-negative *E. coli* was partially functional. It may be expected that a *T. ferrooxidans* RNA polymerase holoenzyme that contains an *E. coli* β subunit may be more efficiently functional than the *M. luteus*-*E. coli* hybrid enzyme. *E. coli* and *T. ferrooxidans* are both Gram-negative organisms. Unless a more suitable marker for use in *T. ferrooxidans* is described, the use of the *E. coli* rif^d allele should be pursued.

The *E. coli* rif^d allele is available on a plasmid and may be cloned into one of the *T. ferrooxidans* plasmids described in Chapter 2. Since the *T. ferrooxidans* plasmids replicate, and are transcribed in *E. coli*, the effect of plasmid copy number on rif^d expression in *E. coli* may be assessed. If the rif^d allele, on a *T. ferrooxidans* plasmid, is expressed in *E. coli*, then the plasmid may be used in attempts to transform *T. ferrooxidans*.

Apart from its use for the isolation of individual genes, a cosmid library of the *T. ferrooxidans* genome is of importance in investigations requiring large segments of cloned DNA. So far, genes isolated from *T. ferrooxidans* have not been spatially mapped on the *T. ferrooxidans*

chromosome. This is mainly due to the small number of T. ferrooxidans genes that have been isolated and identified. With the characterization of more genes, the cosmid bank will be of direct application in that it will provide an opportunity to "walk" along the T. ferrooxidans chromosome. Clones from a cosmid library may be used as hybridization probes to identify overlapping clones and in this way used to map the chromosome. Although chromosomal "walking" is possible using the pEcoR251-based banks, this process is much facilitated using larger fragments of DNA.

CHAPTER 4

CLONING AND CHARACTERIZATION OF A *recA*-LIKE GENE from *T. ferrooxidans*

CHAPTER 4

CLONING AND CHARACTERIZATION OF A *recA*-LIKE GENE from *T. ferrooxidans*

Summary. Three recombinant plasmids, pRSR100, pRSR101, and pRSR102, each containing the functional analogue of the *E. coli recA* gene, were isolated from a genomic library of *T. ferrooxidans* ATCC 33020. The plasmid, pRSR100, was used for further characterization of the cloned *recA*-like gene. pRSR100 complemented defects in DNA repair and homologous recombination in an *E. coli recA*$^-$ strain. Antiserum raised against *E. coli* RecA protein reacted with two protein bands with an apparent M_r of approximately 40 000 and 38 000 in extracts of the *recA* deletion mutant, *E. coli* JK696 containing pRSR100. A single band with an apparent M_r of approximately 40 000 was detected in *T. ferrooxidans* cell extracts with the *E. coli* RecA antiserum.

4.1 INTRODUCTION

In *E. coli*, the *recA* gene product is required in DNA repair (Hanawalt *et al.*, 1979), homologous recombination (Radding, 1978) and for the induction of the SOS response to DNA damage (Radman, 1975). The SOS response, under the control of the *recA* and *lexA* gene products, includes phenomena such as prophage induction, inhibition of cell division (filamentation), abnormal reinitiation of DNA replication, DNA repair, halting of cellular respiration, and enhanced

mutagenesis (Radman, 1974; 1975; Little and Mount, 1982; Walker, 1984; 1985)).

The crucial role of *recA* was indicated by the phenotypes of strains carrying various alleles of this gene. Initially, *E. coli* strains that were defective in homologous recombination were designated *recA*$^-$ (Clark and Margulies, 1965; Clark, 1973). Subsequently, it was shown that in *recA*$^-$ mutants, none of the SOS phenotypes could be induced (Witkin, 1976), and the *recA* gene was not derepressed after inducing treatments (Gudas and Pardee, 1975). Mutations in the *recA* gene were recessive to *recA*$^+$, suggesting that the RecA protein is a positive control element in SOS regulation.

Mutations in the *lexA* gene resulted in either wholly repressed or constitutive expression of SOS genes and led to the discovery of its regulatory function in the SOS system (Radman, 1974; Walker, 1984). Mutants of *lexA*, such as *lexA* (Ind$^-$), were dominant to *lexA*$^+$, and had the effect of preventing the expression of the SOS response (Mount *et al.*, 1972; Gudas and Pardee, 1975). In contrast, *lexA*(Def) strains were constitutive for *recA* and SOS functions (Gudas and Pardee, 1975; Gudas and Mount, 1977). The phenotype of mutant *lexA* alleles suggested that the wild-type LexA protein is a negative control element in SOS induction. The recombination proficiency of *lexA*(Ind$^-$) mutants indicated that a deficiency in SOS induction was not associated with a deficiency in homologous recombination.

The observation that lexA(Def) recA(Def) double mutants constitutively synthesized high levels of RecA protein strongly suggested that LexA functioned as a repressor of the recA gene, and that the RecA protein was involved in the inactivation of the LexA protein during SOS induction (Gudas and Mount, 1977). It was therefore shown that although the SOS response may involve a wide variety of complex biochemical pathways, it is governed by a system of gene regulation involving the products of at least two genes, recA and lexA.

4.1.1 Model of SOS regulation

LexA protein is a repressor that directly regulates the expression of a group of unlinked genes. In an uninduced cell, the repressor binds to operator sequences in front of each gene involved in the SOS response (Little and Mount, 1982; Walker, 1984). The recA and lexA genes are themselves subject to LexA repression. DNA damage or treatments that interfere with DNA replication generate an inducing signal which reversibly activates the small amount of constitutive cellular RecA protein to a proteolytic state. The activated RecA proteins cleave LexA repressor (Little and Mount, 1982; Walker, 1984). Under these conditions, repressors of temperate phages such as λ and P22 are also cleaved, causing induction to lytic growth of these prophages (Craig and Roberts, 1980; Phizicky and Roberts, 1981).

Cleavage by RecA protein results in a decrease in the pool of LexA molecules in the cell. Various SOS genes, including recA, are derepressed, resulting in the expression of SOS functions (Little and Mount, 1982; Walker, 1984). When the threat to the cell is removed and damage is repaired, the level of the signal molecule decreases. RecA protein loses its protease activity and LexA repressor accumulates, exerting its repressor role in the cell. The activity of the SOS system is, therefore, under the strict control of RecA protein in its active protease state (Little et al., 1980)

4.1.2 The recA gene product

The recA gene product in E. coli is a protein of 37 800 daltons (Horii et al., 1980; Sancar et al., 1980). Purified RecA protein can catalyze DNA-dependent hydrolysis of nucleoside triphosphates (NTPs) (Phizicky and Roberts, 1981), proteolysis of regulatory proteins (Little et al., 1981; Phizicky and Roberts, 1980) and DNA strand exchanges analogous to the process in vivo (McEntee et al., 1979).

It has been shown that the cleavage of LexA protein and λ repressor in vivo does not depend on the synthesis of new RecA protein (Baluch et al., 1980; Moreau et al., 1980; Little, 1983), but rather on the activation of RecA to its proteolytic state. This proteolytic state is independent of levels of RecA in the cell, since overproduction of RecA

protein alone does not induce the SOS response (Uhlin and Clark, 1981).

LexA protein, like λ repressor, has been shown to be active in its dimeric form, the formation of which is dependent on an intact carboxyl end (Brent and Ptashne, 1981; Brent, 1983). Cleavage of the LexA protein *in vitro* is dependent on both RecA protein and ATP. LexA protein is cleaved at an -Ala-Gly- peptide bond near the middle of the protein (Horii *et al.*, 1981). In the reaction, intact LexA protein is gradually cleaved into two smaller protein fractions, which can no longer function as a repressor (Little *et al.*, 1981).

Little (1984) has demonstrated RecA-independent cleavage of LexA and λ repressors *in vitro* under alkaline conditions in the presence of a divalent cation. These results suggest that instead of being directly involved in proteolysis, RecA may play an indirect role. Activated RecA protein could act as an effector molecule which, once bound to LexA, results in autodigestion at the -Ala-Gly- cleavage site.

Irrespective of their precise molecular interaction, it is evident that the interplay of the RecA protein and the LexA repressor constitute the primary control mechanism for the SOS system. Proteolysis of LexA protein and some phage repressors requires activated RecA protein. A detailed understanding of the precise mechanism by which the various SOS phenotypes are expressed is lacking.

It is still not clear what agent mediates or initiates the activation of RecA to its proteolytically active form. The current view is that the inducing signal *in vivo* is either ssDNA, or dsDNA containing single-stranded gaps (Walker, 1984; 1985). These molecules would occur in a cell as a result of either, (i) post-damage DNA processing, (ii) replicative arrest, or (iii) abnormal replication fork movement (Roberts *et al.*, 1982; Walker, 1985).

The *ssb* gene, which codes for a major single-strand binding (SSB) protein, has been implicated as a factor in the induction of the SOS response, since mutations in this gene lead to defects in SOS induction (Vales *et al.*, 1980; Whittier and Chase, 1981; Meyer *et al.*, 1982; Lieberman and Witkin, 1983). The function of the SSB protein may be either to preserve ssDNA-signal molecules, by binding to them, or alternatively to enhance the binding of RecA protein to the signal molecules, thereby protecting them from degradation by exonuclease V (RecBC DNase) (Williams *et al.*, 1981). It has also been suggested that the active entity for SOS induction *in vivo* is a quarternary complex of SSB, RecA, ssDNA and a NTP (Cohen *et al.*, 1983).

Little is known about the exact target site for the inducing signal. The most likely target is RecA, since this molecule responds directly to DNA damage and its activation results in the induction of the SOS response (Little and Mount, 1982).

4.1.3 Cellular functions involving RecA protein

Although the RecA protein is involved in a wide variety of cellular functions, only some functions which are tested by investigators for the verification of recA-like genes will be described here.

4.1.3.1 Generalized or homology-dependent recombination. Recombination at the molecular level involves genetic exchange by the breakage and reunion of DNA molecules in a process which occurs often and results in the pairing and rearrangement of genetic material (Radding, 1982).

The recombination process involves the formation of a joint molecule from two homologous DNA molecules. Recombination may occur between two pairs of double helices or between a single DNA strand and parts of a double helix (Holliday, 1974; Das Gupta et al., 1981; Radding, 1982; Howard-Flanders et al., 1984; Cox and Lehman, 1987). The process involves the release of complementary strands from the double-helical conformation and the subsequent coalescence of a hybrid double helix. This intermediate must be converted from the joint structure into a covalently-linked hybrid-DNA double helix. Three repair steps, excision, repair replication, and ligation are required to mediate this process.

To establish the function of RecA protein it was shown that no reaction occurred between two homologous but completely

base-paired helices mixed with RecA protein (Howard-Flanders, 1981; Howard Flanders et al., 1984). However, RecA protein recognized ssDNA and matched it to a complementary sequence in a homologous duplex, simultaneously displacing the resident strand.

In the presence of ATP, the RecA protein may unwind different parts of the duplex in an attempt to locate a sequence complementary to the ssDNA bound by the RecA cluster (Howard-Flanders et al., 1984). Once located, the pairing of complementary sequences is driven by ATP in the 5′ - 3′ direction with reference to the RecA-bound single strand. During pairing and formation of the new hybrid, RecA protein is displaced.

The original discovery of recombinationless mutants of E. coli, which were unable to produce genetic recombinants in conjugational crosses was traced back to mutations in several bacterial genes which were subsequently designated rec (Clark and Margulies, 1965; Witkin, 1969). In conjugation experiments, rec^- mutants conjugated and accepted DNA from the donor, but they were unable to integrate this into the recipient genome unless the rec^+ allele also entered the cell.

Analysis of the recombination system through the use of mutants revealed that the rec genes control the formation of enzymes necessary for the recombination process (Das Gupta et al, 1981; Cox and Lehman, 1987). The product of the recA

gene catalyzes the renaturation of DNA with a concomitant hydrolysis of ATP. The recB and recC genes code for subunits of exonuclease V (RecBC DNase), a DNA dependent-ATPase, that functions as both an exo- and an endonuclease, as well as a helicase (Taylor and Smith, 1980; Telander-Muskavitch and Linn, 1982; Llyod and Thomas, 1983). This enzyme hydrolyzes ssDNA molecules. Exonuclease V is therefore useful in removing excess single-stranded unpaired regions of DNA after homologous-sequence matching and renaturation of DNA during the recombination process.

In addition, the products of four different genes, recF, recJ, recN, and ruv are required (Walker, 1985). Most of these proteins that participate in conjugal recombination, play roles in post-replication repair of daughter-strand gaps, and double-strand breaks (Walker, 1985). It would appear that these types of DNA damage are sufficiently like intermediates encountered in conjugal homologous recombination that certain recombination proteins can process both.

It is known that crossing over or recombination is stimulated by breaks or discontinuities in DNA. Agents that damage DNA to create double-strand breaks or single-strand gaps increase crossing over phenomena (Walker, 1984; 1985; Friedberg, 1985).

4.1.3.2 SOS functions

(i) Identification of SOS genes. Genes involved in the SOS-response have been identified using the Mu dl (Ap *lac*) transducing phage to construct operon fusions *in vivo* (Kenyon and Walker, 1980). Mu dl (Ap *lac*) carries the structural genes for the *lac*-operon and integrates into the *E. coli* chromosome at random (Casadaban and Cohen, 1980). Because the structural *lac* genes, without a promoter, are present on the phage, β-galactosidase can only be expressed if the phage inserts in the correct orientation into a chromosomal transcription unit. Such an insertion creates an operon fusion in which the synthesis of β-galactosidase is under the control of a cellular regulatory locus (Casadaban and Cohen, 1980).

Random screening of Mu dl (Ap *lac*) phage-generated fusions in the *E. coli* chromosome, in the presence of the DNA damaging agent, mitomycin C, revealed a set of at least 17 *din* (*d*amage *in*ducible) genes (Kenyon and Walker, 1980; Walker, 1984; Peterson *et al.*, 1988). Although the physiological roles of most of the *din* genes identified in this way are known, there are several whose nature and function remain to be established (Walker, 1984; Peterson *et al.*, 1988).

(ii) Prophage induction. This phenomenon has been the subject of intensive investigation and is currently the best understood response to DNA damage. It has been shown that

proteolytic RecA alone is sufficient for prophage induction (Craig and Roberts, 1981; Phizicky and Roberts, 1981). The λ repressor exists in both a monomeric and dimeric form. In E. coli λ lysogenic strains, most of the repressor occurs as dimers, the active form that binds to phage operators. It is the amino-terminal end of the monomer which determines recognition of the operator sites, while the carboxy-terminal end is responsible for chemical recognition and dimerization between monomers (Sauer et al., 1979). A hinge region that is sensitive to many proteases lies between these two domains and is the cleavage site for RecA protease. Cleavage by the RecA protein prevents repressor dimerization, thereby inactivating the repressor (Little and Mount, 1982; Slilaty et al., 1986).

Cleavage of λ repressor by purified RecA protein is dependent on the presence of ATP and nucleotide polymers (Craig and Roberts, 1980; 1981). These include oligonucleotide hexamers, circular DNA molecules, polyribonucleotides and polydeoxyribonucleotides (Craig and Roberts, 1980).

(iii) DNA repair. In E. coli, most of the inducible genes coding for DNA repair proteins are members of two major regulatory pathways that can be induced by DNA damage. These pathways are, (i) the adaptive response network, which is controlled by the Ada protein (Lindahl, 1982; Walker, 1984; Walker, 1985), and (ii) the SOS network, which is controlled by the RecA and LexA proteins (Radman, 1974;

1975; Witkin, 1975; Howard-Flanders, 1981; Walker, 1984; 1985). RecA-dependent mechanisms are involved in excision repair and postreplication repair of damaged DNA. Because of the double-stranded nature of DNA, the information necessary to repair a damaged segment is present in the complementary strand (Hanawalt et al., 1979; Grossman, 1981). This is restricted to damage of bases on one or the other strand. Several pathways exist within the SOS response by which a damaged base or segment of DNA is removed or replaced.

Excision repair. Apart from mutations in the photolyase gene (phr), mutations in E. coli rendering it sensitive to UV light (i.e. defective in UV-damage repair), map in three genes; uvrA, uvrB, and uvrC, which are known to be under LexA and RecA control (Sancar and Rupp, 1983). The repair system encoded by these genes senses damage-induced distortion of the DNA helix. The uvrA, uvrB and uvrC genes each encode a single subunit of an enzyme that removes a damaged DNA segment by cutting the strand that contains it (Sancar and Rupp, 1983). UvrABC enzyme does not detect a lesion directly, but acts on the secondary conformation effects that result from the lesions. The functions of the UvrA, UvrB and UvrC subunits in the UvrABC complex have been proposed (Seeberg and Steinum, 1983).

The UvrA subunit binds nonspecifically to DNA. Association of UvrA with UvrB results in the translocation of the UvrAB complex to a site of DNA damage. In the presence of UvrC

the UvrABC complex incises DNA. The UvrABC enzyme cuts on either side of a lesion, e.g. a pyrimidine dimer, specifically removing an oligonucleotide containing the lesion. The corresponding gap is filled by DNA polymerase I, and requires the action of the UvrD protein (DNA helicase II) (Caron et al., 1985). The nick in the repaired strand is sealed by DNA ligase (Cox and Lehman, 1987).

Excision repair, mediated by the UvrABC enzyme system, mostly involves short patches of approximately 20 nucleotides, although patches can be longer than 1 500 nucleotides (Kuemmerle et al., 1981; Cooper, 1982).

Induced recombinational repair. Recombinational repair involves mechanisms whereby cells resume synthesis on templates containing pyrimidine dimers or other bulky lesions and in so doing enhance their potential for survival (Rupp and Howard-Flanders, 1968). Reinitiation of DNA synthesis occurs downstream from the replicative blocks, creating gaps or discontinuities in the newly-synthesized daughter strands.

Evidence for this excision-independent cellular response was provided by the observation that in UV-irradiated excision-repair-defective (uvr$^-$) E. coli cells, the average size of newly-synthesized daughter strands was significantly smaller than that in unirradiated cells (Rupp and Howard-Flanders, 1968; Waldstein et al., 1974). With increasing time after irradiation, the newly-synthesized daughter strands

increased in length, eventually attaining the size of those synthesized in unirradiated cells. It was evident that the DNA synthesized on damaged templates contained discontinuities. The subsequent increase in the sedimentation rate of the daughter strands indicated that the gaps had disappeared (Waldstein *et al.*, 1974).

Gap filling in nascent DNA depends on the recombinase properties of the RecA protein. Smith and Meun (1970) found that *recA*$^-$ cells which were deficient in generalized recombination, failed to convert short, intergap nascent DNA strands into high M_r products.

Evidence for DNA strand exchange during semiconservative replication of damaged DNA comes from the analysis of the distribution of pyrimidine dimers in the progeny of UV-irradiated cells. During successive rounds of DNA replication pyrimidine dimers should remain confined to the original parental strands. However, these lesions are in reality equally distributed between parental and daughter strands (Ganesan, 1974; Ganesan, 1975). This is consistent with the notion that strand exchanges accompany the replicative response in the presence of pyrimidine dimers. During this process the original lesions are not removed from the cell's genetic material but are redistributed within the DNA. This process is therefore a damage tolerance mechanism, allowing the replication of DNA, until the lesion is removed totally from the DNA. Once strand exchange has occurred the lesions in the original strand and

those in daughter strands may be removed by excision repair (Howard-Flanders, 1981; Walker, 1985).

It is thought that double-stranded breaks, such as those caused by X-irradiation, may be repaired using a similar mechanism (Friedberg, 1985; Walker, 1985). Exonuclease degradation from each end of the break could expose single-stranded regions which may independently invade a homologous sequence, forming two adjacent recombination joints. Initiation may also be provided by exonuclease V by extending each free end of the damaged DNA. The two joints may be cut and the parental helices separated, with the resultant gaps being filled by DNA polymerase, and sealed by DNA ligase.

The predominant mechanism of daughter-strand gap repair appears to be mediated through the RecF pathway of recombination (Walker, 1985). RecA, RecF and Ruv proteins function directly in daughter-strand gap repair. Repair of double-strand breaks is thought to require functional RecA and RecN proteins (Picksley *et al.*, 1984). RecB has also been shown to be functional in the repair of certain double-strand breaks (Walker, 1985).

Eight genes, including *recA*, *recF*, *recJ*, *recN*, *recO*, *recQ*, *ruv*, and *uvrD* have been identified as members of the RecF pathway (Walker, 1985; Peterson *et al.*, 1988). The genes, *recN*, *recQ*, *ruv* and *uvrD* are part of the SOS regulon (Peterson *et al.*, 1988). RecF is not and the status of the

remaining genes is unknown. Mutations in the RecF pathway genes have little affect on recombination in otherwise wild-type cells, suggesting that the major role of these recombination proteins is in DNA repair (Walker, 1985).

SOS mutagenesis. One of the coordinately-inducible SOS functions is SOS mutagenesis, or SOS processing (Radman, 1974; Witkin, 1976). This system has until recently been called error-prone repair. However, induced mutagenesis may not be a form of DNA repair, but rather it may be a mechanism of damage tolerance (Peterson *et al.*, 1988). SOS mutagenesis involves a mechanism that processes damaged DNA in such a way that mutations result. This occurs by single-base substitutions at the site of DNA damage. This function was reported to be responsible for the mutagenic action of agents such as UV irradiation, NQO, and aflatoxin B1 (Walker, 1985). SOS mutagenesis was only observed in *recA*$^+$ *lexA*$^+$ cells which indicated that the processing of damaged DNA to give rise to mutations was influenced by these SOS regulatory genes.

Apart from control by the RecA and LexA proteins, UV mutagenesis in *E. coli* is under the control of the *umuC* and *umuD* (for **U**V non**mu**table) genes (Walker, 1984, 1985; Bridges and Woodgate, 1985). Cells carrying a mutation in either of these genes were more sensitive to UV killing than were wild-type cells, and non-mutable with a variety of agents including UV and NQO (Kato and Shinoura, 1977; Steinborn, 1978) and MMS (Walker and Dobson, 1979). This implicated

umuC and umuD in ensuring survival by enhanced mutagenesis. Since umuC and umuD are SOS genes, activated RecA protein is required for the induction of SOS-mutagenesis.

It has been shown that some naturally occurring plasmids have the ability to make strains of E. coli and Salmonella typhimurium susceptible to mutagenesis (Walker, 1984). Some of these plasmids were found to carry two genes, mucA and mucB, which are analogues of the E. coli chromosomal umuD and umuC genes (Walker, 1984).

Current models suggest that during SOS induction the fidelity of DNA polymerase is relaxed, possibly by the UmuDC proteins, permitting DNA synthesis and misincorporation of nucleotides opposite lesions in the template strand (Bridges and Woodgate, 1985; Hutchinson and Wood, 1986; Lu et al., 1986). Another suggestion is that umuC and umuD encode an entirely new DNA polymerase that is more error prone than the normal cellular DNA polymerase (Walker, 1985). Furthermore, the direct involvement of the recA gene product in SOS mutagenesis has been implicated. Lu et al. (1986) demonstrated that RecA binds to UV-induced photoproducts. The binding of RecA to damaged DNA may assist DNA polymerase in by-passing lesions (Lu et al., 1986).

In summary, it has been established that in E. coli a number of genes involved in different modes of DNA repair, mutagenesis, and recombination are under the direct control of the recA and lexA gene products. The RecA protein is a

positive regulatory factor which is capable of functioning as a protease and a recombinase.

4.1.4 *recA*-like genes in other organisms

B. subtilis has been shown to possess an inducible system (SOB) analogous to the SOS response in *E. coli*. The SOB system in *B. subtilis* functions under the control of the product of the *recE* gene (Love and Yasbin, 1986). The *recE* gene product has been shown to be immunologically related to the RecA protein of *E. coli*.

Reports indicate the presence of RecA-like proteins in a wide variety of Gram-negative and Gram-positive heterotrophic bacteria (Clark and Margulies, 1965; Hofemeister, 1977; Notani and Setlow, 1980; Better and Helinski, 1983; Keener *et al.*, 1984; Kokjohn and Miller, 1985; Ohman *et al.*, 1985; Goldberg and Mekalanos, 1986; Goodman *et al.*, 1987; Resnick and Nelson, 1988) and at least three cyanobacteria (Owttrim and Coleman, 1986; Geoghegan and Houghton, 1987; Murphy *et al.*, 1987). These studies have demonstrated that cloned *recA* genes from several prokaryotes are able to functionally complement *recA* mutants in *E. coli*.

