The Development of Genetic Systems For Iron-Oxidizing, Acidophilic Moderate Thermophiles.

by

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Dedication

To Mum and Dad.

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DECLARATION

I declare that this thesis has been composed by myself and has not been used in any previous application for a degree. The results presented here were obtained by myself under the supervision of Dr. J. C. Murrell and Dr. P. R. Norris. All sources of information have been specifically acknowledged by means of reference.

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SUMMARY

Acidophilic, moderately thermophilic, Gram positive bacteria which are able to oxidize iron and solubilize sulphide ores are likely to be of industrial importance for the leaching of metals from mineral ores. Genetic manipulation of these bacteria might produce improved strains with regard to desirable leaching characteristics for industry. This work describes initial attempts to develop a host:vector system for the moderate thermophiles.

The bacteria were found to be microaerophilic and a pour plate technique and filter disc assay were used to assess the comparative sensitivities of strains ALV, BC1 and TH3 to antibiotics and metals. Chloramphenicol and kanamycin were further investigated as potential selection agents for transformation. The latter was found to be unstable at pH 1.7 and 45°C in a ferrous iron medium.

A large plasmid which migrated more slowly than chromosomal DNA during agarose gel electrophoresis was identified in strain LM2, along with comparatively small plasmids in strains LM2, LM1, TH1 and BC1. The latter plasmid (pBC1) was cloned into *E. coli* vectors pACYC177, pBR325 and pMTL20C. These recombinant vectors were used to investigate the host range of pBC1 and *in vitro* expression from the pBC1 DNA in an *E. coli* system. Recombinant vectors containing pBC1 did not transform *B. subtilis* 168 but expressed a polypeptide with apparent M_r of 42,000 as determined by SDS-PAGE following *in vitro* transcription and translation in an *E. coli* system.

The complete nucleotide sequence of pBC1 (2,617 bp) was obtained and encoded four putative open reading frames (ORFs A, B, C and Z) which corresponded to polypeptides of M_r 41,112, 14,227, 8,228, and 6,538 respectively. Analysis of the nucleotide and predicted ORF amino acid sequences indicated that pBC1 belonged to the pC194/pUB110 family of interrelated plasmids from Gram positive bacteria and replicated by a rolling-circle mechanism via a ssDNA intermediate. Evidence for this was the similarity between ORF A of pBC1 and other plasmid replication proteins and the identification of a conserved region within the ORF A product (Rep) containing a tyrosine residue which has been shown elsewhere to bind to DNA during replication. Furthermore, a second possible DNA-binding domain was identified within Rep and single-stranded DNA was isolated from strain BC1. A region of the pBC1 sequence upstream of Rep was similar to the nick-site within the origin of plus strand replication of pC194 and pUB110 and homology with the minus origins (MO) of these plasmids suggested the presence of an MO in pBC1. A large putative secondary structure of about 100 bases was predicted from the pBC1 DNA sequence and was positioned about 250 bases upstream of ORF A and within ORF C.

Methodology was developed for the electroporation of strains ALV and BC1 but no electrotransformants were isolated. However, a novel method was developed which indicated that plasmid DNA was transferred into the bacteria during electroporation.

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ABBREVIATIONS.

A	adenine
Amp	ampicillin
a.r.b.s	atypical ribosome binding site
ATP	adenosine 5'-triphosphate
ATPase	adenosine triphosphatase
bp	base pairs
C	cytidine
^{o}C	degrees celsius
CCC	covalently closed circular
CFU	colony forming units
Ci	Curies
CIAP	calf intestinal alkaline phosphatase
cm	centimetre
Cm	chloramphenicol
cpm	counts per minute
D	Daltons
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dITP	deoxyinosine triphosphate
dNTPs	deoxyribinucleoside triphosphates
dTTP	deoxythymidine triphosphate
dd	dideoxy
DMF	dimethylformamide
DNase	deoxyribonuclease
DTT	dithiothreitol
E	field strength
EDTA	ethylenediaminetetraacetic acid
Fe^{2+}	ferrous iron
Fe^{3+}	ferric iron
Fe/Ye/G	iron/yeast extract/glucose medium

g	gramme
G	guanine
ΔG	free energy of interaction
G+C	guanine plus cytosine
h	hour
HEPES	N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
HMW	high molecular weight
IPTG	isopropylthio-beta-galactoside
Kan	kanamycin
kb	kilobase
kcal/mol	kilocalories per mole
kD	kilodaltons
kV	kilovolts
l	litre
M	molar
mA	milliamperes
mg	milligramme
MIC	minimum inhibitory concentration
ml	millilitre
mM	millimolar
MO	minus origin
M_r	molecular weight
mRNA	messanger ribonucleic acid
ms	millisecond
M_r	relative molecular mass
nm	nanometres
OC	open circular
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PEG	polyethylene glycol
PPO	2,5-diphenyloxazole