Although there is little DNA sequence homology between *recA* genes from different species (as determined by Southern hybridization studies), the recombinase and protease activity of the *E. coli recA* gene product was restored to

varying degrees in E. coli recA$^-$ strains carrying the cloned heterologous recA or recA-like genes (Keener et al., 1984).

4.1.5 The present study

Three clones which exhibited Ap^r and MMS^r were isolated from a plasmid library of T. ferrooxidans ATCC 33020 (Chapter 3). Plasmids from the clones were mapped by restriction analysis and characterized further. In order to prove any similarity between the cloned MMS^r-conferring character and that of the E. coli recA gene, functional tests were carried out. The cloned T. ferrooxidans MMS^r-conferring gene was investigated for its ability to complement protease and recombinase functions in E. coli recA mutants. Structural similarity between the product of the cloned T. ferrooxidans gene and the RecA protein of E. coli was investigated in Western blot studies using antibodies raised against E. coli K12 RecA protein.

4.2 MATERIALS AND METHODS

4.2.1 Bacterial strains, bacteriophages and plasmids

The bacterial strains, bacteriophages and plasmids used in this study are listed in Table 4.1.

4.2.2 Media and reagents

E. coli strains were grown in LB medium (Appendix B). When required, Ap (100 μg/ml) was added to the cultures. MMS, at 0.01% (v/v), was used to select for the recA$^+$ phenotype. Freshly prepared LA medium containing MMS at a final concentration of 0.01% (v/v) was used for initial screening of the recombinant T. ferrooxidans chromosomal library as described in Chapter 3. Minimal medium (Appendix B), to which thiamine (5 μg/ml) Pro (20 μg/ml), Leu (20 μg/ml) and Sm (30 μg/ml) were added as required, was used in bacterial conjugation experiments.

4.2.3 Preparation of DNA and cloning techniques

Construction of the T. ferrooxidans genomic library has been described in Chapter 3. The three Ap^r MMS^r-conferring plasmids isolated from the plasmid-wt library (Chapter 3) were used in this study. Cloning techniques are described where necessary.

Table 4.1 Bacterial strains, bacteriophages and plasmids

Strain, phage or plasmid	Relevant genotype	Source or reference
E. coli BHB2600	803 *supE*$^+$ *supF*$^+$ r_k^- m_k^+ *met*$^-$	Hohn (1979)
E. coli CSH62	HfrH	Cold Spring Harbor Laboratory[1]
E. coli HB101	*leuB6 hsdS20 proA2 supE44 rpsL20* (Sm^r) *recA13*	Boyer and Roulland-Dussoix (1969)
E. coli JK696	*pro endA hsdR recA* (Δ*recA* by Tn*10*::*srb* curing)	J. Konisky[2]
E. coli RR1	as for HB101 but *recA*$^+$	Bolivar *et al.* (1977)
T. ferrooxidans	wild-type	ATCC33020
Phages		
λ	wild-type	H. Echols[3]
λ	P80 cI857 *bio-10* (*red gam*)	Cohen (1983)
P1	wild-type	
Plasmids		
pRSR100	Ap^r MMS^r	this study
pRSR101	Ap^r MMS^r	this study
pRSRE102	Ap^r MMS^r	this study
pRSR500 (cosmid)	Ap^r MMS^r	this study
pDR1453	Tc^r *recA*$^+$	Sancar and Rupp (1979)

[1] Cold Spring Harbor Laboratory: Cold Spring Harbor, New York, U.S.A.
[2] J. Konisky: University of Illinois-Urbana, Champagne, Illinois, U.S.A.
[3] H. Echols: University of California-Berkeley, California, U.S.A.

4.2.4 Restriction endonuclease mapping

Single and double digests of plasmid DNA with a variety of restriction enzymes as described in Appendix A.2, followed by separation of restriction fragments by agarose gel electrophoresis (Appendix A.3) were used to establish the restriction maps of the plasmids. Restriction fragment sizes were estimated by comparison with λ DNA digested with PstI and HindIII endonucleases.

4.2.5 Southern blotting and DNA hybridization

Details of the technique are provided in Appendix A.7. T. ferrooxidans genomic DNA, purified plasmid pRSR100, and the cosmid, pRSR500 (section 3.3.5), were digested with the appropriate restriction enzymes and the digested DNA fragments were fractionated by agarose gel electrophoresis. The gels were denatured in situ and transferred to Hybond-N nylon hybridization membrane (Amersham Corp., Buckinghamshire, U.K.).

Plasmid pRSR100 was nick-translated and labelled with [α-^{32}P]dCTP (Amersham Corp.) to a specific activity of approximately 10^7 cpm/μg. Hybridization was carried out at 60^0C overnight. Solutions of increasing stringencies were used to wash the filters. Autoradiography was performed for 48 h with Kodak XAR 5 X-ray film and intensifying screens.

4.2.6 Assays for inferred protease activity

4.2.6.1 UV, MMS and NQO sensitivity. Cells for UV-sensitivity testing were grown to an OD_{600} of 0.2 in LB medium, harvested by centrifugation and resuspended in an equal volume of Ringer's solution. Samples (5 ml) were placed in a Petri dish and irradiated with UV light, using a Cole-Palmer Series 9815 germicidal lamp (Cole Palmer Instruments Co., Chicago, Illinois, U.S.A.). The UV fluence was determined with a Blak-Ray Ultraviolet Meter (Ultraviolet Products, Inc., San Gabriel, California, USA). The number of survivors was determined by plating on LA medium. All manipulations were carried out in subdued lighting to prevent photoreactivation of DNA damage in the cells. LA plates, spread with cells, were incubated in the dark.

Resistance to MMS and NQO was determined by streaking freshly grown cells onto the surface of LA plates containing one of a range of concentrations of either MMS (between 0,001 and 0,5%, v/v) or NQO (between 0.01 and 10 μg/ml).

4.2.6.2 Prophage induction. Bacteriophage λ lysogens of appropriate strains were constructed using a modification of the method of Arber et al. (1983), as described in Appendix A.9. Lysogens were grown to an OD_{600} of 0,5, harvested by centrifugation and resuspended in LB medium, with or without mitomycin C (5 μg/ml). The cultures were incubated at 37^0C in the dark for 2 h, followed by lysis

with chloroform. Cell debris was removed by centrifugation at 8 000 rpm for 5 min in a Beckman JA20 rotor, and the lysates were titred for phage on LAM plates (Appendix B) using E. coli BHB2600 cells grown in LM medium (Appendix B).

4.2.7 Assays for inferred recombinase activity

4.2.7.1 Bacterial conjugations. E. coli CSH62 (Pro^+ Sm^S) was used as the donor in matings with E. coli RR1, E. coli HB101 and E. coli HB101(pRSR100). The recipient strains are Pro^- auxotrophic mutants. Overnight cultures were diluted 1:10 in LB medium and incubated for 1 h at 37^0C. The cells were mixed in a donor-to-recipient ratio of 1:10 and allowed to conjugate for 60 min at 37^oC, with gentle shaking. Selection for transconjugants (Sm^r recipients that had acquired amino acid prototrophy), was on minimal medium containing Sm. The number of E. coli CSH62 Sm^S donor cells was determined by plating on minimal medium.

4.2.7.2 Efficiency of plating (eop) of phage P1 and λ Fec^- mutants. Fec^- mutants of λ carry the red and gam mutations and grow so poorly that they produce no plaques on E. coli recA$^-$ strains. The eop of phages P1 and λ Fec^- was tested on E. coli RR1 recA$^+$, E. coli HB101 recA$^-$ and E. coli HB101 (pRSR100) strains. Cells were grown in LB medium to an OD_{600} of 0.2, harvested by centrifugation and resuspended in SM buffer (Appendix B) to an OD_{600} of 0.5. The cells (0.1 ml) were mixed with dilutions of phage and adsorption allowed to proceed for 20 min at room temperature. LAM

plates. The number of plaques was determined after 18 h incubation at 37^0C.

4.2.8 Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Soluble protein fractions of T. ferrooxidans ATCC 33020 and strains of E. coli, with or without plasmids, were prepared and separated by PAGE as described in Appendix A.10. Proteins were transferred to GeneScreen hybridization transfer membrane (New England Nuclear Corp., Boston, Massachusetts, U.S.A.) according to the method of Towbin et al., 1979) (Appendix A.11). Purified antiserum, raised against E. coli K12 RecA protein in New Zealand white rabbits (Goodman et al., 1987), was diluted and used for detection of RecA proteins as described in Appendix A.12

4.3 RESULTS

4.3.1 Isolation of a T. ferrooxidans MMS^r-conferring gene

Three distinct plasmid types (Fig. 4.1) were isolated from the 18 Ap^r MMS^r E. coli HB101 transformed colonies described in Chapter 3. Retransformation of E. coli HB101 with these plasmids consistently produced almost equal numbers of colonies on LA plates containing either Ap (100 μg/ml), or both Ap (100 μg/ml) and MMS (0.01%). The recombinant plasmids were designated pRSR100, pRSR101 and pRSR102.

4.3.2 Restriction mapping

Digestion of plasmids pRSR100, pRSR101 and pRSR102 with Pst1 endonuclease illustrates the size of the T. ferrooxidans inserts in these plasmids (Fig. 4.1). Restriction mapping of plasmids pRSR100, pRSR101 and pRSR102 confirmed that they contained cloned fragments of approximately 4.5 kb, 4.0 kb and 3.4 kb, respectively (Fig. 4.2). Based on the restriction maps, each recombinant plasmid shared a common fragment of cloned DNA and therefore represented independent cloning events of the MMS^r-conferring gene. An EcoRV and EcoRI endonuclease deletion of pRSR100, and a BglII-BamHI endonuclease deletion of pRSR102 were constructed to locate the gene.

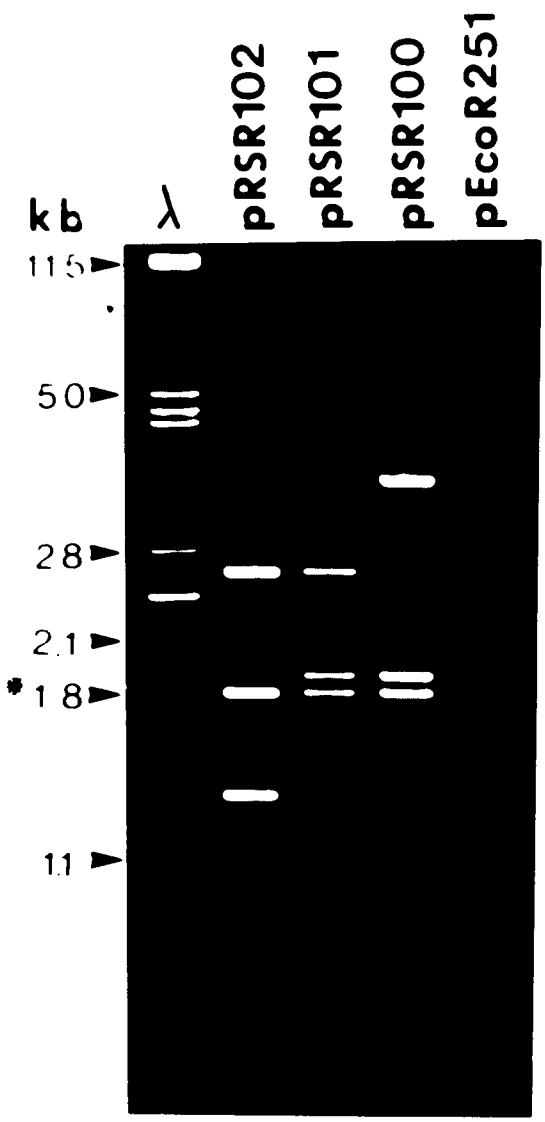

Fig. 4.1 Agarose gel analysis of the three distinct MMS^r-conferring clones, pRSR100, pRSR101 and pRSR102, isolated from the plasmid-wt library. Recombinant plasmids and the vector, pEcoR251, were digested with PstI endonuclease. The 1.8 kb* internal PstI endonuclease vector fragment is evident in each of the clones. The lane containing λ DNA, digested with Pst1 endonuclease, is indicated with size markers in kb alongside.

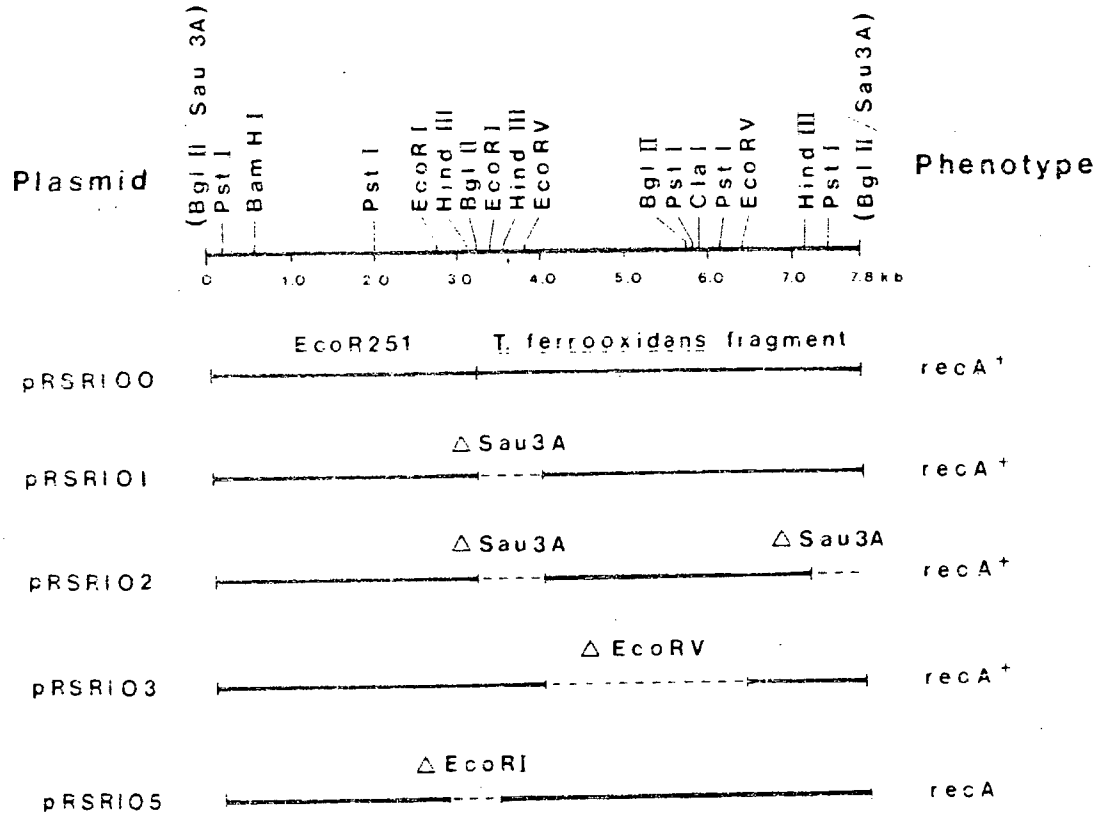

Fig. 4.2 Detailed restriction map of pRSR100 (7.8 kb), constructed by the cloning of Sau3A endonuclease digested T. ferrooxidans chromosomal DNA into the single BglII endonuclease site of pEcoR251. Plasmids pRSR101 and pRSR102 are natural deletions of pRSR100 and were also isolated from the plasmid-wt library.

4.3.3 Southern blotting and DNA hybridization

Gel electrophoresis, Southern blotting and hybridization, were used to confirm that the MMS^r-conferring gene originated from T. ferrooxidans. A positive hybridization signal was obtained with fragments of the T. ferrooxidans chromosomal digest and from the MMS^r-conferring cosmid, pRSR500, which corresponded exactly to fragments of pRSR100 produced from the PstI and EcoRV restriction endonuclease sites internal to the 4.5 kb insert (indicated by open arrow heads in Fig. 4.3). The smallest PstI endonuclease fragment common to pRSR100, pRSR500, and the T. ferrooxidans chromosome (indicated by shaded arrow heads in Fig 4.3) was internal to the cloned gene (refer to Fig 4.2).

4.3.4 Assays for inferred protease activity

4.3.4.1 UV, MMS, and NQO sensitivity. The effect of pRSR100 on UV, MMS and NQO sensitivity of the E. coli HB101 recA$^-$ strain was compared with the untransformed strain and the parental E. coli RRI recA$^+$ strain. As shown in Fig. 4.4, the UV resistance of the E. coli HB101 strain was greatly increased by pRSR100. However, the transformed strain was less resistant to UV than the repair-proficient E. coli RR1 recA$^+$ strain.

Plasmid pRSR100 enabled E. coli HB101 to grow at concentrations of 0.1% (v/v) MMS and 10 μg/ml NQO compared with maximum levels of 0.002% (v/v) MMS and 1 μg/ml NQO for

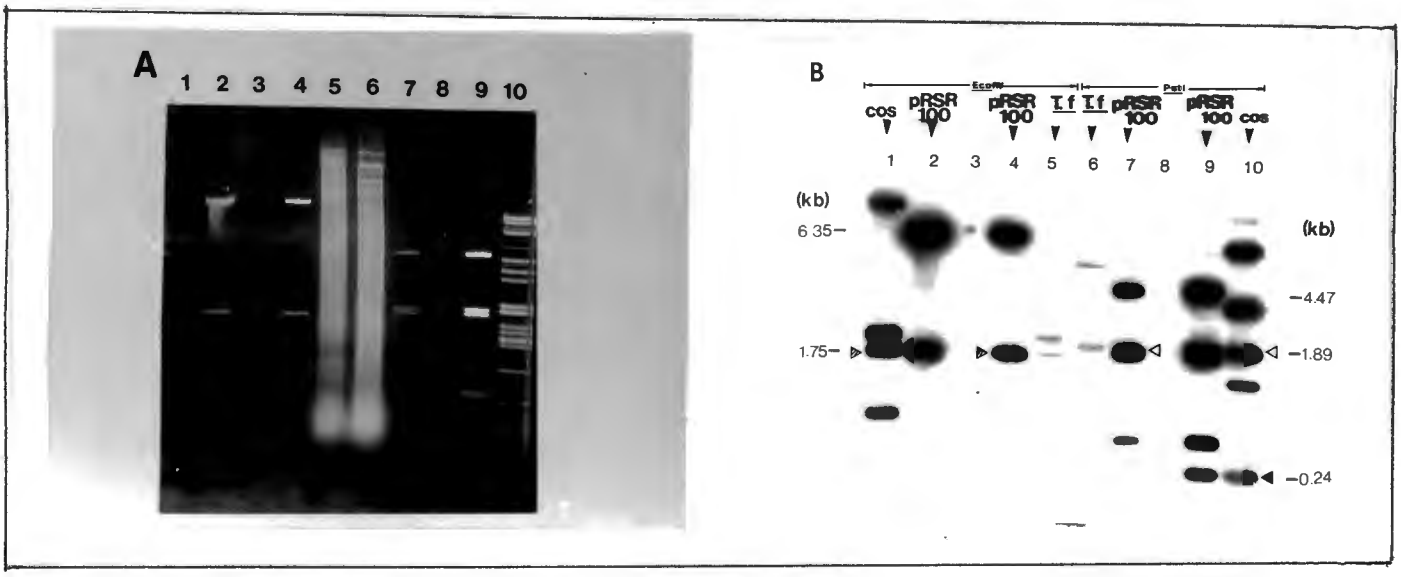

Fig. 4.3 Hybridization of [α-^{32}P]-labelled pRSR100 DNA with total T. ferrooxidans chromosomal DNA, and plasmids.

Photoplate A. Photograph of agarose gel containing:

Lane 1: pRSR500 (Ap^rMMS^r cosmid)
Lane 2: pRSR100 (0.5 ug)
Lane 3: --
Lane 4: pRSR100 (0.5 ug)
Lane 5: T. ferrooxidans chromosomal DNA (10 ug)

} cut with EcoRV endonuclease

Lane 6: T. ferrooxidans chromosomal DNA (10 ug)
Lane 7: pRSR100 (0.2 ug)
Lane 8: --
Lane 9: pRSR100 (0.5 ug)
Lane 10: pRSR500

} cut with PstI endonuclease

Photoplate B. Autoradiograph of **A**

Size markers are indicated in kb and correspond to restriction fragments within pRSR100.

▷: indicates the EcoRV endonuclease fragment internal to pRSR100.
◁: indicates one of the doublet bands when pRSR100 is digested with PstI endonuclease. This fragment is internal to the T. ferrooxidans insert DNA.
◀: indicates the 0.24 kb PstI endonuclease fragment internal to the cloned T. ferrooxidans MMS^r-conferring gene (refer to Fig. 4.2)

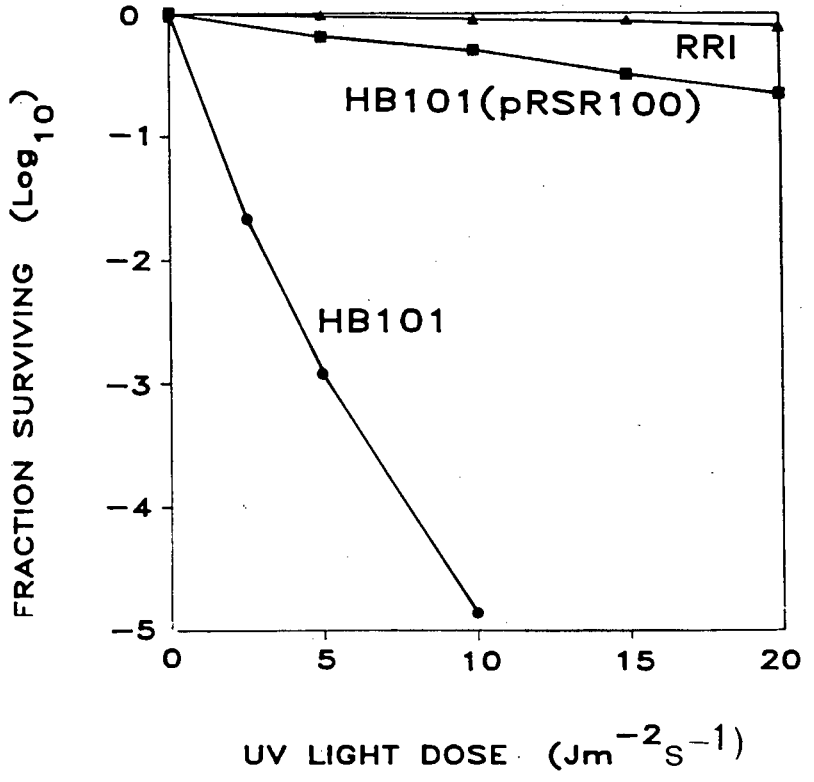

Fig. 4.4 The effect of the recombinant plasmid pRSR100 on the survival of UV-irradiated E. coli HB101. A cell suspension was treated with increasing doses of 254 nm radiation at a dose rate of 1 $J/m^2/s$. Survivors were determined on LA medium after 18 h. (●), E. coli HB101 (recA$^-$), (■), E. coli HB101(pRSR100); (▲), E. coli RR1 (recA$^+$)

the untransformed strain. E. coli HB101, carrying any of the three recombinant plasmids, pRSR100, pRSR101 or pRSR102, was resistant to lower levels of UV irradiation, and similar levels of MMS and NQO as the E. coli RRI recA$^+$ strain.

4.3.4.2 Prophage induction. The quantity of spontaneous and induced phage from recA$^-$ λ lysogens, with and without pRSR100, was determined and compared with E. coli RR1 (recA$^+$) (Table 4.2). Spontaneous prophage induction was evident in E. coli RR1, E. coli HB101(pDR1453) and E. coli HB101(pRSR100). In E. coli, induction of λ lysogens was stimulated by both the cloned T. ferrooxidans recA-like character on pRSR100, and the cloned E. coli recA gene on plasmid pDR1453. Mitomycin C induced the production of λ 26-fold in E. coli RR1, 19-fold in E. coli HB101(pDR1453), and 5-fold in E. coli HB101(pRSR100). Prophage release was negligible in E. coli HB101 containing pBR322. These results indicated that DNA damage was required for induction of protease activity of the cloned RecA-like protein in E. coli.