psi	pounds per square inch
(^{R})	resistant
RBS	ribosome binding site
RCR	rolling-circle replication
RF	replicative form
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal ribonucleic acid
(^{S})	sensitive
SDW	sterile distilled water
sp.	species
subsp.	subspecies
SDS	sodium dodecyl sulphate
ss	single-stranded
T	thymine
Tc	tetracycline
TEMED	N,N,N',N'-tetramethylethylenediamine
TCA	tricarboxylic acid
Tn	transposon
Tris	tris-hydroxymethylaminomethane
tRNA	tranfer ribonucleic acid
u	units
µFD	microfarads
µg	microgramme
µl	microlitre
UV	ultraviolet
v/v	volume by volume
V	volts
w/v	weight by volume
Ye/G	yeast extract/glucose medium
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Chapter 1.
Introduction.

1.1 Mineral Leaching by Bacteria.

Various workers have described the mechanisms involved in the solubilization of metals during mineral leaching (Lundgren & Silver, 1980; Hutchins et al., 1986; Lundgren et al., 1986; Ehrlich, 1986; Kelly, 1988; Bruynesteyn, 1989). In leaching environments oxidizable mineral sulphides occur, and there is a combination of biological and chemical oxidations resulting in the solubilization of metals.

The biochemical reactions which occur when bacteria oxidize sulphide minerals are complex and have been described elsewhere (Kelly, 1988; Bruynesteyn, 1989; Lundgren et al., 1986; Hutchins et al., 1986) and in addition, Hughes and Poole (1989) have discussed different commercial processes used for metal recovery including leaching in dumps, heap leaching, and leaching in reactors or vats.

Bacteria can catalyse the dissolution of metals from ores directly by the oxidation of the sulphide portion of the mineral or minerals can be indirectly attacked by the bacteria when products of reactions catalysed by the organisms oxidize the mineral. Ferric ion is the principal agent of indirect leaching and sulphuric acid produced during direct bacterial leaching maintains a low pH and also indirectly leaches mineral ores (see Lundgren & Silver, 1980).

Before 1972 only two mesophilic organisms were believed to have an important role in mineral leaching, Thiobacillus ferrooxidans and Thiobacillus thiooxidans. Since 1972 a plethora of physiologically and phylogenetically diverse bacteria has been isolated from hot springs and mining environments and some of these bacteria appear to flourish under the extreme conditions prevailing in mineral leaching environments (see Kelly, 1988; Hutchins et al., 1986; Norris, 1988, 1990; Harrison, 1982, 1984; Lane & Harrison, 1989). Differences in the physiology and

biochemistry of these bacteria affect their suitability for industrial processes of metal recovery. Studies have indicated that, in some processes, thermophilic bacteria would result in significant capital and operating cost savings compared to their mesophilic counterparts (Lawrence & Marchant, 1988). In addition, the dissolution of mineral concentrates by thermophilic strains of bacteria is generally more rapid than dissolution by mesophilic mineral-oxidizing bacteria (Norris, 1988; Norris & Barr, 1988; Marsh & Norris, 1983b).

During attempts to optimize bacterial activity in commercial processes a number of extreme conditions may have to be considered, including high acidity, regional temperatures, temperature rises generated by exothermic mineral oxidations, high concentrations of toxic metals and the nature of the target mineral. In hotter climates, moderately thermophilic bacteria are more suitable for the commercial treatment of arsenopyrite concentrates than the mesophile T. ferrooxidans (Spencer et al., 1989). However, it is unlikely that a single organism will fulfil the requirements of the variety of commercial operations.

1.2 Mineral-Oxidizing Bacteria.

The different types of bacteria which have been implicated in the oxidation of minerals have been extensively reviewed elsewhere (Norris, 1988, 1990; Bruynesteyn, 1989; Kelly, 1988; Hutchins et al., 1986) In summary, they can be divided into three main groups: mesophiles, such as Thiobacillus sp. and Leptospirillum ferrooxidans, moderately or facultatively thermophilic bacteria with an optimum growth temperature of about 50°C and extreme thermophiles, archaebacteria with an optimum growth temperature of about 70°C (see later for the classification of thermophiles).