Table 4.2 Spontaneous and mitomycin C-stimulated induction of λ prophage in E. coli

Lysogen	Phage produced	
	Spontaneous	Induced[1]
E. coli HB101	18	26
E. coli HB101 (pBR322)	15	19
E. coli RR1	7.51×10^5	1.96×10^7
E. coli HB101 (pDR1453)[2]	8.63×10^5	1.65×10^7
E. coli HB101 (pRSR100)	4.14×10^5	2.06×10^6

[1] Cells were treated with 5 μg of mitomycin C per ml and incubated in the dark at 37^0C for 2 h.
[2] recA gene of E. coli on a plasmid

4.3.5 Assays for inferred recombinase activity

4.3.5.1 Bacterial conjugations. The ability of *E. coli* to carry out homologous recombination after Hfr-mediated transfer of markers was compared in *E. coli* HB101 *recA*$^-$, *E. coli* HB101(pRSR100), and the parental *E. coli* RRI *recA*$^+$ strain (Table 4.3). The level of homologous recombination in *E. coli* HB101 was increased over 10^3-fold by pRSR100. This value was, however, only 15% of that of the *E. coli* RR1 *recA*$^+$ strain.

4.3.5.2 Replication of phages P1 and λ Fec$^-$ mutants. Strains of *E. coli* containing *recA* mutations are unable to support the growth of phage P1 (Cohen, 1983) or λ Fec$^-$ mutants (Smith, 1983). Fec$^-$ mutants of λ carry the *red* and *gam* mutations and grow so poorly that they produce no plaques on *E. coli recA*$^-$ strains. The eop of the phages on *E. coli* HB101, *E. coli* HB101(pRSR100) and *E. coli* RRI is shown in Table 4.3. The cloned gene restored the eop of phage P1 to levels close to those of the parental *E. coli* RR1 *recA*$^+$ strain. The λ Fec$^-$ mutant produced large plaques with an eop of 1.0 on *E. coli* RRI cells but no plaques were observed on *E. coli* HB101 cells. The ability of λ Fec$^-$ to grow on *E. coli* HB101 was restored by pRSR100. However, in comparison with *E. coli* RRI, the eop of the λ Fec$^-$ mutant on *E. coli* HB101(pRSR100) cells was reduced from 1.0 to 0.21 and the size of the individual plaques was reduced to pin-pricks.

Table 4.3. Recombinational proficiency as determined by genetic recombination following Hfr matings and the eop of phages P1 and λ Fec⁻

E. coli strain	Recombinational proficiency[1]	eop[2]	
		Phage P1	λ Fec-
RR1 (*recA*⁺)	2.20	1.00	1.00
HB101(pRSR100)	0.34	0.79	0.21
HB101 (*recA*⁻)	2×10^{-4}	$<1 \times 10^{-5}$	$<1 \times 10^{-5}$

1 Recombinational proficiency is reported as the number of Sm^r Pro^+ transconjugants per 100 donor cells. *E. coli* CSH62 (Pro^+ Sm^s) was used as donor, *E. coli* RR1 and HB101 recipients are Pro^- Sm^r mutants.

2 Relative to the eop of the *recA*⁺ strain, RR1.

4.3.6 Western blotting

Rabbit antiserum prepared against *E. coli recA* protein (Goodman *et al.*, 1987) was used in Western blots to challenge proteins from crude extracts of *E. coli* HB101, *E. coli* HB101(pRSR100), *E. coli* JK696, *E. coli* JK696(pRSR100), *E. coli* RRI and *T. ferrooxidans* (Fig. 4.5). The *E. coli recA* gene product is a single polypeptide with an M_r of approximately 40,000 (lane G). A relatively weak antibody reaction was detected with a *T. ferrooxidans* protein at an M_r of approximately 40 000 (lane A). The antiserum reacted with a protein of the expected M_r value from both *E. coli* RRI (lane D) and *E. coli* HB101 (lane C), which produced functional and non-functional RecA proteins, respectively. No antiserum reaction was detected with cell extracts from the *E. coli* JK696 *recA* deletion mutant

(lane E). However, a protein extract from E. coli JK696(pRSR100) cells reacted with the antiserum and two protein bands with apparent M_r of approximately 40,000 and 38,000 were obtained (lane F). In E. coli HB101(pRSR100) cell extracts, the inactive E. coli RecA protein and the T. ferrooxidans RecA protein comigrate so that the antiserum reacted strongly with a protein band with an apparent M_r of approximately 40 000, but less strongly with the unique T. ferrooxidans RecA protein band with an apparent M_r of approximately 38,000.

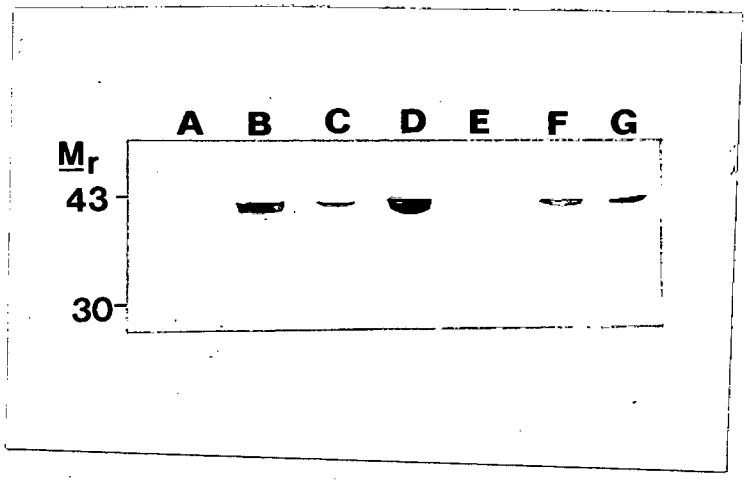

Fig. 4.5 Western blot of purified RecA protein from E. coli (**G**), and crude extracts from T. ferrooxidans ATCC 33020 (**A**), E. coli HB101 (**pRSR100**) (**B**), E. coli HB101 (**C**), E. coli RR1 (**D**), E. coli JK696 (**E**), and E. coli JK696 (**pRSR100**) (**F**). The SDS-fractionated proteins were transferred to nitrocellulose membrane and reacted with antiserum prepared against purified E. coli RecA protein.

4.4 DISCUSSION

Functional similarity of the cloned *T. ferrooxidans* gene to the *E. coli recA* gene was confirmed by its complementation of *recA*$^-$ mutants of *E. coli*. The cloned *T. ferrooxidans* gene product was able to restore RecA-associated protease and recombinase activities to *E. coli* HB101 (*recA*$^-$).

Protease activity of the cloned *T. ferrooxidans* gene was demonstrated in several ways. The cloned gene has the ability to render *E. coli* HB101 (*recA*$^-$) resistant to the otherwise lethal DNA-damaging agent, MMS. In wild-type *E. coli*, the damaging effects of MMS are counteracted by the activity of RecA protein directly or by the action of the products of genes derepressed by the proteolytic RecA protein (Walker, 1984).

The ability of the recombinant plasmid, pRSR100, to confer resistance to the DNA-damaging agent, MMS, was corroborated by the property of *E. coli* HB101 containing either pRSR100, pRSR101 or pRSR102, to tolerate much higher fluences of UV irradiation, and doses of NQO than *E. coli* HB101. Since cleavage of the LexA repressor by the *recA* gene product is required to induce the DNA repair mechanisms of the SOS response, increased survival of treated cells indicates protease activity of the cloned gene product.

That the cloned T. ferrooxidans gene codes for a RecA-like protease is further supported by the observation that excision of λ prophage is enhanced in E. coli HB101 lysogens in the presence of the cloned T. ferrooxidans gene. This is indicative of cleavage of λ repressor by the product of the cloned gene since induced excision of prophage in lysogens of either E. coli HB101 or E. coli HB101(pBR322) was not observed. Prophage induction was stimulated by mitomycin C in E. coli HB101(pRSR100) in a manner similar to that in E. coli RR1. The enhanced excision of prophage is evidence for the necessity of DNA damage for the conversion of the T. ferrooxidans recA-like gene product to its proteolytically active state.

Functional recombinase activity of the cloned T. ferrooxidans gene product restored homology-dependent recombination defects in the E. coli recA$^-$ mutant. This was shown by, firstly, the increase in the number of recombinants obtained by homologous recombination after Hfr-mediated conjugation (Table 4.3). Secondly, growth of phage P1, which requires recombination between terminal repeats for replication, was restored by the cloned gene. Thirdly, the Fec$^-$ phenotype of phage λ, which renders λ incapable of plaque production in the absence of a functional RecA protein, was suppressed.

The level of restoration of both proteolysis and homology-dependent recombination by the product of the cloned T. ferrooxidans gene was less in the E. coli HB101 recA$^-$

background than in the E. coli RR1 recA$^+$ strain. Similar observations have also been reported for the expression of other cloned recA genes in E. coli (Better and Helinski, 1983; Keener et al., 1984; Kokjohn and Miller, 1985; Goodman et al., 1987). It has been suggested that the incomplete restoration of the recA$^+$ phenotype may be due either to the reduced ability of E. coli RNA polymerase to interact with the promoter of the cloned heterologous gene, or because of an inefficient interaction between the cloned recA gene product and E. coli LexA repressor (Better and Helinski, 1983; Kokjohn and Miller, 1985). In either case partial expression of the cloned T. ferrooxidans gene would result.

Further evidence that plasmid pRSR100 codes for a T. ferrooxidans RecA-like protein is provided by experiments using antibody against E. coli RecA protein. These studies demonstrated structural conservation between the E. coli RecA protein and the cloned T. ferrooxidans gene product. The cloned T. ferrooxidans recA-like gene produced two polypeptide bands with an apparent M_r of approximately 40 000 and 38 000 in an E. coli recA deletion mutant. Since a single polypeptide band with an apparent M_r of approximately 40 000 was detected in T. ferrooxidans, it is suggested that the polypeptide with an apparent M_r of 38 000 in E. coli JK696(pRSR100) and E. coli HB101(pRSR100) cells is a degradation product of the larger T. ferrooxidans polypeptide.

From the evidence presented here, it is concluded that the gene isolated and characterized in this study is the *recA* gene of *T. ferrooxidans*. These studies provide evidence to suggest that the RecA protein has been functionally and structurally conserved between *E. coli* and *T. ferrooxidans*. The conservation of key components of the SOS regulon among bacterial genera has been suggested previously (Keener *et al.*, 1984).

The presence of a *recA* gene in *T. ferrooxidans* indicates that the bacterium may have an inducible DNA repair- and a homologous genetic recombination-system. The implication of this latter phenomenon for the genetic manipulation of *T. ferrooxidans* is that once a means of reintroducing DNA into the organism is discovered, it may be possible to recombine manipulated genes back into the chromosome.

However, for the maintenance of homologous traits on reintroduced plasmids, it will be necessary to isolate a *T. ferrooxidans recA*$^-$ mutant. The isolated *T. ferrooxidans recA* gene will enable this to be done by *in vitro* mutagenesis of the *T. ferrooxidans* gene. The gene may then be transferred to *T. ferrooxidans*, where it may recombine with the chromosomal *recA* gene to produce an inactive *recA* gene.

CHAPTER 5

NUCLEOTIDE SEQUENCE OF THE *T. ferrooxidans recA* GENE AND ITS EXPRESSION IN *E. coli*

CHAPTER 5

NUCLEOTIDE SEQUENCE OF THE T. ferrooxidans recA GENE AND ITS EXPRESSION E. coli

Summary. The nucleotide sequence of the T. ferrooxidans recA gene has been determined. No SOS box characteristic of LexA-regulated promoters could be identified in the 196-bp region upstream of the coding region. The T. ferrooxidans recA gene specifies a protein of 346 amino acids that has 66% and 69% homology to the RecA proteins of E. coli and P. aeruginosa, respectively. Most amino acids that have been identified as being of functional importance in the E. coli RecA protein are conserved in the T. ferrooxidans RecA protein. Although some amino acids that have been associated with ATPase and constitutive protease activity have been substituted, the cloned protein has retained these activities. The cloned recA gene was expressed in E. coli from both the λ P_r and lac promoters. However, no expression from the 2.2 kb T. ferrooxidans DNA preceding the gene was evident.

5.1 INTRODUCTION

Promoters are transcription start sequences that direct the binding of RNA polymerase to DNA (Chamberlin, 1976; Gilbert, 1976). Comparisons of wild-type promoter sequences recognized by E. coli RNA polymerase showed that two common sequences of six nucleotides occurred about 10 bp and about 35 bp before the transcription start (Pribnow, 1975; Youderian et al., 1982; Hawley and McClure, 1983). These

1983). These are referred to as the -35 and -10 sequences: -35 (5'-TTGACA-3') and -10 (5'-TATAAT-3'). Few promoters have perfect -10 and -35 consensus sequences, but most differ from the consensus sequence by only a few nucleotides.

Initiation of transcription in E. coli involves RNA polymerase binding a promoter in two steps (Rodriguez and Chamberlin, 1982; Gottesman, 1984). First, is the initial recognition between RNA polymerase and DNA, and their association to form a closed complex in the vicinity of the -35 region. Mutations that alter nucleotide bases in the -35 region impede binding to the promoter. Next, is the conversion of the closed complex to an open complex in which RNA polymerase is bound more tightly to the DNA. During the shift from a closed to an open complex, about 17 bp beginning in the -10 region are unwound, exposing the template strand against which RNA nucleotides are polymerized. This region is rich in AT bp which 'melt' more easily than GC pairs. Mutations that hinder the formation of the open complex map in the -10 region (Gottesman, 1984; Watson et al., 1987). Many of these mutations change the -10 sequence but preserve its AT content. This suggests that besides being inherently susceptible to melting, this region must have a specific shape to be recognized by RNA polymerase (Gottesman, 1984). Initiation of transcription at most E. coli promoters may be accomplished by the major cellular RNA polymerase (Rodriguez and Chamberlin, 1982).

Some genes require the participation in the initiation process of a protein factor, which is not always present or not always active in the cells in which it is required. Several classes of such proteins, called activators, exist (Raibaud and Schwartz, 1984). Many activators bind together with RNA polymerase so as to stimulate its binding to promoters. Sigma (σ) factors are a class of activators that alter the affinity of RNA polymerase for a promoter (Raibaud and Schwartz, 1984). New σ factors replace "standard" σ factors, in a fraction of the RNA polymerase molecules. These σ factors alter the RNA polymerase recognition specificity so that promoters distinct from the "standard" consensus promoters are recognized. In some cases activators may be wholly new RNA polymerases (Raibaud and Schwartz, 1984).

Besides σ factors, transcription regulatory proteins such as repressors act by binding DNA near to or within the promoters of genes they regulate (Watson *et al.*, 1987). Operators, the binding sites of repressors, may overlap promoters and directly prevent RNA polymerase binding.

5.1.1 Transcription of the *E. coli recA* gene

Regulation of the genes involved in the coordinated SOS response is through the binding of LexA repressor protein to their operator sequences, as described in Chapter 4. The nucleotide sequence of SOS-gene operators (or the SOS box), gives an indication of the way LexA repressors work (Howard-

Flanders, 1981; Walker, 1984). The SOS box has, to varying degrees in different genes, the inverted repeated form that is characteristic of repressor-binding sites (Little and Mount, 1982). LexA repressor binds to the SOS gene operators, of about 20 bp, possibly interacting with bases in the major groove of the DNA (Brent, 1982). The operators overlap the promoter regions in these genes. LexA bound to the operator, therefore, prevents RNA polymerase from interacting with the DNA (Howard-Flanders, 1981).

The exact location of the operator regions, relative to the promoter, may differ between SOS genes (Fig. 5.1) (Brent, 1983). Operators may cover the -35 hexamer (as in uvrA), the -10 hexamer (as in lexA), or both -35 and -10 hexamers (uvrB and recA) (Fig. 5.1) (Howard-Flanders, 1981). Other operators lie within the transcribed region (as in cle1). The site of RNA polymerase binding extends beyond either the -35 or the -10 consensus hexamer on either side. Therefore, the variety of locations at which operators are found within the vicinity of the promoter does not detract from their effectiveness in impeding gene transcription (Watson et al., 1987).

Most LexA-regulated genes contain a single operator near to their promoters (Howard-Flanders, 1981; Walker, 1984). However, some of the SOS genes such as lexA (Fig. 5.1), umuDC, and the cle1 gene have two LexA-binding sites adjacent to one another (Walker, 1984). Studies have shown that the strength with which LexA protein binds to the

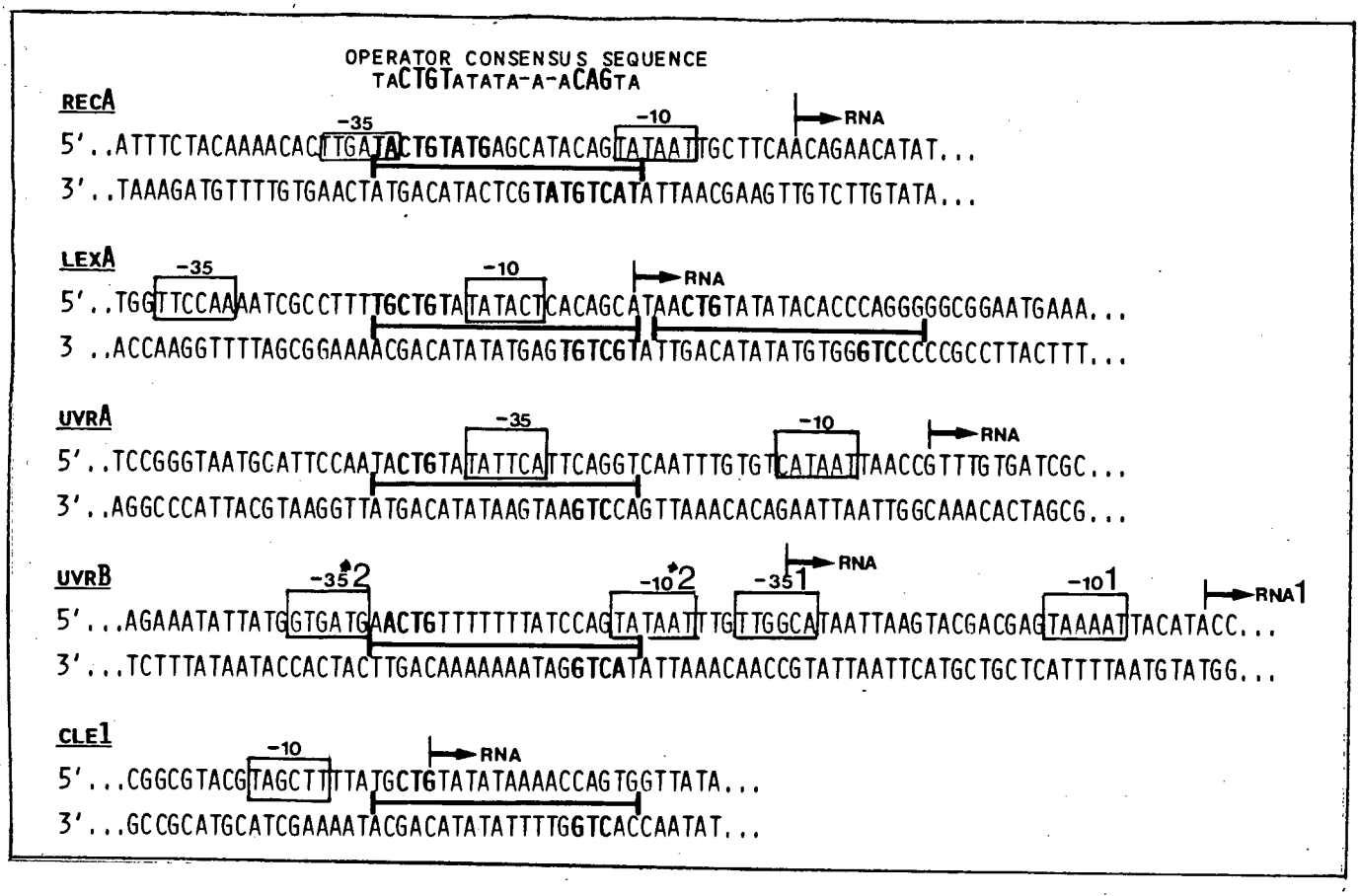

Fig. 5.1 Nucleotide sequence near the beginning of some SOS genes showing the relative position of promoters and operators. Bold lines indicate the LexA-binding site. Boxed regions indicate -35 and -10 promoter sequences. Conserved, inverted repeat sequences in the operator regions are indicated in bold typeface. Two adjacent operators are associated with the lexA gene. Of the two promoters shown for uvrB, only promoter *2 is repressed by LexA (After Howard-Flanders, 1981; Sancar et al., 1982)

The cle1 gene of plasmid ColE1 encodes colicin E1, an antibacterial toxin.

various SOS-gene operators varies considerably. Brent and Ptashne (1981) reported that the binding affinities of LexA repressor to operators of the recA, uvrB and lexA genes, and mutants of these, was due to variations in sequence between the operator sites. Nucleotide sequencing studies have shown that several operator-constitutive mutants contained single bp changes within the region containing the homologous sequence (Clark, 1982; Uhlin et al., 1982). These findings were more recently reinforced by a statistical analysis of the binding affinity of several LexA-binding operators (Berg, 1988).

Repressor-binding affinity is important in determining the chronological order of expression of the genes under LexA repression (Walker, 1984). Genes whose operators have a stronger affinity for LexA protein will only be derepressed after genes whose operators bind LexA protein less strongly. This mechanism is likely to be involved in the fine tuning of the expression of the SOS response (Walker, 1984).

An interesting mechanism of regulation occurs in the uvrB gene which contains two adjacent non-overlapping promoter sequences (Fig 5.1) (Sancar et al., 1982). In addition to the promoter, P2, which is susceptible to LexA repression, uvrB has a LexA-independent promoter region, P1, downstream from the LexA-regulated promoter. A third promoter, P3, occurs 320 bp upstream from P2. Although transcription from P3 is directed towards the uvrB structural gene, it is terminated in the region of the LexA-binding site, even in

the absence of LexA repressor. The role of this promoter and its transcript is as yet unknown. The function of the unregulated promoter, P1, is to produce a basal level of the uvrB gene product, even in uninduced cells (Walker, 1984). This is useful for low level excision repair which occurs where DNA damage is at levels insufficient to elicit the entire SOS response.

Dual control has been shown in another SOS gene, himA, which is regulated by both LexA and its own product (Miller et al., 1981). The himA gene product is required for site specific recombination which is most studied in λ integration into the E. coli chromosome. The LexA-binding site of the himA gene differs from the established consensus sequence. It is located right next to a binding site for integration host factor (IHF), one subunit of which is encoded by the himA gene. The binding of LexA to the site requires the simultaneous binding of the IHF molecule to the adjacent IHF-binding site (Sedgwick and Yarranton, 1982; Walker, 1984) .

5.1.2 Organization of the functional domains of the recA gene product

RecA protein has ATPase, protease and recombinase activity and is involved in a wide variety of cellular functions (Walker, 1984). In addition, RecA binds to NTPs (Craig and Roberts, 1981; Phizicky and Roberts, 1981) and to ssDNA (McEntee et al., 1981; Bryant et al., 1985). It was shown

that the active species in DNA recombination and proteolytic cleavage of repressor proteins is a ternary complex of RecA, NTP, and ssDNA (Craig and Roberts, 1981; Phizicky and Roberts, 1981).

Interspecific complementation of E. coli recA mutants with cloned chromosomal fragments from a wide selection of bacteria provides evidence for the functional conservation of RecA. In addition, structural conservation was shown using polyclonal antibodies prepared against the E. coli RecA protein. These antibodies cross react with RecA proteins from Erwinia carotovora, Proteus vulgaris, Shigella flexneri (Keener et al., 1984), Bacteroides fragilis (Goodman et al., 1987) and T. ferrooxidans (Ramesar et al., 1988; Chapter 4, Fig.4.5). The structural and multifunctional similarities of RecA proteins from different organisms suggests that the RecA protein contains distinct conserved functional domains.

RecA mutants defective in one or a combination of its known phenotypes have been isolated (Castellazzi et al., 1972; Ogawa et al., 1978; Phizicky and Roberts, 1981). The assigning of functional domains was made possible by correlating the observed phenotype in several classes of E. coli recA mutants with the amino acid change/s in their RecA proteins (Kawashima et al., 1984; Ogawa and Ogawa, 1986; Wang and Tessman, 1986). Alterations in the properties of proteins might be due to changes in the reactive sites, alteration of the tertiary structure of the

protein, or decreased activity of the protein due to a net charge change. A list of some *recA* alleles and their characteristics is presented in Table 5.1.