1.2.1 Moderately Thermophilic Mineral-Oxidizing Bacteria.

Bacteria which are Gram positive, acidophilic and moderately thermophilic have been isolated from numerous sites including hot springs (Le Roux et al., 1977), sulphide ore deposits (see Karavaiko et al., 1988), low grade ore leaching dumps (Brierley, 1978), coal spoil heaps (Marsh & Norris, 1983a) and a simulated copper leaching system (Brierley & Lockwood, 1977; see review by Brierley & Brierley, 1986). All are able to oxidize iron and mineral sulphides (see Norris, 1990) and the mol% GC values of isolates range from about 45 to 70% (Table 1.1). These bacteria are probably representatives of several genera (Harrison, 1984; 1986a; Lane & Harrison, 1989) and of those isolates studied more extensively all have several modes of nutrition (see later).

The bacteria investigated during the course of this work fall into this 'group' of mineral-oxidizing bacteria and all are obligate aerobes, with an optimum growth temperature of about 45 to 50°C and optimum pH for growth of about 2.0. As the bacteria have not been named they will be referred to collectively as the 'iron-oxidizing moderate thermophiles' or the 'moderate thermophiles' and separately by their strain designation.

Strain TH1 was isolated from a hot spring in Iceland (Le Roux et al., 1977; referred to as Thiobacillus TH1, Brierley et al., 1978 and thermophile TH1, Norris et al., 1980) and strain BC1 was isolated from a washed coal pile at Birch Coppice Colliery, Warwickshire in 1981 (referred to as Birch Coppice isolate, Marsh & Norris, 1983a). Strains LM1 and LM2 were obtained from Lake Myvam, Northwest Iceland (LM1, unpublished; LM2 was referred to as Lake Myvam isolate, Marsh & Norris, 1983a). Strains ALV and NAL were isolated from a coal spoil heap near Alvecote, Warwickshire (ALV was referred to as Alvecote isolate, Marsh & Norris, 1983a; NAL, unpublished). Finally, strain TH3 was isolated from samples taken from the Chino Mine copper leach dump in New Mexico (Norris & Barr, 1985).

Comparative whole cell electrophoretic protein patterns as well as morphological and physiological studies have indicated that strains TH1,

Table 1.1 Moderately Thermophilic Mineral Leaching Bacteria.

Strain	Mol% GC	Morphology*	Reference
LM2	59.8	rods in pairs	Harrison, 1986b.
ALV	56.6	rods in filaments	"
BC1	50.0	rods in pairs	"
TH1	49.3	rods in pairs	"
TH3	68.5	rods in long 'hair-like' filaments	"
NAL	ND	rods in filaments	unpublished.
LM1	ND	rods in pairs	"
NMH-6	50.4	NG	Holden *et al.*, 1988.
Sulfobacillus thermosulfidooxidans	47.2	rods single, paired or in short chains	see Karavaiko *et al.*, 1988.

ND = not determined, NG = not given.

* The morphology of the bacteria varies depending on the mode of nutrition and bacteria predominating as rods in pairs may occur as single cells or in short chains (about 4 or 5 cells long). During heterotrophic growth strains TH1, BC1 and LM1 produce swollen, more coccoid shaped cells joined in chains (Norris *et al.*, 1980, 1986b). Strain TH3 produces filaments the length of which reflects the concentration of yeast extract in the medium (Norris *et al.*, 1986b).

LM1 and BC1 are probably of the same species; strains TH1 and BC1 share over 90% DNA:DNA homology (Harrison, 1986a). Likewise, strains ALV and NAL are probably of the same bacterial species but of a different genus from the TH1-type, strain LM2 or strain TH3.

Organisms with similar growth characteristics and morphology to the TH1-type appear to be particularly widespread e.g. strain NMW-6 isolated in Australia (Holden et al., 1988) and Sulfobacillus thermosulfidooxidans isolated in Russia (see Karavaiko et al., 1988).

16S ribosomal RNA analysis by the method of Lane et al. (1985) has indicated that strains ALV and BC1 were probably affiliated with the 'low' G+C Gram positive bacteria and were more closely related to one another than to other bacteria in this division. In contrast strain TH3, which has a long filamentous morphology (Table 1.1) was affiliated with the 'high' G+C division of Gram positive bacteria (Figure 1.1; Lane et al., 1988; Smida et al., 1988, Lane & Harrison, 1989).