Table 5.1 Summary of alleles of *recA* and their properties

Allele	Phenotype	Biochemical alteration
wild-type (*recA*$^+$)	resistant to UV irradiation able to express SOS functions	wild-type protein
Defective (*recA1*)	recombination-deficient cannot induce SOS functions very sensitive to DNA damage	defective protein
Protease constitutive (*recA441*)	constitutive expression of SOS functions and λ induction at 42^{0}C	protease easily activated
split function (*recA430*)	recombination proficient cannot induce λ or SOS functions	reduced proteolytic activity

The product of the *recA*$^-$ or *recA*(Def) gene is unable to hydrolyze ATP, bind to DNA in the presence of ATP or change conformation in the presence of ATP (Ogawa *et al.*, 1978). *recA441* mutants show constitutive SOS functions at 42^{0}C (Castellazzi *et al.*, 1972). RecA441 protein binds more tightly to polynucleotides and to ATP than the wild-type protein (Phizicky and Roberts, 1981). In contrast, the *recA44* mutant is defective in most SOS responses and in recombination at 42^{0}C. Another mutant, *recA430*, shows a separation of RecA functions. This mutant is defective in the SOS response and cleavage of phage repressor, but is proficient in homologous recombination. The RecA44 protein

is able to hydrolyze ATP and anneal ssDNAs (Roberts and Roberts, 1981).

Kawashima et al. (1984) showed that the mutant properties of each of the above mutants was due to single amino acid substitutions in the wild-type protein. Knight et al. (1984), however, found that the RecA441 protein contains two amino acid substitutions. Nevertheless, Kawashima and coworkers proposed the functional domains of RecA protein in two ways, (i) by predicting the affinities of certain regions on the protein for known effector molecules on the basis of their electrostatic charge and amino acid content, and (ii) by relating the changes in the amino acid sequence to the functional properties of the mutant protein.

The involvement of the amino-terminal end of the E. coli RecA protein in ssDNA binding is indicated by several observations (Kawashima et al., 1984). A comparison of the amino acid sequence of the E. coli wild-type RecA protein with those of the E. coli SSB and T4 gene-32 proteins, both of which have been shown to bind ssDNA, showed a high degree of homology at the amino-terminal end (Kawashima et al., 1984). In addition, the bias of basic amino acids in this region results in a positive charge, which is likely to enhance interactions with the negatively charged effectors (Kawashima et al., 1984).

Furthermore, the RecA441 mutant protein, in which Glu^{38} is substituted with Lys, had an increased affinity for ssDNA.

Increased positive charge in an already basic region enhanced the affinity of RecA441 for the ssDNA effector, possibly explaining the constitutive expression of RecA functions by the recA441 mutant (Kawashima et al., 1984). The change at amino acid position 38 was shown to be responsible for the constitutive protease activity of RecA441, whereas the change at amino acid position 278 resulted in temperature sensitive suppression of the effects of the substitution at position 38 (Wang and Tessman, 1986).

In addition, the ssDNA-binding activity of a RecA protein was impaired when 33 amino acids from the amino-terminal end were removed (Kawashima et al., 1984). The ssDNA-binding affinity of the amino-terminal end has also been confirmed by Wang and Tessman (1986), using protease constitutive (Prt^C) mutants.

The defect in the SOS and recombination functions in the recA1 mutant was due to the amino acid substitution, Gly^{160}-Asp, in the RecA1 protein (Kawashima et al., 1984). The acidic replacement resulted in an increased negative charge in the protein which, in turn, decreased the affinity of the protein for the effectors, ATP and possibly also ssDNA. The amino acid substitution Gly^{204}-Ser, in recA430 mutants, resulted in a local conformational change of the RecA430 protein (Kawashima et al., 1984). This affected the proteolytic activity of RecA. Recombination proficiency was retained, indicating a separate active site for recombinase activity.

Location of a protease domain in the amino-terminal half of the E. coli RecA protein was suggested by the presence of the total complement of Cys and His residues in this region (Sancar et al., 1980). These amino acids are components of the active site of most serine proteases (Dayhoff, 1972). Furthermore, the amino acid sequences -Asp-Ile-Ala-Leu-, Ala-Glu-His-Ala-, and -Gly-Asp-Ser-, found in this half of the protein, are also similar to active sites in several serine proteases (Sancar et al., 1980).

The constitutive protease activity of the recA5327 mutant, which lacked 25 amino acids at the carboxy-terminal end of RecA, led Ogawa and Ogawa (1986) to suggest that the carboxy-terminal region might be involved in regulation of protease activity. This suggestion was supported by the temperature sensitive suppression of the Prt^{C} activity that resulted from the Ile^{228}-Val change, by the Gly^{38}-Lys change in recA441 mutants (Blanar et al., 1984). However, since the carboxy-terminal is highly acidic, an increase in the net positive charge is possible in the truncated protein (Ogawa and Ogawa, 1986). Whether the greater constitutive protease activity of the truncated protein is due to the excision of a regulatory region, as suggested by Ogawa and Ogawa (1986), or whether it is the result of a net gain in positive charge by the RecA protein, is not known. The latter possibility may result in a greater affinity by the truncated RecA molecule for effector molecules, as proposed for the RecA441 protein.

The nucleotide-binding site predicted by Kawashima *et al.* (1984) involved the residues Cys^{90}, Cys^{129}, and Cys^{116}, and occupies the domain between the 90^{th} and 129^{th} amino acid residues. The amino acid residue, Cys^{116}, was shown to be responsible for ATPase activity (Kuramitsu *et al.*, 1984). Tyr^{264} has also been implicated in NTP binding (Knight and McEntee, 1985). An analysis of various ATP-binding proteins led to the identification of a possible consensus sequence: Gly/Ala X X Gly X Gly Lys Thr/Ser (where X represents variable amino acids) (Walker *et al.*, 1982; Gill *et al.*, 1986). A similar sequence was located in both *E. coli* and *P. aeruginosa* RecA proteins, from Gly^{65} to Ser^{72} (Sano and Kageyama, 1987).

Wang and Tessman (1986) showed that some *recA* Prt^{C} mutants were insensitive to negative effectors such as G and C nucleosides. These workers concluded that the insensitivity of the mutant RecA protein to these negative effector molecules was due to an alteration in the nucleoside-binding domain. Correlation with the site of nucleotide replacement in mutants led to the assigning of the middle third of the RecA protein as the NTP-binding domain (Wang and Tessman, 1986). The exact mechanism of nucleotide interaction with the proposed NTP-binding sites is not known, but may involve cooperative binding and processing of NTP in the domain between Gly^{65} and Cys^{129}.

RecA protein has been reported to be capable of dsDNA binding (Howard-Flanders *et al.*, 1984; Cox and Lehman, 1987). However the domain on RecA protein and the mechanism of interaction between RecA protein and dsDNA is not clear. Rusche *et al.* (1985) demonstrated that RecA binds to dsDNA such that the rate of ligation of dsDNA fragments was enhanced by T4 DNA ligase. Heterologous ssDNA, when added to the reaction, resulted in aggregation of ssDNA, dsDNA and RecA, indicating a cross reaction, or that the RecA molecule has both ssDNA- and dsDNA-binding sites. The region on RecA responsible for recombinase activity has not yet been identified.

5.1.3 The present study

Isolation and characterization of a *recA*-like gene from *T. ferrooxidans* was described in Chapter 4 (Ramesar *et al.*, 1988). *T. ferrooxidans* has a unique physiology, distinct from other bacteria from which *recA* has thus far been cloned and characterized. It is of particular interest to compare the nucleotide and derived amino acid sequences of the *T. ferrooxidans recA* gene with those of other organisms to determine whether genes other than those involved in nitrogen metabolism are conserved. In this chapter, the sequencing of the *T. ferrooxidans recA* gene and flanking regions is reported. The derived amino acid sequence of the *recA* gene product is compared with the RecA proteins of *E. coli* and *P. aeruginosa*. Sano and Kageyama (1987) reported a high degree of nucleotide sequence conservation

(71%) between the *recA* genes of *E. coli* and *P. aeruginosa*, particularly in regions coding for functional domains of the RecA protein. These workers also located a typical SOS-box region upstream of the *P. aeruginosa recA* gene.

The regulation of the *T. ferrooxidans recA* gene was examined to determine whether regulatory regions similar to those associated with the *recA* genes from *E. coli* and *P. aeruginosa* are present. To ascertain whether the *T. ferrooxidans recA* gene is transcribed in *E. coli* from its own promoter, the expression of the *T. ferrooxidans recA* gene in the absence of vector promoter influence was investigated.

5.2. MATERIALS AND METHODS

5.2.1 Bacterial strains, vectors and plasmids.

Plasmid pRSR100 (Chapter 4; Ramesar et al., 1988) which contained the cloned T. ferrooxidans recA gene was used as the primary source of DNA. The plasmid vectors, Bluescript SK (Stratagene, San Diego, California, U.S.A.), pUC18 and pUC19 (Yanisch-Perron et al., 1985) were used for subcloning and for nucleotide sequencing. E. coli LK111 (Table 3.1 for genotype and source) was used as a host for pUC and Bluescript SK plasmids. E. coli HB101 and E. coli JK696 are recA$^-$ strains, described in Table 4.1

5.2.2 Media and buffers.

Media and buffers not described in the text are listed in Appendix B.

5.2.3 Growth conditions.

E. coli LK111 harboring pUC- and Bluescript SK-based recombinant plasmids was plated on LA plates containing Ap (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-galactoside (X-gal; Appendix B). LA plates containing X-gal and Ap (100 μg/ml) will hereafter be referred to as XG plates.

5.2.4 DNA Sequencing

5.2.4.1 Sequencing strategy. Standard molecular genetic techniques (Maniatis et al., 1982) were used to subclone DNA fragments from pRSR100 into pUC18, pUC19 and Bluescript SK vectors. DNA for sequencing was prepared by a combination of subcloning from available restriction sites, and the construction of ordered deletions using the enzyme BAL-31 (Mishra, 1985). The restriction map of pRSR100 and the sequencing strategy is shown in Fig. 5.2.

5.2.4.2 BAL-31 exonuclease shortening of DNA cloned into pUC19. A BAL-31 exonuclease reaction was prepared by combining linearized plasmid (10 μg), 5 X BAL-31 reaction buffer (Appendix B) and water to a final volume of 140 μl and equilibrated at 37^0C for 5 min. The reaction was started by the addition of BAL-31 exonuclease (Gray et al., 1975) (10 μl, 5 units; New England Biolabs, Inc., Beverly, Massachusetts, U.S.A.), and 25 μl samples were removed at 1 min intervals to microfuge tubes containing 75 μl of TE buffer and 10 μl of TE buffer-saturated phenol (Appendix B). Each sample was extracted with chloroform-isoamyl alcohol (24:1) (100 μl), and the DNA precipitated with ethanol. The DNA pellet was resuspended in restriction buffer (Appendix B) and digested with the appropriate restriction enzyme (Appendix A.2) to release the shortened fragments. The digested DNA samples were analyzed by agarose gel electrophoresis (Appendix A.3) and the results were used to determine the appropriate concentration of BAL-31

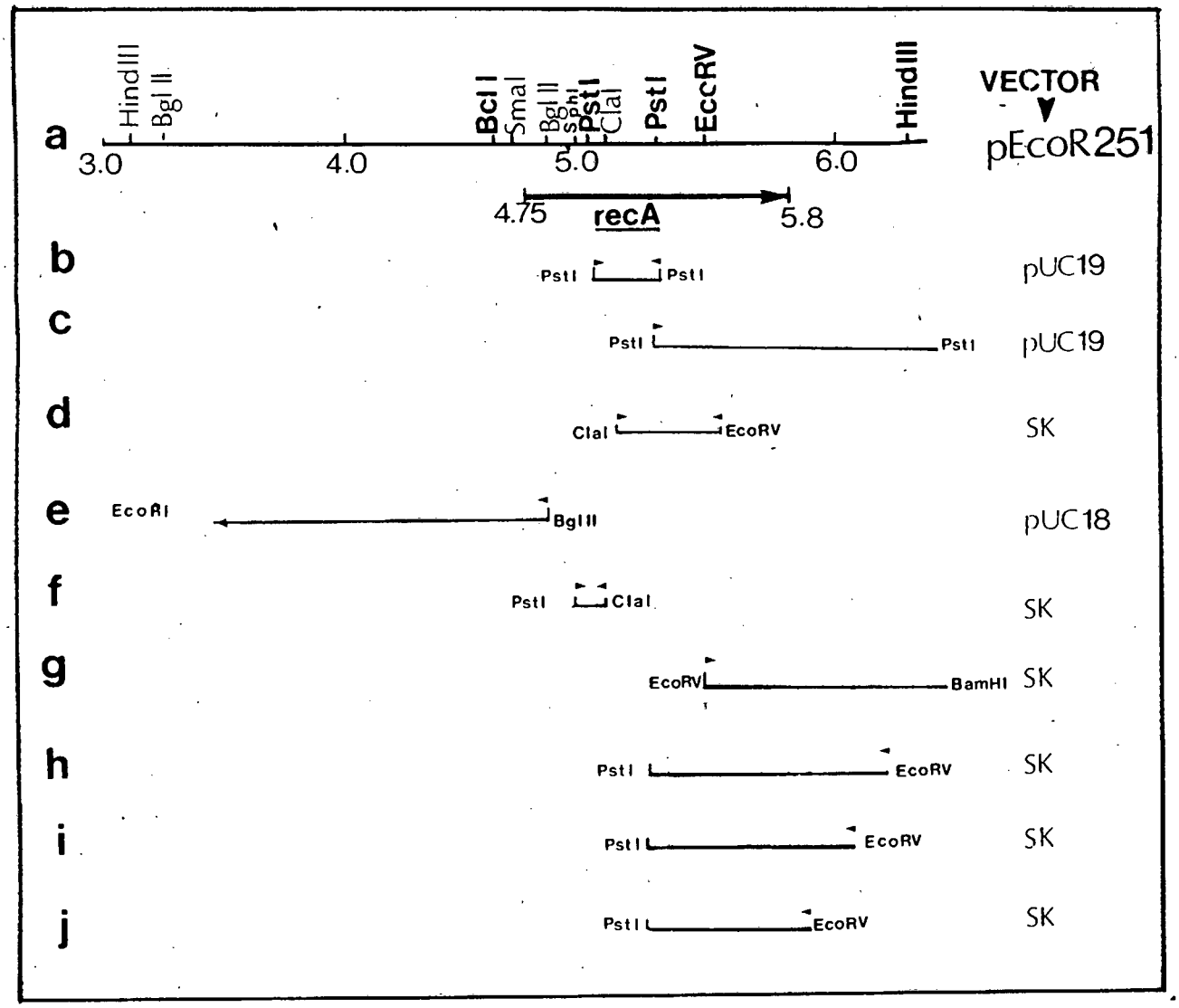

Fig. 5.2 The sequencing strategy used in this study to determine the nucleotide sequence of the T. ferrooxidans recA gene. Subclones are designated **a-j**. Clone **a** is pRSR100, with only the relevant portion of DNA shown. Sequencing was carried out in the direction indicated by little arrow heads (forward and reverse). Clones **g-j** were shortened from the indicated restriction sites (on the right hand side of the page) using BAL-31 restriction enzyme. The cloning vectors used for subcloning are indicated.

5.2.4.4 Preparation of template DNA. Template (ssDNA) derived from pUC and Bluescript SK recombinant plasmids was prepared as follows: Plasmid DNA (2-4 μg in 20 μl H_2O) was denatured by adding 2 μl of NaOH (2 M) and left at room temperature for 5 min. The reaction was neutralized by adding 5 μl of sodium acetate (3 M), diluted with 25 μl water and the DNA precipitated by adding 150 μl cold (-20^{0}C) ethanol and held at -70^{0}C for 5 min. The DNA was collected by centrifugation in a microfuge (30 min at 4^{0}C), washed with 70% cold ethanol, air dried and resuspended in 8 μl of sterile distilled water.

5.2.4.5 Primers and annealing reactions. Primers for reverse and forward annealing for pUC and Bluescript SK-recombinant plasmids were obtained from Bethesda Research Laboratories, Inc. (BRL, Rockville, Massachusetts, U.S.A.). Primers for single-stranded pUC and Bluescript SK DNA were annealed to the respective plasmid DNA by adding 1,4 μl 10 X DNA polymerase I buffer (Appendix B) and 4,5 μl primer (4,0 ng/μl) to the 8 μl of ssDNA template prepared above, and incubated for 15 min at 40^{0}C. The DNA was used directly for sequencing or stored at -70^{0}C.

5.2.4.6 Sequencing reactions. DNA sequences were determined using the dideoxy chain termination procedure (Sanger *et al.*, 1977). Reagents for the DNA sequencing reactions were obtained in kit form (Sequencing kit, Amersham Corp., Buckinghamshire, U.K.) and used according to

the manufacturer's specifications. The DNA chains were radiolabelled with [^{35}S]dCTP (400 Ci/mmol) or [^{35}S]dATP (410 Ci/mmol), supplied by Amersham Corp.

5.2.4.7 Polyacrylamide gel electrophoresis (PAGE). The sequencing reactions were analyzed on denaturing polyacrylamide gels. Polyacrylamide (8%, w/v) wedge gels (0,4 mm spacers at the top, and 0,8 mm spacers at the bottom) were cast in a BRL apparatus (BRL model SO) (34 X 40 cm). A sharktooth comb was used to form the loading wells (BRL 1045 SC, 24 teeth, 0,4 mm thick, 6 mm point-to-point). Although this comb had the capacity for 24 samples (6 templates), only 4 templates were routinely loaded in the central wells of the gel to improve resolution and minimize "smiling" effects. The composition of the gels and running conditions were as described in the manual supplied with the sequencing kit. After electrophoresis the gels were dried onto filter papers (Whatman No. 3) using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco, California, U.S.A.).

5.2.4.8 Autoradiography. Gels containing [^{35}S]-labelled DNA were placed under Cronex 4 film (Dupont-Rŏntgen autoradiographic film) in an X-Ray cassette and exposed for 5-24 h. The autoradiographs were developed using Kodak GBX X-RAY developer and fixer according to the manufacturer's instructions.

5.2.4.9 Sequence analysis. Nucleotide and derived amino acid sequences were analyzed using the DNA Tools and Genepro (version 4.1; Hoefer Scientific Instruments) computer programmes. The deduced T. ferrooxidans RecA amino acid sequence was aligned and compared with the RecA sequences of E. coli and P. aeruginosa with the aid of the Beckman Microgenie sequence analysis programme. Stem and loop secondary structure stability was calculated according to Salser et al. (1977).

5.2.5 Expression of the T. ferrooxidans recA gene in E. coli

A series of experiments was devised to examine the expression of the cloned T. ferrooxidans recA gene, located on plasmid pRSR100.

5.2.5.1 Construction of an EcoR1 deletion of pRSR100. Excision of the λ P_r promoter from pRSR100 was carried out to determine its effect on T. ferrooxidans recA gene expression. The λ promoter was excised on a 640 bp EcoR1 restriction endonuclease fragment and the expression of T. ferrooxidans recA, located on pRSR100, and that on the deletion plasmid, were compared.

The plasmid, pRSR100, was digested with EcoR1 restriction endonuclease and the restriction fragments were separated by electrophoresis in low melting point agar. The major EcoR1 restriction endonuclease fragment was excised and ligated in the low melting point agarose as described in Appendix

A.4.2. Competent E. coli HB101 cells (Appendix A.5) were transformed with the ligation mixture (Appendix A.6) and plated onto LA plates containing Ap (100 μg/ml). Transformants were grown in 5 ml LB medium, harvested and plasmid isolated using the miniprep method (Appendix A.1.1). The successful construction of the EcoR1 deletion of pRSR100, designated pRSR105, was confirmed by endonuclease digestion (Appendix A.2) and agarose gel electrophoresis of plasmid preparations (Appendix A.3). Expression of the recA gene in E. coli strains was assayed using UV survival and MMS^r as described in Chapter 4.

5.2.5.2 Repression of the λ P_r promoter on pRSR100. Plasmid pcI857 (Km^r), which codes for a temperature sensitive λ repressor (Remaut et al., 1981) and which is compatible with pRSR100, was transformed into E. coli HB101(pRSR100). At the permissive temperature (30^0C), λ repressor is produced and binds to the λ promoter on the vector, upstream of the cloned recA gene. The effect of the λ promoter on the recA gene was ascertained by growth at the non-permissive temperature, i.e. 42^0C. At this temperature active λ repressor is not produced.

5.2.5.3 Cloning of the recA gene into Bluescript SK. Bluescript SK is a cloning vector which carries a multiple cloning site within its lacZ gene. Cloning within this site effectively inactivates the lac operon and renders selectivity of recombinants a simple task by differentiating between white recombinants and blue parentals on XG plates.

Plasmid pRSR100 was restricted with HindIII endonuclease and electrophoresed through low melting point agarose. The appropriate HindIII fragment, containing the recA gene, was excised from the gel and ligated to Bluescript SK, which had already been restricted with HindIII endonuclease.

Competent E. coli LK111 cells were transformed with the ligation mixture, plated on XG medium and incubated at 37^0C overnight. White colonies were chosen at random and grown overnight in 5 ml LB medium. The cultures were harvested and plasmid DNA was isolated using the miniprep method, restricted and resolved by agarose gel electrophoresis. Plasmids containing the T. ferrooxidans HindIII fragment, carrying recA in both orientations, were then transformed into E. coli HB101 (recA$^-$) cells, and tested for their ability to confer resistance to MMS and UV.

5.2.5.4 Western blotting. Details of the technique are provided in Appendix A.7. Soluble protein fractions of crude extracts of E. coli and T. ferrooxidans were prepared by SDS-mercaptoethanol lysis, and separated by electrophoresis through a 10% (w/v) polyacrylamide gel. Separated proteins were transferred to nitrocellulose filters. Rabbit antiserum prepared against E.coli K12 RecA protein (Goodman et al., 1987) was used to challenge the proteins.

5.3 RESULTS AND DISCUSSION

5.3.1 Nucleotide sequence of the recA gene.

A 1 406 bp fragment of DNA containing the T. ferrooxidans recA gene and flanking regions is shown in Fig. 5.3. The sequence contained a 1 038 bp ORF preceded by a -GGAGAAGGAAA- sequence 5 bp upstream from the presumptive start codon at nucleotide position 196. This AG-rich region contains sequences which resemble other Shine-Dalgarno (SD) sequences (Shine and Dalgarno, 1976) and is situated the correct distance from the ORF to be regarded as a putative SD-like region.

E. coli and P. aeruginosa sequences have clearly identifiable SOS boxes (Sano and Kageyama, 1987) which contain a consensus LexA binding site (Walker, 1984) within 57 and 41 bp of the ATG start codon, respectively. No distinct E. coli- or P. aeruginosa-like SOS box is present in the 196-bp sequence upstream of the T. ferrooxidans recA gene, and there is also no -35, -10 consensus promoter sequences evident.

The most notable structural features in the T. ferrooxidans DNA sequence, upstream of the recA gene, are two 13 bp directly repeated sequences in tandem (nucleotides 23 to 48), and a 10 bp complementary inverted repeat sequence (nucleotides 160 to 169) 31 bp from the ATG start codon. Downstream from the recA ORF are two 14 bp directly repeated

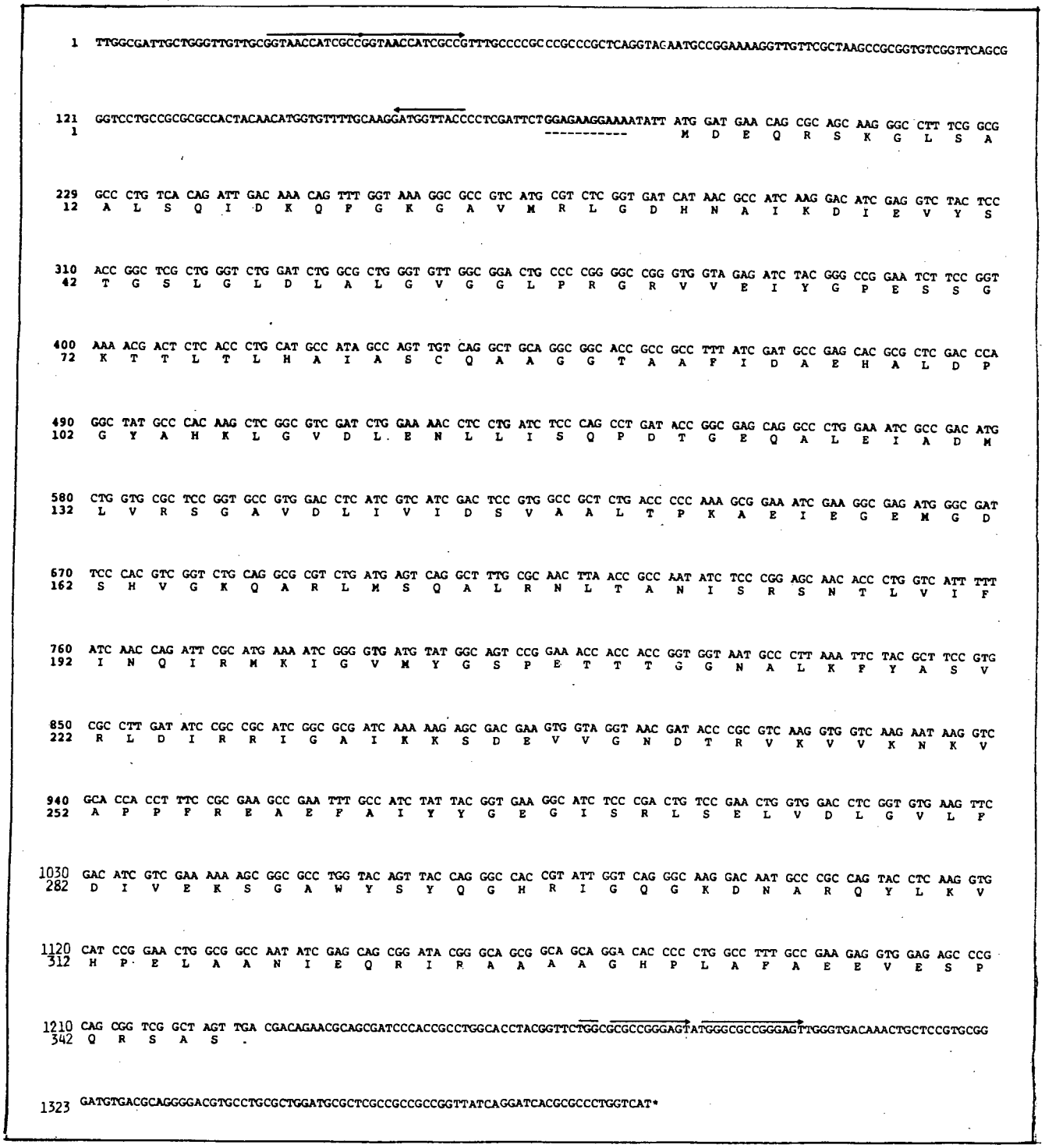

Fig. 5.3 Nucleotide sequence of the T. ferrooxidans recA gene and flanking regions. Only the DNA strand with the polarity of the mRNA is shown. The deduced amino acid sequence is given below the coding region. The single letter amino acid code used is explained in Appendix D. Arrows above the nucleotide sequence indicate sets of tandem or inverted repeat sequences. A SD-like region is shown as a broken line below the nucleotide sequence.

sequences that have a single bp mismatch. Within the ORF are two 14 bp complementary inverted repeat sequences (nucleotides 1 107 -1 147) which have two single bp mismatches and are capable of forming a single-stranded stem-loop structure (ΔG = -23.95 kcal/mole).

5.3.1.1 Codon usage. Gene expression and its control at the translational level is affected by codon usage. A very different codon usage could prevent the expression of the foreign gene when present in a particular host. Table 5.2 shows a comparison of the codon usage in the recA genes of T. ferrooxidans, E. coli, and P. aeruginosa. Overall, the codon usage pattern in the recA genes of the three organisms is similar.

However, T. ferrooxidans and P. aeruginosa use mostly either G or C in the codon wobble position, where possible. This may be expected from the relatively higher G+C ratios of these organisms, 58-59 mol% for T. ferrooxidans (Harrison, 1984) and 67 mol% for P. aeruginosa (Krieg, 1984), compared to 48-52 mol% for E. coli (Krieg, 1984). For Lys, G is favoured over A in both T. ferrooxidans (G:A=10:8) and P. aeruginosa (G:A=22:2). In E. coli, there is a preference for A in the wobble position (G:A=6:21). For Arg: T. ferrooxidans and P. aeruginosa preferentially use the codon CGC, whereas in E. coli CGT is used.

Where a choice of either C or G exists in the wobble position, one of the bases is often favoured. In

Table 5.2 Comparison of the codon usage in recA genes between T. ferrooxidans (Tf), E. coli (Ec) and P. aeruginosa (Pa).

		Tf	Ec	Pa			Tf	Ec	Pa
Ala	GCA	5	11	2	Leu	CUA	0	0	0
	GCC	22	4	26		CUC	8	2	4
	GCG	8	19	9		CUG	19	24	23
	GCU	4	4	1		CUU	3	3	0
Arg	AGA	0	0	0		UUA	1	0	0
	AGG	0	0	0		UUG	1	2	2
	CGA	1	0	0	Lys	AAA	8	21	2
	CGC	10	2	12		AAG	10	6	22
	CGG	6	0	1	MET	AUG	7	10	8
	CGU	3	12	3	Phe	UUC	3	6	7
Asn	AAC	6	14	10		UUU	5	4	0
	AAU	5	1	2	Pro	CCA	2	1	0
Asp	GAC	10	11	18		CCC	3	0	3
	GAU	9	9	3		CCG	4	9	8
Cys	UGC	0	1	1		CCU	2	0	0
	UGU	1	2	1	Ser	AGC	5	5	4
Gln	CAA	0	1	2		AGU	4	0	0
	CAG	14	12	14		UCA	1	2	0
Glu	GAA	15	21	12		UCC	10	6	9
	GAG	8	9	13		UCG	3	1	4
Gly	GGA	2	1	1		UCU	1	6	0
	GGC	18	16	31	Thr	ACA	0	0	0
	GGG	2	1	0		ACC	11	9	15
	GGU	13	17	6		ACG	1	5	0
His	CAC	5	2	2		ACU	1	3	0
	CAU	3	0	1	Trp	UGG	1	2	1
Ile	AUA	2	0	0	Tyr	UAC	7	7	7
	AUC	19	25	25		UAU	3	0	1
	AUU	4	2	1	Val	GUA	2	5	2
						GUC	10	2	14
						GUG	12	10	12
						GUU	1	4	1

T. ferrooxidans C is used more often than G, e.g. for Arg, Gly, Ser and Thr. The nucleotide, G, is favoured in Leu. For Ala, the codon GCC is used more often than GCG (GCC:GCG=22:8). A similar trend is evident in P. aeruginosa. In E. coli, GCC is favoured over GCA, GCU or GCG.

Interestingly, there are instances where either U or A are used preferentially over either G or C in T. ferrooxidans. For Glu, GAA:GAG=15:8. In some cases U and A are used at a similar frequency to G and C. In T. ferrooxidans, for Asp GAC:GAU=10:9, which is similar to E. coli (C:U=11:9). However, in P. aeruginosa C is favoured over U (18:3). Similarly, in T. ferrooxidans, the codons for Lys AAA:AAG=8:10. In P. aeruginosa the use of G in the wobble position is strongly favoured (G:A=22:2). In E. coli A is favoured over G (G:A=6:21).

The codon usage between T. ferrooxidans and P. aeruginosa is more similar than that between T. ferrooxidans and E. coli. To establish significant similarities, a comparison of codon usage in the recA gene of T. ferrooxidans with recA genes from a wider range of organisms is necessary. However, the recA sequences of E. coli and P. aeruginosa were the only ones available at the time of this study.

5.3.2 Comparative analysis of *recA* gene products

A comparison of the derived amino acid sequence between the completely sequenced *recA* genes of *T. ferrooxidans*, *E. coli* (Horii *et al.*, 1980; Sancar *et al.*, 1980), *P. aeruginosa* (Sano and Kageyama, 1987) and a partial sequence from the cyanobacterium *Synechococcus* (Murphy *et al.*, 1987) is shown in Fig. 5.4. The predicted amino acid sequence of the *T. ferrooxidans* RecA protein is 346 amino acids in length. This is the same as that of *P. aeruginosa* and six residues shorter than the *E. coli* RecA protein (Sancar *et al.*, 1980). The *T. ferrooxidans* RecA protein shares a high degree of homology with both the *E. coli* (66%) and *P. aeruginosa* (69%) RecA proteins. A striking degree of homology was found to extend throughout the proteins, except for two regions. There is a region of poor homology at the amino terminus (amino acid residues 30-41 of the *T. ferrooxidans* protein), and a larger region of limited homology (of approximately 40 amino acids) at the carboxy terminus. The greatest amount of conservation between the three eubacterial RecA proteins is in the region from Ile^{189} to Arg^{277}. The amino acid sequences of the *E. coli* and *P. aeruginosa* RecA proteins are perfectly conserved within this region and there is a single substitution (*E. coli*, Phe^{203} to *T. ferrooxidans*, Tyr^{203}) in the case of the *T. ferrooxidans* RecA protein.

Of particular interest is the degree of conservation in the regions already identified as functional domains. Although there is little homology evident between the RecA proteins

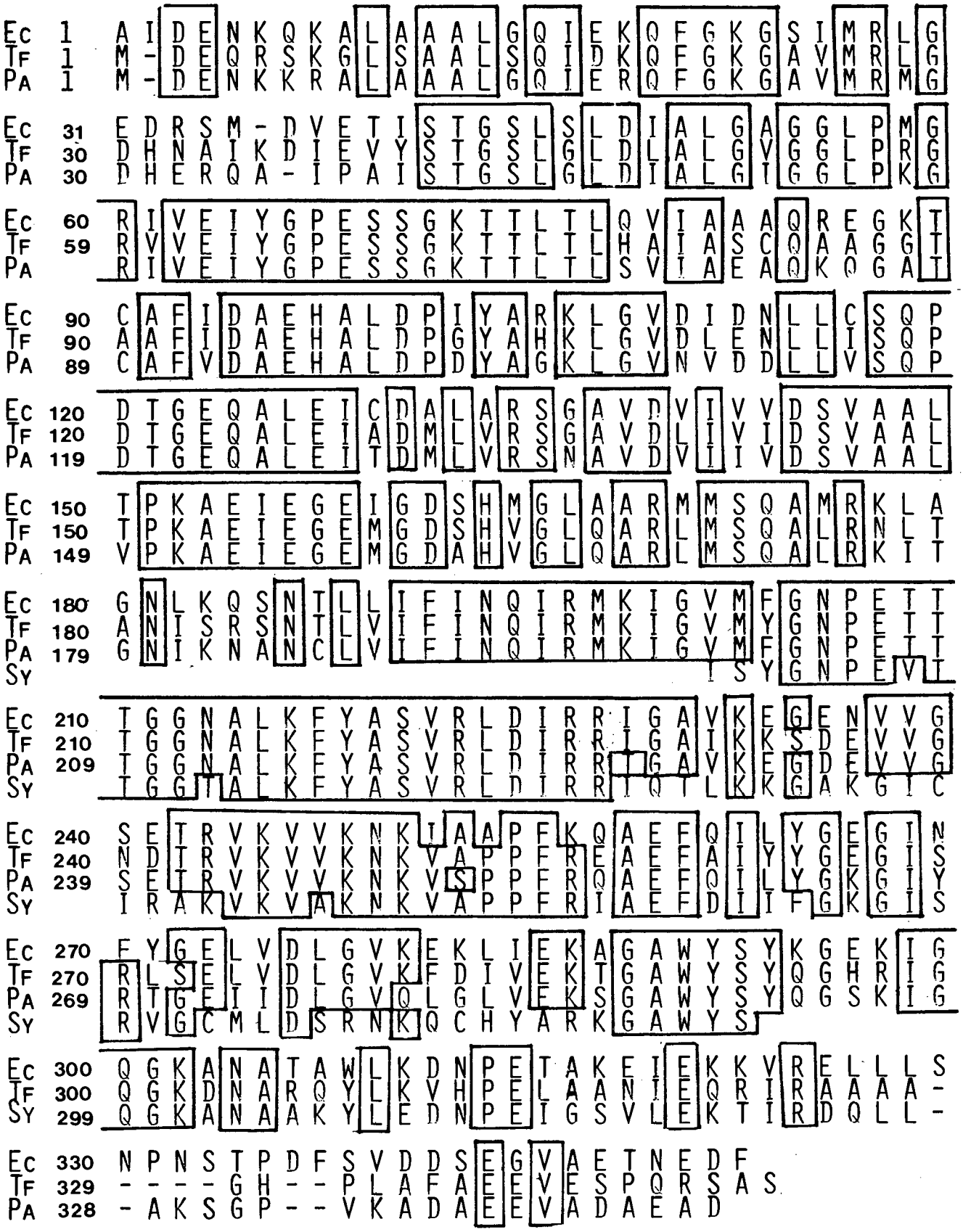

Fig. 5.4 A comparison between the amino acid sequence of the RecA proteins of E. coli (Ec), T. ferrooxidans (Tf) and P. aeruginosa (Pa), and a partial sequence of Synechococcus (Sy). Boxed residues are identical in at least three out of the four proteins.

of T. ferrooxidans and the other organisms in the amino-terminal region, a net positive charge has been retained. It has been suggested that the amino-terminal region is likely to be the ssDNA-binding domain (Kawashima et al., 1984; Wang and Tessman, 1986; Sano and Kageyama, 1987).

The effect of mutations in the E. coli recA gene on the amino acid sequence and protein activity has been studied by several workers as described in section 5.1. Most of the single amino acid substitutions which affected the function of the E. coli RecA protein are conserved in the T. ferrooxidans protein (Table 5.3). Exceptions are the presence of T. ferrooxidans Ile^{116} in place of E. coli RecA Cys^{116} and several changes in the amino acids associated with constitutive protease activity (Wang and Tessman, 1986). These are E. coli amino acids Ser^{25}, Thr^{39}, Ala^{179} and Gln^{184} which have been replaced by Ala^{24}, Val^{39}, Thr^{179} and Arg^{184} in the corresponding positions of the T. ferrooxidans RecA protein (Fig. 5.4 and Table 5.3).

It was essential to assess whether these amino acid changes in the T. ferrooxidans RecA protein have resulted in a protein that is constitutively proteolytic in E. coli. The ability of the cloned T. ferrooxidans recA gene product to inactivate the repressor of phage λ and the LexA repressor of the E. coli recA gene were used to indicate protease activity.

Table 5.3 A comparison of amino acid substitutions that affect *E. coli* RecA function with the corresponding amino acids in *T. ferrooxidans* (Tf)and *P. aeruginosa* (Pa)

Change in *E. coli*	Function affected	Amino acid in an equivalent position	
		Tf	Pa
Gly^{160}--Asp	all functions	Gly^{160}	Gly^{160}
Gly^{204}--Ser	defective protease activity	Gly^{204}	Gly^{204}
Gly^{229}		Gly^{229}	Gly^{229}
Cys^{116}	ATPase	Ile^{116}	Val^{115}
Ser^{25}--Phe		Ala^{24}	Ala^{24}
Glu^{38}--Lys		Glu^{38}	Pro^{37}
Thr^{39}--Ile		Val^{39}	Ala^{38}
Glu^{158}--Lys		Glu^{158}	Glu^{158}
Ala^{179}-Val		Thr^{179}	Thr^{178}
Gln^{184}--Lys		Arg^{184}	Asn^{183}
Arg^{169}--Cys	Prt^{C}	Arg^{169}	Arg^{168}
Gly^{301}--Ser	Rec^{-}	Gly^{301}	Gly^{300}
--Asp	Rec^{-}		
Val^{246}--Met	Thermostability	Val^{246}	Val^{245}
Ile^{298}--Val	Thermostability	Ile^{298}	Ile^{297}

It was previously shown that the cloned T. ferrooxidans recA gene product in E. coli was able to inactivate prophage λ repressor (Chapter 4). However, maximal phage release was only evident after induction with mitomycin C (section 4.3.4.2). This suggested that the cloned recA product was not constitutively proteolytic and that it required DNA damage for maximal protease activity.

The inducible protease activity of the cloned T. ferrooxidans RecA protein, indicated by its ability to cleave E. coli LexA repressor, was shown using Western blot studies (Fig. 5.5). E. coli HB101 produces a defective RecA protein that, unlike the E. coli RR1 RecA, has no protease activity and is not induced by DNA damage (Fig 5.5, lanes C and D). Induction of the defective E. coli HB101 RecA protein as a result of DNA damage was detected in the presence of the cloned T. ferrooxidans recA gene on pRSR100 (lane B). No increase in the quantity of T. ferrooxidans RecA protein as a result of DNA damage was detectable in protein preparations from E. coli HB101(pRSR100) (lane B) or T. ferrooxidans cells (lane A). The inability of the cloned T. ferrooxidans RecA protein to be induced after UV damage was confirmed using the E. coli JK696 recA deletion strain which lacks RecA protein and does not partially obscure the T. ferrooxidans RecA protein (lane E).

Maximal prophage induction and increased synthesis of the defective RecA protein in E. coli HB101 after DNA-damaging treatment indicated that the T. ferrooxidans RecA protein

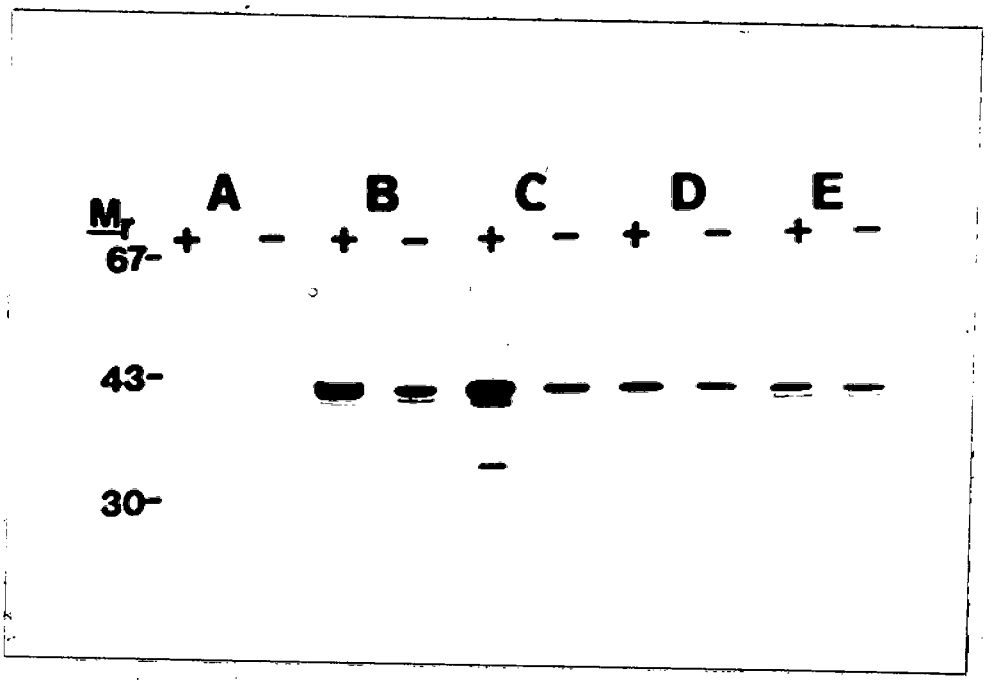

Fig. 5.5 Western blot of crude protein extracts from mitomycin C -induced (+) and -uninduced (-) cultures of:

Lane **A:** *T. ferrooxidans*
B: *E. coli* HB101 (pRSR100)
C: *E. coli* RR1
D: *E. coli* HB101
E: *E. coli* JK696 (pRSR100)

was not constitutively proteolytic; DNA damage was required for complete activation of protease function. The amino acid changes in the T. ferrooxidans RecA protein, therefore, did not result in a RecA protein that was constitutive for protease activity in E. coli.

Induction of the native RecA protein in response to DNA damage in E. coli RR1 and E. coli HB101(pRSR100) was indicative of derepression of the respective recA genes through inactivation of LexA repressor. However, unlike the E. coli recA gene, the T. ferrooxidans recA gene may not be regulated by a LexA repressor. This was suggested by the absence of an E. coli-like LexA-binding site within 196 bp upstream of the T. ferrooxidans recA ORF. The lack of induction of the T. ferrooxidans RecA protein as a result of DNA damage in T. ferrooxidans, E. coli HB101(pRSR100) and E. coli JK696(pRSR100) (Fig. 5.5; lanes A, B and E, respectively), was additional evidence for the absence of LexA-like regulation of the T. ferrooxidans recA gene.

With respect to the highly conserved region in the RecA proteins of E. coli, P. aeruginosa and T. ferrooxidans, it is interesting that the amino acid substitution, E. coli Phe^{203} to T. ferrooxidans Tyr^{203} did not result in a RecA protein that lacked protease activity. Kawashima et al. (1984) found that the recA430 mutant had a single conservative amino acid substitution (Gly^{204} to Ser) in this region which resulted in a protein with nearly normal recombination function but defective SOS functions. The

protease activity of the T. ferrooxidans RecA protein was retained probably because of the charge and aromatic conservation that accompanied the Phe^{203} - Tyr^{203} change. The E. coli Phe^{203}- T. ferrooxidans Tyr^{203} change is a conservative amino acid substitution with no accompanying change in charge.

The location of the protease domain in the first half of the T. ferrooxidans RecA protein is suggested by the presence in this region of five of a total of eight His residues, and the only Cys residue. These amino acids are found in the active sites of a large proportion of serine proteins (Dayhoff, 1972). In addition, the sequences, -Ala^{98}-Glu-His-Ala^{101}-, and -Gly^{163}-Asp-Ser-His^{166}-, common in active sites of serine proteases (Dayhoff, 1972), are evident in the first half of the T. ferrooxidans RecA protein. Similar sequences were also shown in the E. coli RecA protein (Sancar et al., 1980).

An amino acid consensus sequence common to ATP-binding proteins (Walker et al., 1982; Gill et al., 1986) was also found in E. coli (Gly^{66}-Thr^{73}) and P. aeruginosa (Gly^{65}-Thr^{72}) RecA proteins (Sano and Kageyama, 1987). The presence, in T. ferrooxidans, of an identical consensus sequence, -Gly^{66}-Pro-Glu-Ser-Ser-Gly-Lys-Thr^{73}-, suggests that this conserved region has a similar role in this organism. Cys^{85}, which occurs close to this area may play a cooperative role in binding ATP.

It is not known whether the limited changes in the conserved regions of the T. ferrooxidans RecA protein have contributed to its lower activity in E. coli. Furthermore, although the major domains within the T. ferrooxidans protein are conserved, the changes in the unconserved regions may affect the tertiary structure of the RecA protein. These changes could have resulted in the observed lower activity of the cloned RecA protein when compared with that of the native E. coli RecA protein (reported in Chapter 4). The level of activity of the cloned RecA protein in E. coli is, however, not necessarily an indication of the level of activity within T. ferrooxidans. Evolution of the recA gene and its product within T. ferrooxidans has most likely occurred so as to function optimally within the natural host under the selective pressures and physiological conditions unique to this organism.

5.3.3 Expression of the cloned recA gene in E. coli

As no promoter sequences were apparent from the nucleotide sequence in the region immediately upstream of the recA ORF (Fig. 5.3), the expression of the cloned gene in E. coli was investigated. The T. ferrooxidans recA gene on three independent clones pRSR100, pRSR101 and pRSR102 (Chapter 4; Ramesar et al., 1988), had the recA ORF in the identical orientation, between 2.1 and 2.5 kb downstream from the λ P_r promoter of the pEcoR251 cloning vector. A plasmid, pRSR105, was constructed in which the λ P_r promoter was deleted

(Fig. 5.6). E. coli HB101(pRSR105) was sensitive to similar levels of MMS and UV irradiation as E. coli HB101 (recA$^-$) (Table 5.4). To confirm that the T. ferrooxidans recA gene on pRSR100 was read from the λ P_r promoter, plasmid pcI857 which codes for a temperature-sensitive λ P_r repressor was transformed into E. coli HB101 (pRSR100). At the non-permissive temperature (42^0C), expression of the cloned recA gene was the same as that for the strain lacking the temperature sensitive repressor, whereas at 30^0C, expression of the recA gene was repressed (Table 5.4). These two observations indicated that the cloned recA gene on pRSR100 was mainly expressed from the λ P_r promoter of the cloning vector.

To determine whether the T. ferrooxidans recA gene was expressed when cloned in the opposite orientation with respect to a vector promoter, a 3.3 kb HindIII restriction endonuclease fragment containing the recA ORF was cloned in both orientations into the Bluescript SK plasmid. A recombinant plasmid, pRSR106, in which the cloned recA gene was situated 2.0 kb downstream from the lac promoter of the Bluescript SK vector (Fig. 5.6), resulted in approximately the same levels of MMS and UV resistance as conferred by pRSR100 (Table 5.4). In contrast, when the recA gene was cloned in the opposite orientation, in pRSR107 (Fig. 5.6), gene expression was reduced to that of pRSR105 (Table 5.4). These results indicated that the 2.2 kb DNA fragment upstream of the cloned T. ferrooxidans recA gene did not contain a promoter that was functional in E. coli. It is

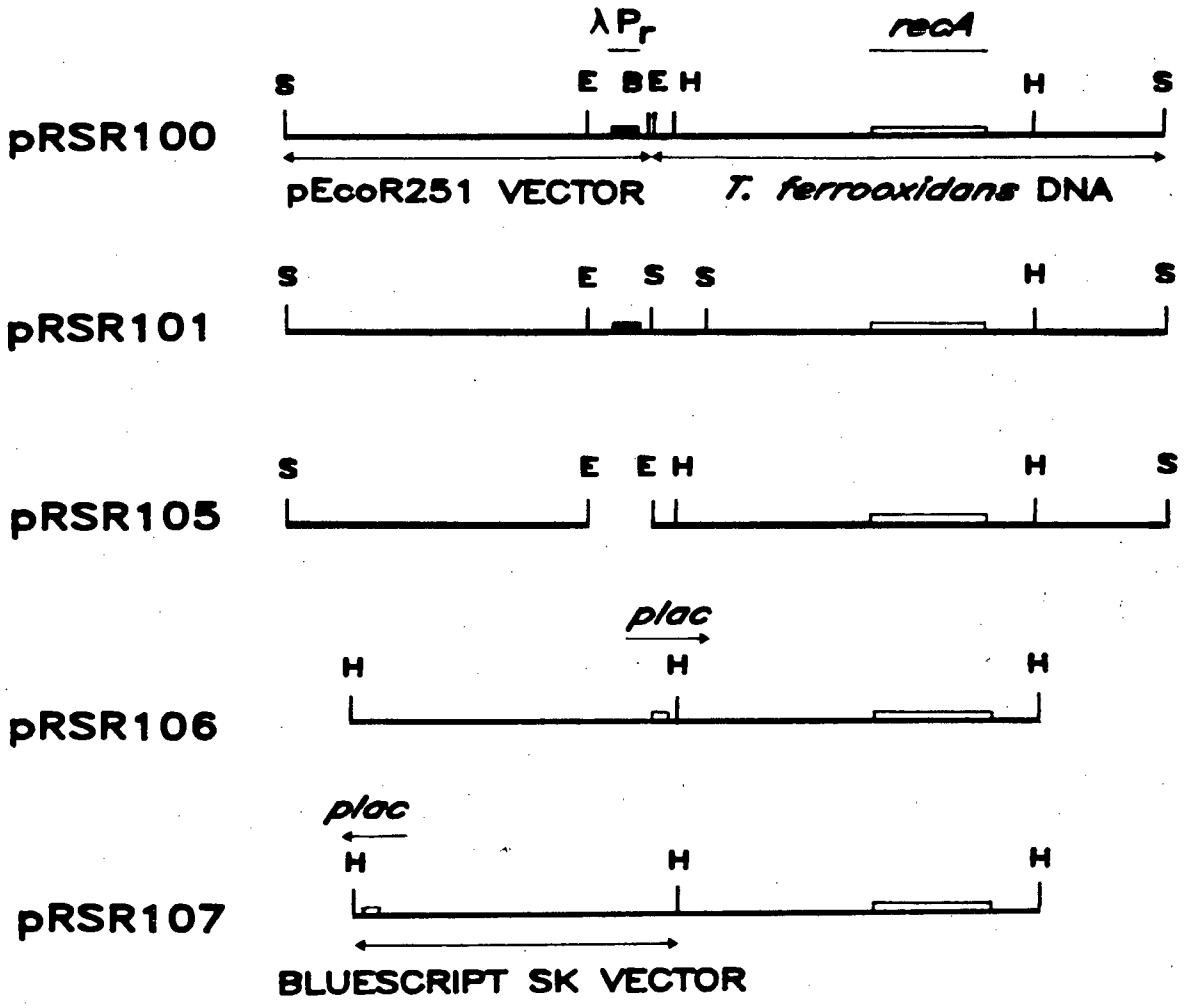

Fig. 5.6. A restriction map of pRSR100 and related subclones showing the relationship between the T. ferrooxidans recA gene and the λ P_r and Bluescript SK lac vector promoters. The restriction enzyme sites indicated are : B, BglII; E, EcoRI; H, HindIII; S, Sau3A.

not known whether this is because the T. ferrooxidans recA gene has a promoter that is not recognized in E. coli or whether, unlike E. coli and P. aeruginosa, its promoter is located more than 2.2 kb upstream of the structural gene. These findings confirmed the absence of E. coli-like -35, -10 promoter sequences associated with the cloned T. ferrooxidans recA gene (Fig. 5.3).

Table 5.4 Expression of the cloned T. ferrooxidans recA gene in E. coli

Strain and plasmid	MIC of MMS (% v/v)	UV dose for 10% survival[1] (s)
E. coli HB101	0.02	2
E. coli RR1	0.50	80
E. coli HB101 (pDR1453)[2]	0.40	55
E. coli HB101 (pRSR100)	0.30	30
E. coli HB101 (pRSR101)	0.30	30
E. coli HB101 (pRSR105)	0.02	3
E. coli HB101 (pRSR106)	0.20	25
E. coli HB101 (pRSR107)	0.02	2
E. coli HB101 (pRSR100 pc1857 at 42^0C)	0.30	30
E. coli HB101 (pRSR100 pc1857 at 30^0C)	0.02	3

[1] UV irradiation was at 1 $J.m^{-2}$
[2] pDR1453 is the cloned E. coli recA gene (Sancar and Rupp, 1979)

Several possibilities may be considered in trying to explain the absence of consensus -35, -10 promoter and SOS box-like sequences associated with the T. ferrooxidans recA gene.

(i) Consensus sequences may occur more than 196 bp upstream of the T. ferrooxidans recA gene. However, lack of expression of the T. ferrooxidans recA gene from the 2.2 kb of T. ferrooxidans chromosomal DNA upstream of the cloned gene (Table 5.4) indicates the lack of promoter sequences that can be recognized in E. coli.

(ii) The recA gene in T. ferrooxidans may be the second or subsequent member of an operon that is controlled from a promoter more than 2.2 kb upstream of the structural gene. If this situation existed, it would be unique for the recA gene among Gram-negative prokaryotes.

(iii) The T. ferrooxidans recA gene may have a unique promoter sequence that is not recognized in E. coli. Such a promoter may be peculiar either to T. ferrooxidans or to a set of genes in T. ferrooxidans, switched on only when RecA-type protein functions are required. Alternatively, expression of the T. ferrooxidans recA gene could require an activator that is not present in E. coli. Such activators have been shown to be necessary for expression of some genes in E. coli (Grossman et al., 1984; Raibaud and Schwartz, 1984; Cowing et al., 1985).

(iv) The regulatory mechanism governing the expression of the recA gene and the SOS response in E. coli may have undergone a major change during the evolution of T. ferrooxidans. A comparison between the T. ferrooxidans

recA gene product and those of *E. coli* and *P. aeruginosa* indicates strong structural and functional conservation of the *recA* gene and its product during the evolutionary process. However, structural and functional conservation of genes and gene products need not necessarily mean that the regulation of gene expression has been equally well conserved.

CHAPTER 6

GENERAL CONCLUSIONS

Progress towards the development of a genetic system and an understanding of the molecular biology of T. ferrooxidans as a result of the work presented in this thesis is reviewed in this chapter.

With respect to the development of a genetic system the two main problems are the lack of a DNA transfer technique, and the absence of a suitable marker for T. ferrooxidans. In this study conjugation as a means of DNA transfer to T. ferrooxidans was investigated. Neither direct nor multistage matings between E. coli and T. ferrooxidans were successful. However, expression of the mobilization and replication functions of a recombinant T. ferrooxidans plasmid was demonstrated by intergeneric transfer of the plasmid to the non-iron-oxidizing thiobacilli. Since the broad-host-range plasmid, pTF-FC2, which is incorporated in the recombinant plasmids, originated from T. ferrooxidans, it is not unreasonable to assume that it must have been mated into T. ferrooxidans or be able to be mated out of the bacterium. T. ferrooxidans is therefore likely to be capable of mating.

Failure to detect plasmid transfer could have been due to the use of the incorrect donor strain, unsuitable mating conditions, or an unsuitable genetic marker. The expression

of heterologous antibiotic-resistance markers in T. ferrooxidans, was considered a major factor in the inability to detect plasmid transfer. In these studies Tc was shown to have limitations when used with T. ferrooxidans. Attempts to identify expression of Cm^r were also unsuccessful. However, Cm may be more workable than Tc since the Cm^r marker is known to function in a wide range of prokaryotic and eukaryotic cells.

With regard to alternative markers, homologous chromosomal genes from T. ferrooxidans were considered. Although T. ferrooxidans was successfully mutated to Rif^r, attempts to isolate a Rif^r-conferring gene from a cosmid and a plasmid library of this strain were unsuccessful. Because Rif is an effective antibiotic in iron- and sulphur-based media, and because no other homologous marker has been identified, the potential of the E. coli rif^d allele needs to be tested as a marker for T. ferrooxidans . Whether the heterologous gene will give rise to an active Rif^r-RNA polymerase in T. ferrooxidans is not known. Although this approach would still not involve the use of an homologous character, the E. coli chromosomal Rif^r character has advantages over the heterologous plasmid-derived Tc^r and Cm^r markers used in this study.

Genomic libraries of T. ferrooxidans constructed in the course of this work will be useful for the further study of the genetics of this organism. The complementation of E. coli auxotrophic mutants with T. ferrooxidans genomic

libraries indicates that some T. ferrooxidans genes may be expressed in E. coli. Isolation and characterization of genes from T. ferrooxidans that complement mutants of E. coli or other organisms, is useful for the elucidation of the molecular biology of T. ferrooxidans. Expression of unique genes associated with chemolithotrophy, such as those involved in ferrous-iron- and reduced sulphur oxidation, however, require a different means for their detection, and can only be studied in T. ferrooxidans or possibly other chemolithotrophic bacteria.

The present investigation has shown that T. ferrooxidans possesses a recA gene. Since the cloned gene product has recombinase and protease functions when cloned in E. coli, it presumably has these properties in T. ferrooxidans. However, substantial differences in gene expression and regulation between the cloned recA gene and the E. coli recA gene were apparent. The findings from this study indicate that the recA gene in T. ferrooxidans does not have a -35, -10 promoter sequence typical of the recA gene in E. coli. Furthermore, the cloned gene appears not to be associated with a SOS-box or to be regulated by a LexA-like repressor. If the T. ferrooxidans gene has a -35, -10 promoter region, and is controlled by a LexA-like repressor, then the recognition sites for RNA polymerase and the LexA repressor are further upstream than the 196 bp that were sequenced in this study.

Since no expression of recA from 2.2 kb of DNA upstream of the gene was detected in E. coli, mapping the transcription initiation site, using the S1 nuclease mapping or the primer extension technique (Aiba et al., 1981), was considered impractical. The use of T. ferrooxidans mRNA may prove useful in detecting a unique transcription initiation site that may be functional in T. ferrooxidans. The difficulty in obtaining workable cell yields and adapting techniques used for the isolation of mRNA from E. coli to T. ferrooxdans, makes this a formidable exercise, and was not attempted.

In characterizing the genetic system of T. ferrooxidans it is useful to compare regulatory regions of T. ferrooxidans genes. The regulatory sequences associated with nitrogen metabolism genes from T. ferrooxidans, so far characterized, are similar for analogous genes in other bacteria (D.E. Rawlings, personal communication). The nitrogen metabolism genes, however, may represent a special case as their characteristic -24, -12 promoters are conserved in a wide range of organisms. However, the present investigation on the recA gene showed that regulatory regions for genes other than those involved in nitrogen metabolism may be different. A study of genes from other metabolic pathways, e.g. proline and leucine biosynthesis, may be necessary to identify T. ferrooxidans promoters. However, some T. ferrooxidans promoters may not be recognized in E. coli screening strains. The use of vectors, e.g. pEcoR251, containing strong promoters upstream of cloned genes is

therefore useful for the preliminary isolation of genes. A means of identifying unique heterologous promoters, not recognized in E. coli, involves primer extension studies using mRNA from the original host bacterium. However, the problems associated with applying these techniques to T. ferrooxidans were outlined earlier. Strong promoters from T. ferrooxidans will be useful in the construction of vectors to enhance the possibility of foreign genes being expressed in T. ferrooxidans.

To determine whether the T. ferrooxidans recA gene is part of a SOS-like regulatory network, it may be necessary to attempt to isolate genes from T. ferrooxidans which are analogous to other SOS-genes in E. coli, such as uvrB and uvrC. An examination of the DNA sequences upstream of these genes may provide evidence regarding transcription and regulation of SOS-like genes in T. ferrooxidans. Clarity on the function and regulation of recA in T. ferrooxidans could best be obtained by carrying out experiments in the organism itself. However, this requires functional assays for recombinase and protease activity in T. ferrooxidans. The necessary mutants required in such a study, and which are available for E. coli, have not yet been identified in T. ferrooxidans. Attempts at the construction of mutants in T. ferrooxidans are limited by the growth conditions and physiology of the organism. The lack of identifiable T. ferrooxidans mutants is a major stumbling block in attempts to study the genetics of this organism.

The demonstration of a functional *recA* gene in *T. ferrooxidans* is useful because it provides the potential for chromosomal gene manipulation in *T. ferrooxidans*. The recombinase system could be used to recombine manipulated DNA back onto the *T. ferrooxidans* genome. This may be used to introduce desirable genes onto the chromosome or for the construction of mutants in specific genes. For example, it may be possible to inactivate the cloned *T. ferrooxidans glnA* gene by deletion of a DNA fragment, or by transposon mutagenesis in *E. coli*. The modified gene could be recombined back into the *T. ferrooxidans* chromosome and so produce a *T. ferrooxidans glnA* mutant. The unmanipulated cloned *glnA* gene could then serve as an homologous marker. The construction of *recA* mutants in *T. ferrooxidans* may also be possible in this way. However, the major problem to be addressed before this is possible is the introduction of DNA into *T. ferrooxidans*. Electroporation, which has been successfully used to introduce DNA to previously non-transformable bacteria may provide a breakthrough and is presently being investigated.

APPENDIX A

STANDARD TECHNIQUES

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STANDARD TECHNIQUES

A.1 EXTRACTION OF DNA

A.1.1 Miniprep (small scale) plasmid isolation. The method used was essentially that of Ish-Horowicz and Burke (1981). The bacterial strain was grown with selection overnight in 2 ml LB medium (Appendix B), and pelleted by centrifugation for 2 min in a microfuge. All subsequent centrifugations in this preparation were also performed in a microfuge. The cell pellet was resuspended in 200 ul of Solution I (Solutions I, II and III are described in Appendix B) and maintained at room temperature for 5 min. Solution II (400 ul) was added, mixed gently and the tube was placed on ice for 5 min. Precooled (4^{o}C) Solution III (300 ul) was added to the mixture, mixed gently, and placed on ice for 10 min. Centrifugation for 5 min resulted in pelleting of precipitated protein, SDS and chromosomal DNA. The supernatant was removed, mixed with two volumes of 95% ethanol and held at room temperature for 2 min. DNA was pelleted by centrifugation for 5 min. The pellet was resuspended in 200 ul TE buffer (Appendix B) and the DNA reprecipitated by addition of sodium acetate to a final concentration of 300 mM, and 2 volumes of 95% ethanol. After centrifugation, the ethanol was decanted and the pellet was dried in a Speedyvac Concentrator (Savant

Instruments, Inc., Hicksville, New York, U.S.A.) and resuspended in 20 ul TE buffer. Plasmid DNA prepared in this way was sufficiently pure for restriction endonuclease digestion. Five ul of DNA solution was used per restriction digest.

A.1.2 Maxiprep (large scale) plasmid DNA isolation. A 400 ml LB medium culture, grown overnight at 37^0C in the presence of the appropriate selection, was harvested by centrifugation at 5 000 rpm for 5 min in a Beckman JA14 rotor. Cells were resuspended in 4 ml of Solution I and held at room temperature for 5 min. Solution II was added, mixed gently and the sample was held on ice for exactly 5 min. Precooled Solution III was added, mixed gently and the mixture left on ice for 5 min. Precipitated protein, SDS and chromosomal DNA was removed by centrifugation at 14 000 rpm for 20 min in a Beckman JA14 rotor. Two volumes of 95% ethanol were added to the supernatant fluid. The sample was maintained at room temperature for 2 min, and the precipitated nucleic acid was pellet by centrifugation at 27 000 rpm for 15 min in a Beckman JA20 rotor. The pellet was washed with 70% ethanol, resuspended in 4,2 ml TE buffer and purified by isopycnic ultracentrifugation. Cesium chloride (CsCl; 4,2 g) was added to the DNA solution, followed by ethidium bromide (EtBr) to a final concentration of 20 ug/ml. The sample was then adjusted to a refractive index of 1.396, sealed in a Beckman Quickseal ultracentrifugation tube and centrifuged overnight at 55 000 rpm at 15^0C in a Beckman vTi65 rotor. After visualizing the

plasmid band in the gradient with the aid of a long wavelength (350 nm) UV light, the band was removed in as small a volume as possible. The EtBr was removed by repeated extraction with one volume of salt-saturated isopropanol (Appendix B) until the pink colour had disappeared. DNA was precipitated by adding to the sample, two volumes of sterile water and three volumes of isopropanol. After standing at room temperature for 30 min, the samples were centrifuged in a microfuge for 10 min. The DNA pellet was dried in a Speedyvac Concentrator and resuspended in TE buffer. The DNA concentration was determined spectrophotometrically by monitoring its absorbance at the (UV) wavelength of 260 nm and by calculating the concentration using the the relationship: A_{260} = 1 for 50 ug/ml of DNA (Maniatis et al., 1982).

A.1.3 Extraction of total DNA from E.coli (after the method of Kirby and Wotton, 1979). Cultures were propagated in 200 ml LB medium at 37^0C overnight, harvested by centrifugation at 5 000 rpm for 10 min in a Beckman JA14 rotor. The cell pellet was resuspended in 10 ml of a buffer containing 25% sucrose, 10 mM Tris-HCl (pH 8.00), 10 mM EDTA. Lysozyme, (stock 100mg/ml) was added to a final concentration of 1 mg/ml and the sample was incubated for 1 h at 37^0C. After chilling on ice, 5 ml of 0.25 M EDTA (pH 8.00) was added and held on ice for a further 5 min. After addition of 10 ml of a 2% SDS solution (made up in 10 mM Tris-HCl, pH 8.00; 100 mM EDTA), the cell mixture was maintained at room temperature until lysis was complete.

The lysed mixture was treated with RNase (stock 50 ug/ml, pancreatic RNase, DNase-free) for 30 min at 50^{0}C, followed by a Proteinase K (50 ug/ml) treatment for 1 h at 50^{0}C. Proteins were removed from the sample by extractions with phenol-chloroform-isoamyl alcohol (25:24:1), followed by centrifugation at 10 000 rpm for 10 min in a Beckman JA20 rotor. This facilitated the partitioning of phases. The top phase (after 3 extractions and 3 centrifugations) was removed and supplemented with 0.1 volume sodium acetate (3 M), two volumes of ice-cold 95% ethanol and held on ice for 1 h. The precipitated DNA was harvested by centrifugation at 12 000 rpm for 10 min in a JA20 rotor. The DNA was dialysed against TE buffer, at room temperature, and at this stage was sufficiently pure for further manipulation.

A.1.4 Extraction of total DNA from T. ferrooxidans. T. ferrooxidans was inoculated into 9K aqueous medium (10 l) (Appendix B) and incubated at 30^{0}C with vigorous aeration until the ferrous iron was completely oxidized (5-7 d). The cells were harvested by centrifugation at 10 000 rpm for 10 min in a Beckman JA14 rotor, and then washed twice in dilute sulphuric acid (pH 2.00). The pellet was resuspended in 4 ml of a 25% (w/v) sucrose solution (made up in 2 mM EDTA; 50 mM Tris-HCl, pH 8.00), and incubated at -20^{0}C for 1 h. Proteinase K was added to the frozen suspension at 1 mg/ml, and shaken gently at room temperature until thawed. SDS was added to a final concentration of 1% and the sample was kept on ice for 15 min, followed by RNase (50 ug/ml) digestion for 20 min at 37^{0}C. The viscous opaque sample was then

dialysed at room temperature against TE buffer (which was changed frequently until the sample cleared; between 20 to 60 h). Proteins were removed from the sample by three extractions with TE-buffered phenol , followed by two extractions with chloroform. An overnight dialysis against TE buffer at room temperature rendered the DNA sample sufficiently pure for use in further manipulations.

A.2 Restriction endonuclease digestion

Techniques for general recombinant DNA work, as established and described by Maniatis et al. (1982) were followed and adapted as specified. Restriction endonucleases were purchased from Boehringer-Mannheim GmbH (Mannheim, West Germany); Amersham Corp. (Buckinghamshire, UK) and New England Biolabs, Inc. (Beverly, Massachusetts, U.S.A.). Restriction endonuclease digestion of plasmids and total cellular DNA was carried out in either low, medium or high salt restriction buffers (Appendix B) as specified by the suppliers. Digestions with two or even three enzymes could be carried out simultaneously if the salt and temperature requirements of the enzyme was compatible. If the conditions were dissimilar, digestion was carried out sequentially using the enzyme with the lowest salt optimum and the highest temperature tolerance first. The salt concentrations and temperature of incubation were then adjusted for the second and third enzymes. All digestions were incubated at 37^0C except Bcl1 which was optimally active at 70^0C. Plasmid DNA digestion was routinely carried

out in a volume of 20 ul using 4 u of restriction enzyme per 1 ug of DNA. Total cellular DNA was digested in a proportionally scaled-up volume in which 10 ug of DNA was digested. Plasmid DNA was digested for 1 h, whereas total cellular DNA was digested for approximately 4 h. For electrophoretic analysis of digested products, the digestion reaction was terminated with 10 ul of DNA loading buffer (Appendix B). The sample could be stored in this form for extended periods. In cases where it was to be used in subsequent enzymatic reactions, the digestion mix was extracted three times with TE-buffered phenol, followed by three extractions with chloroform. The DNA was precipitated using two volumes of ethanol and 0.1 volume of 3 M sodium acetate and dried in a Speedyvac Concentrator. Following resuspension in TE buffer, the DNA could be either dephosphorylated or ligated efficiently.

A.3 DNA Gel electrophoresis

Electrophoresis of DNA was carried out using a horizontal gel system with TBE buffer (Appendix B), or TAE buffer (Appendix B) if DNA was to be immobilized onto a membrane. Sigma Type I agarose (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), made up in TBE or TAE buffer, containing EtBr (0.5 ug/ml), was used. The concentration of agarose used varied from 0.5% (w/v) for the analysis of large DNA fragments to 1.0% for the detection and sizing of smaller DNA fragments (Maniatis et al.,1982). An agarose concentration of 0.8% was used for routine applications.

DNA was electrophoresed through these gels at 2,7 V/cm overnight. The DNA was visualized using a 254 nm wavelength Transilluminator (Chromato-Vue Model TS-15; UV Products Inc., San Gabriel, California, U.S.A.). The gels were photographed using a Polaroid CU-5 Land camera and Polaroid Land Pack 667 film. Exposures were generally of 1 to 2 s duration at an aperture of f-4.5. The sizes of the DNA fragments were calculated by extrapolation from a standard curve. In this, the mobility of a standard series of fragments were plotted against the log of their molecular masses. DNA standards used were λ DNA digested with either HindIII or PstI endonucleases. The amount of DNA loaded per lane was about 200 ng for every fragment expected from digests of phage λ or plasmid DNA. Approximately 10 ug of total cellular DNA was loaded per lane for detection by hybridization.

A.4 Ligation reactions

A.4.1 Normal ligations. Ligation reactions and conditions outlined by Maniatis et al. (1982) were adhered to, or were adapted as required. Vector to insert ratios were determined by the size of inserts to be cloned. Circle closure reactions using foreign DNA inserted into vector DNA molecules, were carried out at a DNA concentration of 5 pmol/ml. Where endonuclease mediated deletion reactions, followed by circle closure was required , dilute ligation reactions containing DNA, ligation buffer (Appendix B), water to the appropriate volume, and T4 DNA ligase (1 u),

were incubated at 15^{0}C for 10-15 h. Blunt-end ligations were incubated at room temperature for approximately 10 h using approximately 10-fold more T4 ligase than in complementary-ended ligation reactions.

A.4.2 Low melting point (LMP) agarose ligations. Subcloning was carried out using a modification of the method of Struhl (1985). DNA was digested with the required endonuclease, and the DNA fragments separated by electrophoresis through low melting point agarose (Seaplaque^R, Marine Colloids, Rocklands, Maine, U.S.A.) in Tris-acetate (TA) buffer (50 mM, pH 8.2). The gel was visualized on a 310 nm wavelength transilluminator, after staining with EtBr. The required gel fragments were excised from the gel in as small a volume as possible, melted at 70^{0}C for 5 min, and combined with the other fragment, or maintained as a self ligation in a total volume of 10 ul. The required volume of 10 X ligation buffer was added to the ligation, followed by T4 ligase, mixed quickly, and incubated at 15^{0}C for 15 h. Prior to transforming E. coli the gel was liquefied at 70^{0}C for 5 min, and diluted with 10 volumes of 0.1 M $CaCl_2$.

A.5 Preparation of competent E. coli cells

Modifications of the methods of Cohen et al., (1972) and Dagert and Ehrlich (1979) were used. A 1/100 dilution of an overnight LB medium culture was inoculated into 50 ml of prewarmed LB medium, and incubated at 37^{0}C, with vigorous

shaking, until the culture had reached the early exponential phase (OD_{600} of 0.2). A 1/50 dilution of this culture was inoculated into 400 ml prewarmed LB medium and incubated at 37^0C with vigorous aeration until an OD_{600} of 0.2 was reached. The culture was cooled on ice for 5 min and cells were harvested by centrifugation at 4 000 rpm for 5 min at 4^0C in a Beckman JA14 rotor. The cell pellet was resuspended in 200 ml of ice-cold 0.1 M $CaCl_2$ and left for 20 min on ice. Cells were harvested from the $CaCl_2$ solution by centrifugation at 3 000 rpm for 5 min at 4^0C in a Beckman JA14 rotor and resuspended in 4 ml 0.1 M $CaCl_2$. The cells were maintained on ice and used either after ageing for 1 h or after ageing overnight, to improve their competence. If required, competent cells were stored at -70^0C after treatment with glycerol to a final concentration of 15% (v/v). Cells stored in this form were suitable for use for more than 6 months.

A.6 Transformation of competent cells

DNA in TE buffer was added to competent cells (2 ul DNA solution / 100 ul competent cells) and the sample held on ice for 10 min, heat shocked at 42^0C for 3 min and maintained on ice for 10 min. Transformation mixes were routinely diluted with 1 ml LB medium and incubated at 37^0C for 1 h to allow expression of transferred DNA.

Where pEco251 was used as a vector in ligations, the following steps were carried out for selection of

recombinants. Transformation mixes were expressed at 42^0C for 30 min to allow for Ap^r marker expression. The transformation mixes were pooled, diluted (1/20) into prewarmed (37^0C) LB medium containing Ap (100 ug/ml), and incubated at 37^0C with vigorous shaking for 1 h to allow expression of the EcoR1 gene (for counterselection of parental plasmids). The cells were pelleted by centrifugation at 6 000 rpm for 5 min in a Beckman JA20 rotor, resuspended in 4 ml LB medium and 100 ul samples were spread onto LA (Appendix B), containing Ap (100 ug/ml). Plates were incubated overnight at 37^0C.

A.7 Preparation of radioactively-labelled DNA probes

Radioactively labelled DNA probes were prepared by nick-translation using kit PB5025 supplied by Amersham Corp., (Buckinghamshire, UK). The Manufacturer's instructions were followed. Unincorporated [α-^{32}P]-labelled nucleotides were separated from the labelled probe by molecular sieving through Sephadex G-50 spin columns (Maniatis et al., 1982).

A.8 Transfer of DNA from agarose gels to membranes and hybridization with radioactive probes

The method used, was a modification of the methods Grunstein and Hogness (1975) and Smith and Summers (1980). DNA fragments were transferred from agarose gels to Hybond-N nylon hybridization membranes (Amersham Corp., Buckinghamshire, UK). Agarose gels were washed in 2 volumes

of 0.25 M HCl with gentle agitation for 15 min and rinsed in distilled water. The DNA fragments were denatured by two 15 min washes in 2 volumes of 0.5 M NaOH, 1.5 M NaCl and the gels were neutralized by two 30 min washes in 2 volumes of 1 M ammonium acetate, 0.02 M NaOH. The transfer membrane was placed over the gel. Three sheets of Whatman 3 MM filter paper were laid on the membrane, followed by a 5 cm thick layer of absorbent paper. A light weight was placed on top of this, and transfer left to continue for 2 h. The membrane was air dried and placed on a UV (254nm) transilluminator for 5 min to immobilize DNA fragments

Membranes were gently shaken in 100 ml of prehybridization solution (Appendix B) for 2 h at 60^0C. The radioactively-labelled probe to be used was denatured by boiling for 10 min and added to the prehybridization solution. Hybridization was continued overnight at 60^0C. Solutions of increasing stringencies were used to wash the filters. The first two washes were carried out at room temperature for 5 min each, using an excess volume of 0.3 M NaCl, 0.06 M Tris-HCl (pH 8.00), 0.002 M EDTA. Subsequent washes were carried out at 60^0C in 0.2 X SSC (Appendix B), 0.1% SDS (two 30 min washes); 0.01 X SSC, 0.1% SDS (1 h); and 0.05 X SSC, 0.1% SDS (1 h). After sealing in a plastic bag, the filters were laid flat under Kodak XAR-5 autoradiographic film in an X-ray cassette equipped with a Fuji X-ray intensifying screen. Exposure was continued for 2 to 7 d at -70^0C. The film was processed using Kodak GBX X-ray developer and fixed according to the manufacturer's instructions.

A.9 Construction of the packaging system for cosmids

Recombinant cosmid (or λ) DNA was packaged *in vitro* (Hohn, 1979; Maniatis *et al.*, 1982). Genotypes of *E. coli* strains used are listed in Table 3.1. Single colonies of *E. coli* strains BHB2688 and BHB2690 were streaked out on LA plates and grown overnight at 30^0C. Controls were plated and grown at 42^0C to check temperature sensitivity. Single colonies were inoculated into 500 ml prewarmed (37^0C) LB medium to an OD_{600} of not more than 0.15 and incubated with shaking until an OD_{600} of 0.3 was attained. Prophages were induced by incubating the cultures at 42^0C for 15 min. The cultures were transferred to 37^0C and incubated for 3 h with vigorous shaking. A small sample of each culture, which is lysis-inhibited as a result of a mutation in the *S* gene was checked for induction. Upon addition of a drop of chloroform the culture cleared.

Cells from each 500 ml culture were harvested separately at 4 500 rpm in a precooled Beckman JA14 rotor. To avoid dilution of packaging buffers to be added subsequently, the supernatant was decanted and the tubes dried thoroughly.

A.9.1 Preparation of sonication extracts from *E. coli* BHB2690 (λ *E* mutant). Throughout the preparation of cell extracts it is important that the cells and cell extracts are maintained at 4^0C (on ice).

The cell pellet was resuspended in 3.6 ml of freshly prepared sonication buffer (Appendix B), and transferred to a clear Falcon tube. The cell suspension was immersed in an ice bath and sonicated, using a MSE Soniprep 150 sonicator, at maximum power. Sonication was applied in 5 s bursts with intervals of 25 s to allow cooling, until the suspension showed visible signs of clearing, indicative of cell lysis. The suspension was transferred to a cold SS34 Oakridge-type polyallomer centrifuge tube and centrifuged at 18 000 rpm for 10 min at 4^0C in a Beckman JA20 rotor. The supernatant (3 ml) was recovered and diluted with an equal volume of cold sonication buffer, followed by addition of 1 ml of packaging buffer (Appendix B). Aliquots (20 ul) of the sonicated extract were dispensed to cold microfuge tubes which were immediately immersed in liquid nitrogen.

A.9.2 Preparation of freeze-thaw extract from E. coli BHB2688 (λ D mutant). The cell pellet was resuspended in 4 ml of sucrose solution (10% sucrose (w/v) in 50 mM Tris-HCl, pH 8.00), and dispensed in 0.5 ml aliquots to microfuge tubes. A freshly prepared lysozyme solution (2 mg/ml in 25 mM Tris-HCl, pH 8.00) was added to each tube at $1/20^{th}$ of the volume and immediately frozen in liquid nitrogen.

The frozen extracts were thawed on ice and each supplemented with 25 ul of freshly prepared packaging buffer. The contents of the tubes were pooled, mixed and centrifuged at 20 000 rpm for 1 h at 4^0 in a precooled Beckman JA20 rotor. The supernatant was dispensed in 20 ul aliquots to the

frozen microfuge tubes held in liquid nitrogen and which already contained 20 ul of the sonication extract from E. coli BHB2690. The microfuge tubes were immediately immersed in a liquid nitrogen bath. The ready for use packaging mixes were maintained stably in a liquid nitrogen freezer for longer than 9 months.

A.9.3 Packaging. Three packaging mixes were transferred from storage to liquid nitrogen in a portable Thermos flask. The mixes were placed in an ice bath to thaw and assayed as follows:

(i) determination of packaging efficiency

0.5 ug λ DNA

2 ul 10 mM $MgCl_2$

1 ul 40 mM ATP

(ii) background control

2 ul TE buffer

2 ul 1 mM $MgCl_2$

1 ul 40 mM ATP

(iii) recombinant cosmid packaging.

5 ul ligated cosmid-chromosomal DNA mixture

1 ul 40 mM ATP

The still partially frozen contents of the tubes were microfuged for 5 s and again placed on ice. The contents of the tubes were mixed well using a capillary tube, immediately centrifuged for 5 s and incubated at 37^0C for 60

min. The reaction was terminated by adding 0.5 ml SM buffer (Appendix B), and a drop of chloroform. Debris was removed by centrifugation for 30 s in a microfuge.

A.9.4 Titration of packaged particles

(i) determination of packaging efficiency. A fresh colony of an indicator strain, E. coli BHB2600, was inoculated into 5 ml LAM medium supplemented with 0.4% (w/v) maltose and grown to late exponential phase. Cells were pelleted and resuspended in 10 mM $MgSO_4 \cdot 7H_2O$ to an OD_{600} of 1.0

The λ packaged particles were diluted 10^{-6}-fold in SM buffer. Transduction was carried out by adding 0.1 ml of the phage suspension to 0.1 ml of E. coli BHB2600 cell suspension. After adsorption at 30^0C for 30 min, the suspension was added to 3 ml LAM overlay (Appendix B), vortexed gently and poured onto an LA plate. Plates were incubate overnight at 37^0C.

(ii) background packaging. To determine whether packagable λ DNA was still present in the packaging mixes, 0.1 ml of undiluted packaging reaction was used for transduction to E. coli BHB2600, and plated. If present, the λ DNA would effect the efficiency of recombinant cosmid packaging.

(iii) cosmid packaging.

Titration of packaged recombinant cosmid DNA is described in Chapter 3, section 3.2.6.4.

A.9.5 Storage of recombinant cosmids. Because only a fraction of a cosmid bank is used at a time in screening procedures, suitable means had to be found for storage of cosmids.

(i) in cells. Colonies (0.5-1 mm) on plates were collected to create pools containing approximately 1 000 colonies each. Eight such pools were produced from the initial cosmid transduction. Colonies were washed off LA plates with LB medium. The pools were supplemented with glycerol (15% v/v), dispensed in 200 ul samples and stored at -70^{0}C.

(ii) plasmid DNA. Plasmid DNA was harvested from the remainder of the cells in each pool, using the maxiprep method (Appendix A.1.2), without the CsCl purification step. DNA was extracted with phenol, precipitated with ethanol and redissolved in TE buffer at 1 mg/ml. The DNA suspension was stored at 4^{0}C to be used in subsequent transformation experiments.

(iii) diluted original packaging mix. Packaged recombinant cosmid is reported to be a stable means of storing cosmid DNA. The infectious particles may be used for the transduction of suitable E. coli strains.

A.9.6 *In vivo* propagation of the cosmid library. *In vivo* packaging allows reproducible preparation of cosmid gene banks in the form of a cosmid lysate (R. Curtiss III, personal communication).

An overnight culture (2 ml) of *E. coli* χ2819, grown at 30^0C in thymidine supplemented (50 ug/ml) TYM (Appendix B) was used to inoculate a fresh culture at a dilution of 1 to 20 in the same medium, supplemented with 10 mM $MgSO_4 \cdot 7H_2O$. The 250 ml Ehrlenmeyer flask containing the inoculated medium was shaken vigorously at 30^0C for 3 h.

Transduction was carried out by adding 0.2 ml of this cell culture to 0.1 ml *in vitro* packaged recombinant cosmid. After a 30 min adsorption period at 30^0C, 0.7 ml Superbroth (Appendix B) was added to the mixture and incubated for 60 min at 30^0C to allow expression of Ap^r. LA plates containing thymidine (50 ug/ml) and Ap (100 ug/ml) were spread with 50 ul samples of the expression mix and incubated at 30^0C until colonies reached 0.5-1.0 mm in diameter. Colonies were washed off agar plates with 2 ml PG (Appendix B), supplemented with thymidine (50 ug/ml) and stored in this form at -70^0C.

A.9.7 Amplification of packaged recombinant cosmids. LB medium (500 ml) supplemented with thymidine (50 ug/ml), 0.4% (w/v) maltose and Ap (100 ug/ml) was inoculated with transduced *E. coli* χ2819 (described above) to an OD_{600} of

not more than 0.05, and incubated with moderate shaking at 30^0C until the culture reached an OD_{600} of 0.3. Prophages were induced by incubation of the cultures at 45^0C for 15 min while standing. Thereafter they were transferred to 37^0C and incubated for an additional 2.5 h with vigorous aeration, followed by 15 min in an ice bath. Induced cells looked enlarged and elongated under the phase contrast microscope and lysed in the presence of chloroform.

Cells were pelleted by centrifugation at 6 000 rpm for 10 min at 4^0C in polypropylene tubes in a Beckman JA14 rotor, resuspended in 7 ml TMGS (Appendix B), and transferred to a 50 ml SS34 polyallomer centrifuge tube. To lyse induced cells, 0.2 ml chloroform was added and the tube vortexed for 1 min and kept at room temperature for 10 min, with occassional vortexing. To digest DNA liberated from lysed cells (which results in the suspension becoming very viscous), 100 ul of DNase (100 ug/ml) was added and the tube incubated at 37^0C for 10 min. Cell debris was pelleted by centrifugation at 10 000 rpm for 10 min at 4^0C in a Beckman JA20 rotor. Supernatant fluid was collected and stored over chloroform.

The number of antibiotic resistant particles and background pfu was determined by titrating on *E. coli* strains HB101 and BHB2600, respectively.

A.10 Construction of λ lysogens

A suspension (0.2 ml) of the bacteria to be lysogenized was plated in a 3 ml LAM overlay (Appendix B) on a LAM plate (Appendix B) and left to solidify. A drop of phage λ suspension (10^8 pfu/ml) was deposited on the surface, left to dry, and incubated overnight at 37^0C. Bacteria were picked from turbid plaques with a sterile loop, suspended in a small vloume of sterile LM broth (Appendix B), and streaked onto LAM plates to obtain single colonies.

The successful formation of lysogens was confirmed by the release of λ upon induction and by immunity against superinfection. Bacteria from single colonies were resuspended in LM medium and incubated at 37^0C, until a density of approximately 10^8 cells/ml was reached. Approximately 10^8 E. coli LE392 indicator bacteria were plated in 3 ml of LAM overlay on a predried LAM plate. Small drops of the bacterial culture to be tested were deposited on top of the plate, left to dry, and the plate incubated at 37^0C. Phage production by lysogenic bacteria resulted in lysis of the indicator strain.

To test for immunity, a suspension of approximately 10^6 λ phage particles was streaked with a sterile loop across a predried LAM plate. The bacterial cultures to be tested were streaked across the phage streak. The plates were incubated at 37^0C overnight. Lysogens grew confluently over the entire length of the streak, whereas nonlysogens were lysed by the phage.

A.11 SDS-Polyacrylamide gel electrophoresis (PAGE) of proteins

SDS-polyacrylamide gels were prepared according to the method of Laemmli (1970) using a Hoefer gel apparatus (SE600; Hoefer Scientific Instruments, San Francisco, California, U.S.A.) with 1.5 mm spacers assembled according to the manufacturer's specifications. All buffers and a preparation table for resolving (10%) and stacking gels are given in Appendix B. The resolving gel was prepared and degassed before pouring. Butanol was layered on the gel to promote a sharp interface. After the gel had polymerized (about 30 min at room temperature), the butanol was removed by rinsing with stacking gel buffer, and the stacking gel cast. The gel was submerged into the electrophoresis tank before loading the samples.

Samples were prepared by SDS-2-mercaptoethanol (PAGE sample buffer, Appendix B) lysis of 2 ml of cell suspension (OD_{600} of 0.5), and placed in a boiling water bath for 2 min. Samples were centrifuged (in a microfuge) for 3 min to remove debris and membrane bound material. The samples were loaded and electrophoresed through the gel at 35 mA (constant current) per gel (10 lanes) until the dye front had migrated to the end of the gel (4 to 5 h).

After electrophoresis, the gels were either stained for 3 h in staining solution with gentle agitation, destained and dried, or used for Western blotting (Appendix A.12). The

protein molecular mass markers, with a size range of 14 400 to 94 000 D, were obtained from Pharmacia, Uppsala, Sweden (Electrophoresis calibration kit, Cat.No. 17-0446-01).

A.12 Western blotting

Proteins were transferred from SDS polyacrylamide gels to GeneScreen hybridization transfer membrane (New England Nuclear Corp., Boston, Massachusetts, U.S.A.) according to the method described in the GeneScreen manual. The membrane was cut to the exact size of the gel and wet in blot buffer (Appendix B). Two Scotch-Brite pads and two pieces of filter paper (Whatman 3 MM) were also wet in the blot buffer. The GeneScreen membrane was placed on the gel, taking care not to entrap any air bubbles between the membrane and the gel. Two sheets of wet filter paper were placed onto the membrane, and one on the opposite side of the gel. The "sandwich" arrangement was then placed between the two Scotch-Brite pads and inserted into the Hoefer transfer apparatus with the nitrocellulose membrane between the gel and the anode. The transfer reservoir was filled with 4.5 l of blot buffer and the protein transfer was achieved by electroblotting at 100 V for 2 h.

A.13 Detection of proteins with enzyme-labelled antiserum

The membrane was separated from the gel and immersed in 300 ml block buffer (Appendix B) and incubated at 30^0C overnight with constant agitation. The membrane was washed once in

wash saline (Appendix B). Affinity purified RecA antiserum (Goodman et al., 1987) was added to the membrane in as small a container as possible (with maximum contact between the membrane and antiserum suspension) and incubated at 37^0C for 90 min with gentle agitation. The membrane was washed in wash saline (4 X 8 min), and supplemented with 50 ml block buffer (free of azide) containing 30 ul of goat-anti-rabbit peroxidase conjugate (Kirkegaard and Perry Laboratories, Inc., Maryland, U.S.A.) and incubated at room temperature for 90 min with gentle agitation. The solution was decanted, and the membrane rinsed with wash saline (4 X 8 min). Substrate buffer (Appendix B) (45 ml), was supplemented with 30 mg of 4-chloro-1-naphthol (which had been dissolved in 10 ml of methanol), 30 ul of H_2O_2 and incubated at room temperature until the protein bands were evident. The intensity of the bands was enhanced by storing the membrane in distilled water at 4^0C for 2 to 3 h.

Appendix B

MEDIA, BUFFERS and SOLUTIONS

9K (iron) medium

Solution A (acidified salts) :

$(NH_4)_2SO_4$	3 g
KCl	0.1 g
KH_2PO_4	0.1 g
$MgSO_4.7H_2O$	0.1 g
Distilled water	800 ml

The solution was adjusted to pH 1,9 with H_2SO_4

Solution B :

$FeSO_4.7H_2O$	33 g
Distilled water	200 ml

The solution was adjusted to pH 1,3 H_2SO_4.

Solutions A and B were autoclaved, and mixed together once they had cooled to about 40^0C.

9KD

$(NH_4)_2SO_4$	3.0 g
$MgSO_4.7H_2O$	0.5 g
KH_2PO_4	0.5 g
$Ca(NO_3)_2$	0.01 g
$FeSO_4.7H_2O$	0.001 g
Distilled water (liquid medium)	900 ml
or	
(solid medium)	700 ml

The pH of the salts solution was adjusted to 3.0 with H_2SO_4

Glucose	10.0 g
Distilled water	100 ml

Solid medium

Agar	20 g
distilled water	200 ml

The solutions were autoclaved separately and mixed once they had cooled to about 40^0C.

Luria-Bertani (LB) medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Distilled water	1 000 ml

Luria-Bertani agar (LA) medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	15 g
Distilled water	1 000 ml

Luria-Bertani top agar

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Agar	7,5 g
Distilled water	1 000 ml

Luria Mg (LM) medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
maltose	2,5 g
Distilled water	1 000 ml

Luria Mg agar (LAM) medium

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
$MgSO_4 \cdot 7H_2O$	2,5 g
Agar	15 g
Distilled water	1 000ml

LAM soft agar overlay

Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
$MgSO_4 \cdot 7H_2O$	2,5 g
Agar	7.5 g
Distilled water	to 1 000 ml

Minimal medium

Salts solution A :

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
Sodium citrate	4.5 g
Distilled water	290 ml

Salts solution B :

$MgSO_4 \cdot 7H_2O$	0.2 g
Distilled water	10 ml

Nitrogen source :
$(NH_4)_2SO_4$.....1.0 g
Distilled water.....10 ml

Carbon source :
Glucose.....2.0 g
or
Sodium succinate.....1.0 g
Distilled water.....10 ml

Vitamin :
Thiamine (B1).....0.005 g
Distilled water.....1 ml
(The vitamin solution was filter sterilized before use)

Agar :
Bacto-agar.....15.0 g
Distilled water.....679 ml

All solutions were made up separately and sterilized. Once they had cooled to 50^0C the solutions were mixed together and poured into Petri dishes.

PG
Peptone.....10 g
Glycerol.....50 ml
Distilled water.....to 1 000 ml

Superbroth
Tryptone.....32 g
Yeast extract.....20 g
NaCl.....15 g

Tetrathionate (TA) medium
(TAM plates)

Solution A :
KH_2PO_4.....3.0 g
$(NH_4)_2SO_4$.....3.0 g
$MgSO_4 \cdot 7H_2O$.....0.5 g
Distilled water.....400 ml
pH adjusted to 4.0 with H_2SO_4

Solution B :
$K_2S_4O_6$.....3.2 g
Distilled water.....500 ml
pH adjusted to 4.0 with H_2SO_4

Solution C :
Agar (Oxoid Agar No. 1).....4.0 g
Distilled water.....100 ml

Solutions A, B and C were sterilized by autoclaving, and allowed to cool to about 40^{0}C before they were mixed and poured into petri dishes.

TM solution

NH_4Cl.....1.0 g
$MgSO_4 \cdot 7H_2O$.....0.5 g
K_2HPO_4.....0.8 g
KH_2PO_4.....0.2 g
$CaCl_2 \cdot 2H_2O$.....0.01 g
Trace elements solution.....1 ml
$Na_2S_2O_3 \cdot 5H_2O$.....10 g
Distilled water.....1 000ml

TMB
TM solution plus 0.01 g D-biotin
(biotin was filter sterilized in a minimal volume of distilled water and added to autoclaved TM solution)

TMYE
TM solution plus 5 g yeast extract

Where solid media was required, agar was added at 1.5% (w/v).

TYM
Tryptone.....10 g
Yeast extract.....5 g
NaCl.....5 g
Maltose.....4 g
Distilled water.....1 000 ml

TMGS
Tris-HCl.....(pH 8.0).....10 mM
$MgSO_4 \cdot 7H_2O$.....10 mM
Gelatin.....1 g
NaCl.....100 mM

MEDIA ADDITIVES

Media were cooled to about 50^{0}C before addition of antibiotics or X-gal. Plates containing these additives were stored for no longer than one week at 4^{0}C.

Antibiotics

Ap(sodium salt).....1 g
Distilled water.....10 ml

Cm.....1 g
Ethanol.....10 ml

Km.....1 g
Distilled water.....10 ml

Nalidixic acid.....1 g
Distilled water.....10 ml

Rif.....1 g
0.1 M NaOH.....10 ml

Sm.....1 g
Distilled water.....10 ml

Tc.....1 g
Distilled water.....5 ml
Ethanol.....5 ml

All antibiotic solutions were filter sterilized and stored at -20^{0}C.

X-gal (5-bromo-4-chloro-3-indolyl-β-galactoside)
X-gal (2%, w/v).....0.2 g
Dimethylformamide.....10 ml
The solution was stored at -70^{0}C. For use, 40 ul was spread onto the surface of LA plates and left to dry before inoculation with bacteria.

B) BUFFERS AND SOLUTIONS

BAL-31 dilution/storage buffer. The buffer was made according to the following table and stored at -20^{0}C:

Stock solution		final conc.	per 10 ml
Tris-HCl	(1 M, pH 8.0)	20 mM	0.2 ml
$CaCl_2$	(1 M)	5 mM	50 ul
$MgCl_2$	(1 M)	5 mM	50 ul
EDTA	(0.5 M, pH 8.00)	1 mM	20 ul
NaCl	(5 M)	0.1 M	0.2 ml
Glycerol		44% (v/v)	4.4 ml
Distilled water			5.58 ml

BAL-31 reaction buffer (5X). The buffer was made according to the following table and stored at 4^{0}C:

Stock solution		final conc.	per 10 ml
Tris-HCl	(1 M, pH 8.0)	0.1 M	1 ml
$CaCl_2$	(1 M)	60 mM	0.6 ml
$MgCl_2$	(1 M)	60 mM	0.6 ml
EDTA	(0.5 M, pH 8.0)	5 mM	0.1 ml
NaCl	(5 M)	3 M	6 ml
Distilled water			1.7 ml

Denhardt's Solution (X 10) (Maniatis et al., 1982)

Ficoll	(1%, w/v)	1 g
Polyvinylpyrrolidone-40	(1%, w/v)	1 g
BSA (Fraction V)	(1%, w/v)	1 g
Distilled water		to 100 ml

DNA sample loading solution (X6)

Bromophenol blue	0,25g
Sucrose	40g
Distilled water	to 100ml

The solution was stored at 4^{0}C

DNA polymerase 1 (X 10) (Klenow). The buffer was made according to the following table and stored at -20^{0}C:

Stock solution		final conc.	per 10 ml
Tris-HCl	(1 M, pH 7.6)	0.1 M	1 ml
$MgCl_2$	(1 M)	0.1 M	1 ml
NaCl	(5)	0.5 M	1 ml
2-mercaptoethanol		0.7 M	50 ul
Distilled water			6.95 ml

Ethidium bromide solution (2,7-diamino-10-ethyl-9-phenyl-phenanthridinium bromide). A solution of 10 mg/ml distilled water was made up and stored in a dark bottle at 4^{0}C.

Ligation buffer (X 10) (Maniatis et al., 1982)
Tris-HCl.....(pH 7.4).....0.5 M
$MgCl_2$.....0.1 M
dithiothreitol.....0.1 M
spermidine.....10 mM
ATP.....10 mM
BSA.....1 mg/ml

Packaging buffer
Tris-HCl.....(pH 8.00).....6 mM
spermidine.....50 mM
putrescine.....50 mM
$MgCl_2$.....20 mm
ATP....(prepared according to Maniatis et al. (1982).....30 mM
β-mercaptoethanol.....30 mM

Phenol (TE-buffered). Phenol (200 g; E. Merck, Darmstadt, West Germany) was melted at 65^{0}C and 0.3 g of hydroxyquinoline was added. The phenol was extracted three times with 1 M Tris-HCl (pH 8.0) or until the aqueous phase was approximately pH 7.6. The phenol was stored under 0.1 M Tris-HCl (pH 8.0) at 4^{0}C.

SDS-polyacrylamide gel electrophoresis reagents (Laemmli, 1970) **and preparation.**

Acrylamide-bis-acrylamide stock solution
Acrylamide.....29.2 g
Bis-acrylamide.....0.8 g
Distilled water.....to 100 ml
The solution was filtered through Whatman's paper (No. 1) and stored at 4^{0}C.

Running gel buffer
Tris-HCl.....(1.5 M, pH 8.8).....36.3 g
Distilled water.....to 200 ml

Stacking gel buffer
Tris-HCl.....(0.5 M, pH 6.8).....3.0 g
Distilled water.....to 50 ml

PAGE sample treatment buffer
Stacking gel buffer.....2.5 ml
SDS.....(10%, w/v solution).....4 ml
Glycerol.....2 ml
2-mercaptoethanol.....1 ml
Distilled water.....0.5 ml
The solution was stored at -20^{0}C

Tank buffer
Tris base.....(0.025 M).....12 g
Glycine.....(0.192 M).....57.6 g
SDS.....(0.1%, w/v).....4 g
Distilled water.....to 4 000 ml

Polyacrylamide gel preparation table (10% gels)

Stock solution	running gel	stacking gel
Acrylamide	20 ml	2.66 ml
Running gel buffer	15	--
Stacking gel buffer	--	5 ml
SDS (10%, w/v solution)	0.6 ml	0.2 ml
Distilled water	24.1 ml	12.2 ml
Ammonium persulphate* (10%, w/v solution)	0.3 ml	0.1 ml
TEMED	20 ul	10 ul

* made immediately before use

Prehybridization solution

SSC.....6 X
SDS.....0.5%
Denhardt's solution.....5 X
EDTA.....0.01 M
Denatured salmon sperm DNA.....100 μg/ml

Restriction buffers (X 10)

low salt buffer
Tris-HCl.....(pH 7.5).....100 mM
$MgCl_2$.....100 mM
dithiothreitol.....10 mM

medium salt buffer
Tris-HCl.....(pH 7.5).....100 mM
$MgCl_2$.....100 mM
dithiothreitol.....10 mM

high salt buffer
Tris-HCl.....(pH 7.5).....500 mM
$MgCl_2$.....100 mM
NaCl.....1 M
dithiothreitol.....10 mM

Salmon sperm DNA
A 10 mg/ml solution was made in TE buffer. The DNA was sonicated at full power (20 microns) for 10 min in a MSE Soniprep sonicator. The DNA solution was stored in 1 ml aliquots at -20^{0}C. Immediately before use the DNA was denatured by boiling for 10 min followed by cooling on ice.

Salt-saturated isopropanol solution. Isopropanol was saturated with aqueous 5 M NaCl, 10 mM Tris-HCl and 1 mM EDTA (pH 8.5) (Maniatis et al., 1982).

Solutions I, II, and III (Ish-Horowicz and Burke, 1981)

solution I
Glucose.....50 mM
Tris-HCl.....(pH 8.0).....25 mM
EDTA.....10 mM

solution II
NaOH.....0.2 M
SDS.....(prepared fresh before use).....1% (w/v)

solution III
Potassium acetate..(pH 4.8).....5 M

SSC (X 20)
NaCl.....121 g
Sodium citrate .....88.2 g
Distilled water.....to 1 000 ml
The solution was adjusted to pH 7.0 with NaOH and sterilized by autoclaving

SM buffer (for phage storage and dilution; Maniatis et al., 1982)
NaCl.....5.8 g
$MgSO_4 \cdot 7H_2O$.....2 g
Tris-HCl.....(pH 7.5).....50 ml
gelatin.....(2%, w/v).....5 ml
The solution was sterilized by autoclaving and stored at 4^0C.

Sonication buffer
Tris-HCl.....(pH 8.0).....20 mM
EDTA.....1 mM
mercaptoethanol.....5 mM (or 3 ul/10ml)

Tris-acetate (TAE) buffer (X 50)
Tris-base.....242 g
Acetic acid.....57.1 ml
EDTA.....(0.5 M, pH 8.0).....100 ml
Distilled water.....to 1 000 ml

Tris-borate (TBE) buffer (X 10)
Tris-HCl.....108 g
Boric acid.....55 g
EDTA.....9.3 g
Distilled water.....to 1 000 ml
The solution was adjusted to pH 7.8, autoclaved and diluted as required.

Tris-borate (TE) buffer (X 100)
Tris-HCl.....121 g
EDTA.....(0.5 M, pH 8.0).....200 ml
Distilled water.....to 1 000 ml
The buffer was adjusted to pH 7.8, autoclaved and diluted as required.

Western blotting buffers, reagents and solutions

Blocking buffer stock solution
Tris-HCl.....(pH 7.4).....1.21 g
NaCl.....9 g
Distilled water.....to 1 000 ml

Blocking buffer

Blocking buffer stock solution	500 ml
Fat-free milk powder	10 g
Tween-20	0.25 ml
Sodium azide	0.1 g

Blot buffer

Tris-base (25 mM)	15 g
Glycine (0.192M)	72 g
Methanol	1 000 ml
Distilled water	to 5 000 ml

GARP solution (Goat-anti-rabbit IgG conjugated horseradish peroxidase; Kirkegaard and Perry Laboratories). The serum was diluted 1/1 500 in blocking buffer, without sodium azide, immediately before use.

Horseradish peroxidase substrate solution

4-chloro-1-naphthol (Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U.S.A.)	30 mg
Methanol	10 ml
Substrate buffer	50 ml
H_2O_2	30 ul

Substrate buffer

Tris-HCl (0.2 M, pH 7.4)	6 g
NaCl (0.2 M)	11.6 g
Distilled water	to 1 000 ml

Wash saline

NaCl (0.9%, w/v)	9.0 g
Tween-20 (0.05%, v/v)	0.5 ml
Distilled water	to 1 000 ml

APPENDIX C

RESTRICTION MAPS OF PLASMID VECTORS USED IN THIS STUDY

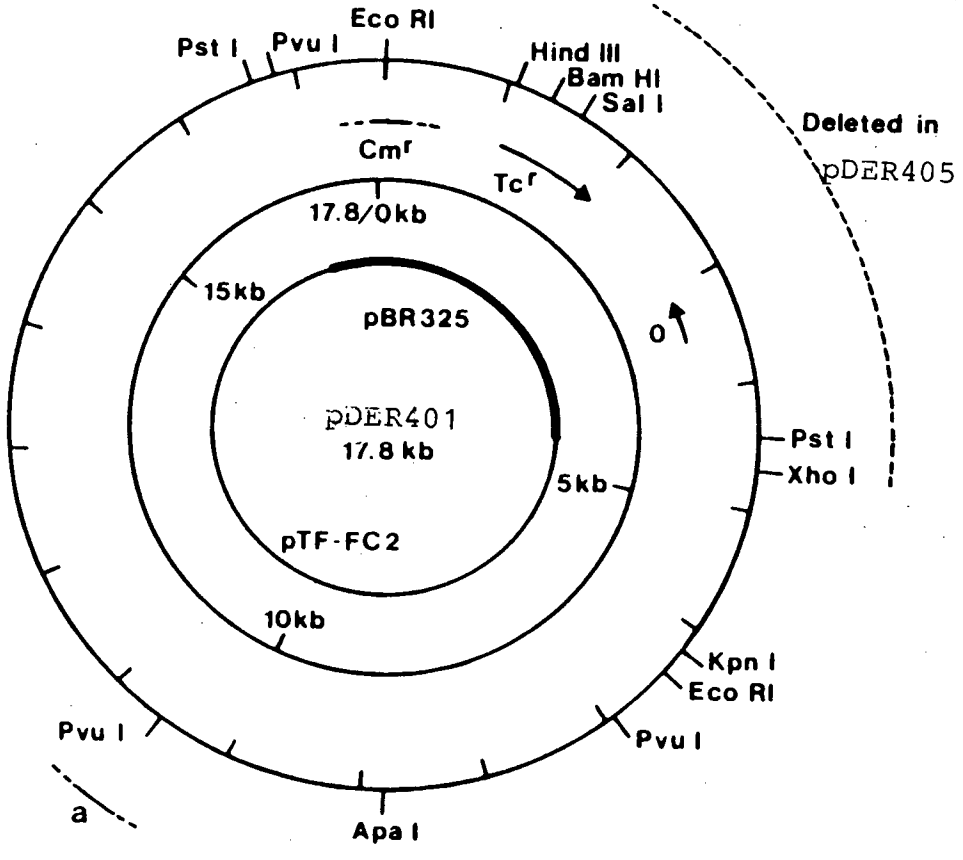
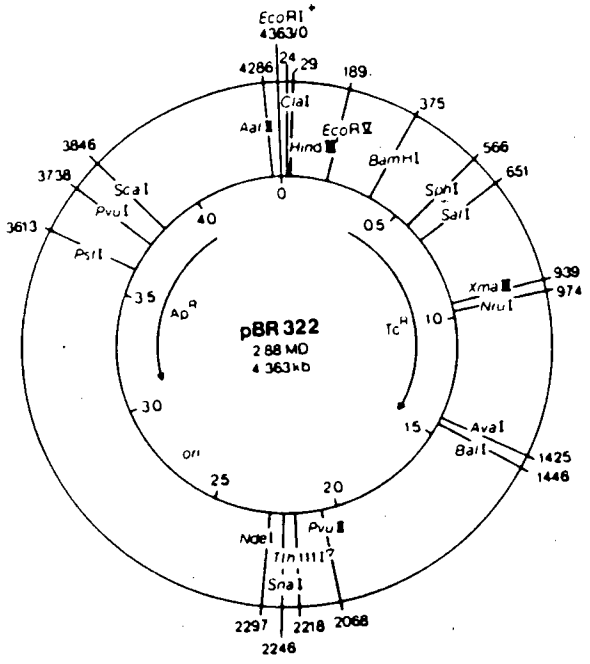


Fig. C1 Plasmid pDER401 (Rawlings and Woods, 1985), the cloning vector used in conjugation studies, described in Chapter 1.



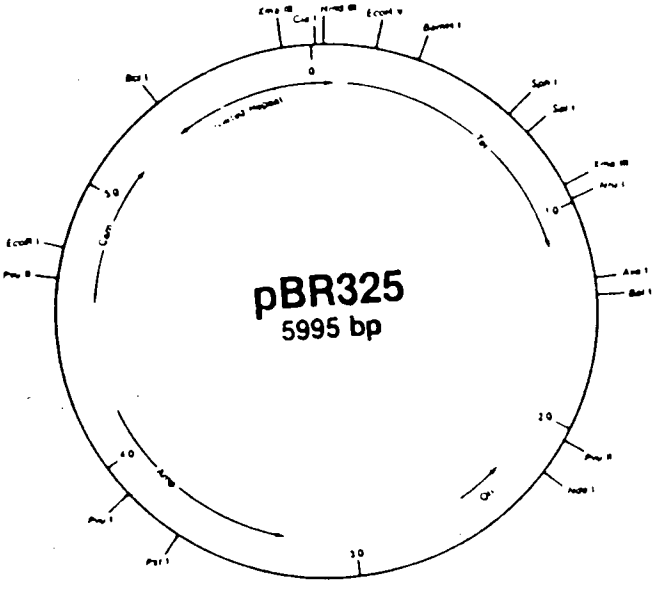


Fig. C2 Plasmid pBR322 (Bolivar et al., 1977), which was used in transformation controls, and pBR325 (Bolivar, 1978), which was used in the construction of pDER401 and pDER405 (and used in Chapter 2).

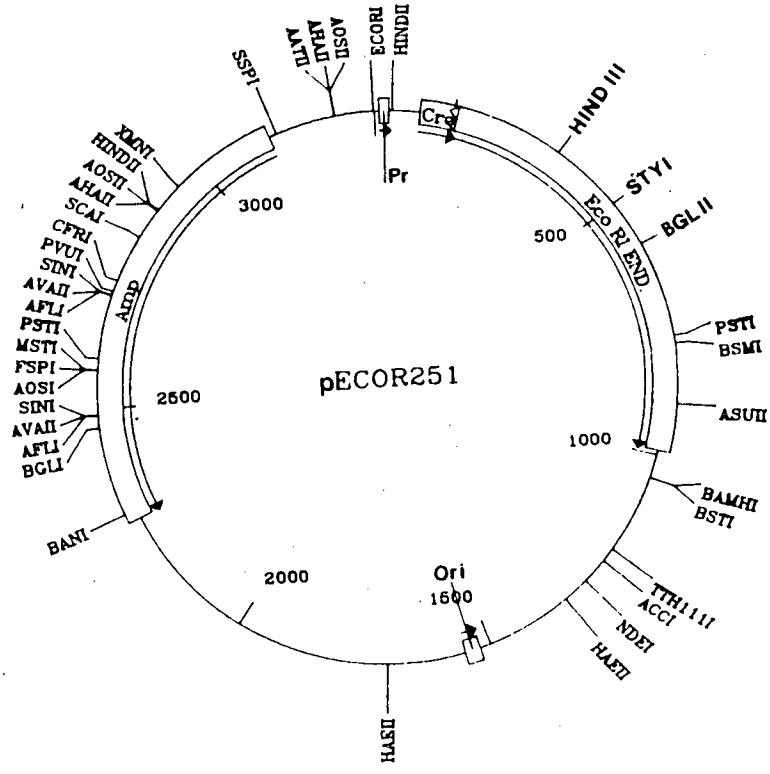


Fig. C3 The plasmid cloning vector, pEcoR251 (Zabeau and Stanley, 1982), used for the construction of plasmid based libraries of T. ferrooxidans

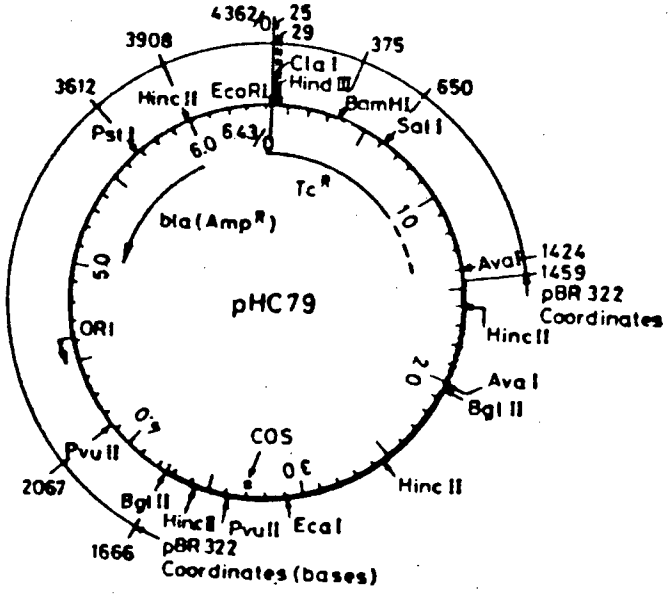
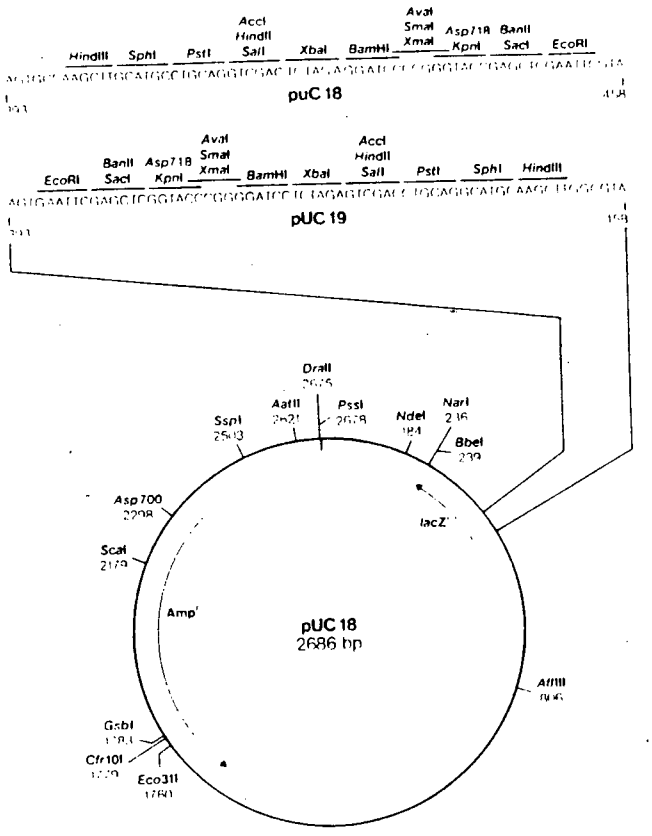


Fig. C4 The cosmid vector pHC79 (Hohn and Collins, 1980), used in the construction of a cosmid library of T. ferrooxidans.



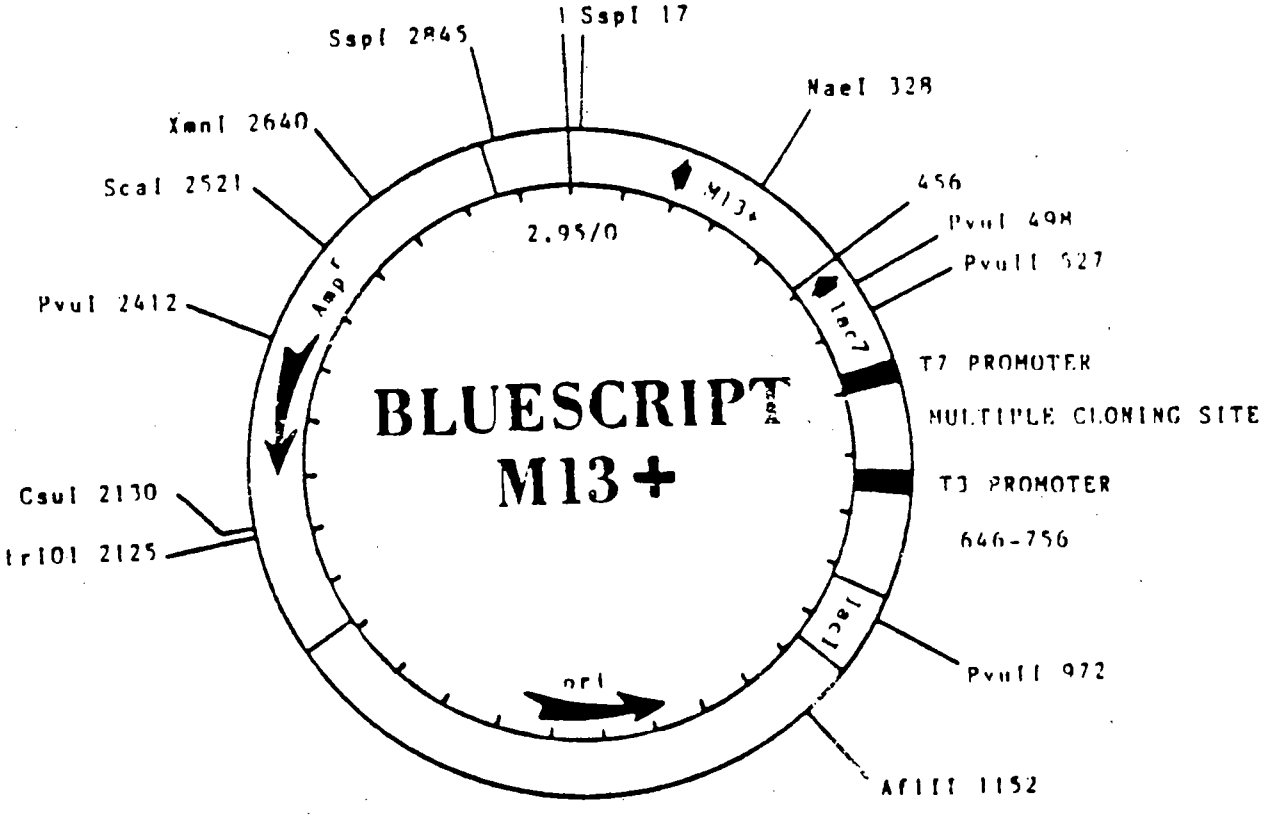


Fig. C5 Plasmid vectors pUC18, pUC19 (Yan isch-Perron et al., 1985) and Bluescript (SK) (Stratagene, California, U.S.A.) used in sequencing and expression studies of the T. ferrooxidans recA gene.

Appendix D

One- and three-letter codes used for amino acids

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

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