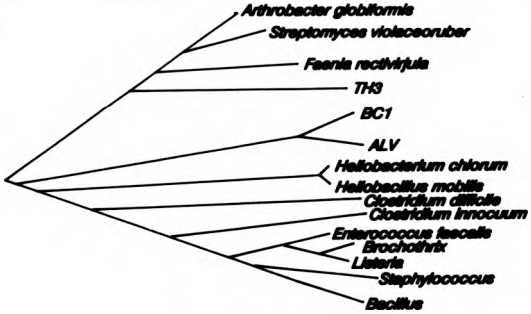


Figure 1.1 Partial Gram Positive Phylogenic Tree Derived From 16S rRNA Analysis.
(A. P. Harrison Jr., pers. comm.).

Introduction

The moderate thermophiles are among the most nutritionally versatile mineral leaching eubacteria and are facultative chemolithotrophs (Matin, 1978). They will grow autotrophically, heterotrophically, chemolithoheterotrophically and mixotrophically (see Norris, 1990), but the organisms preferentially use the latter two types of metabolism with doubling times of about 8-10, 7-11, 3-4, and 5-8 hours respectively (Wood & Kelly, 1985; Marsh & Norris, 1983a). The moderate thermophiles are capable of wholly autotrophic growth on ferrous sulphate in atmospheres enriched in CO_2 but a reduced form of sulphur is required for biosynthesis by all strains except strain ALV (see Norris, 1990; Wood & Kelly, 1983, 1985; Marsh & Norris, 1983a, 1983b; Norris & Barr, 1985). They grow heterotrophically when supplied with 0.1 to 0.5 g yeast extract per litre alone, but higher concentrations of yeast extract inhibit growth (Norris et al., 1980). During chemolithoheterotrophic growth of the moderate thermophiles, cell carbon and energy can be derived from yeast extract and ferrous iron respectively without significant utilization of carbon dioxide (Wood & Kelly, 1983). Finally, during mixotrophic growth in enhanced CO_2 atmospheres, the moderate thermophiles can assimilate carbon simultaneously from both CO_2 and sugars (glucose, fructose or sucrose), and energy can be derived from the oxidation of ferrous iron, but again a reduced source of sulphur is usually required for biosynthesis (Wood & Kelly, 1983, 1985).

1.3 The Mechanism of Iron Oxidation.

The Gram negative, mesophilic, obligate autotroph T. ferrooxidans is the most extensively studied iron-oxidizing bacterium. T. ferrooxidans is able to derive energy from the oxidation of reduced sulphur compounds through to sulphuric acid and from the oxidation of ferrous iron (Fe^{2+}) to ferric iron (Fe^{3+}) using oxygen as the oxidant (for reviews see Ingledew, 1982; Ingledew, 1986; Norris, 1990). Figure 1.2 shows a model proposed by Ingledew (1986) which describes electron transport in T. ferrooxidans. Oxidative phosphorylation and the generation of a

transmembrane proton gradient are coupled to electron transport when ferrous ions are oxidized at the cell surface, with electron transfer ultimately to a terminal cytochrome oxidase in the cytoplasmic membrane.

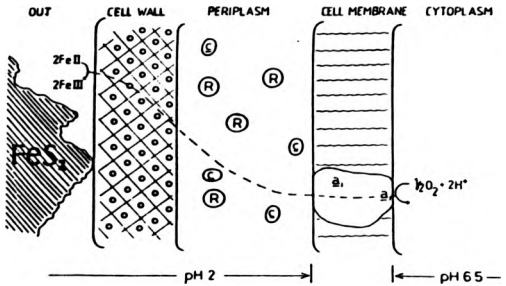


Figure 1.2 A Diagrammatic Representation of Iron Oxidation in T. ferrooxidans.
The possible route of electrons from Fe^{2+} via cell-surface-bound Fe^{3+} (small circles in cell wall), electron carriers rusticyanin (R) and cytochrome c to a cytochrome a-type oxidase (a_1). Figure taken from Ingledew (1986)

The conclusive identification of the primary electron acceptor of T. ferrooxidans has eluded workers for many years. Dugan and Lundgren (1965) suggested the involvement of a lattice of iron and sulphate ions and an iron oxidase in the cell envelope of T. ferrooxidans, whilst Ingledew (1986) implicated an Fe^{3+} polynuclear layer (as above). Rusticyanin, a blue-copper protein (Cox & Boxer, 1978), iron-sulphur proteins (Fry et al., 1986; Fukumori et al., 1988; Sato et al., 1989) and an acid-stable cytochrome c (Blake et al., 1988) have all been suggested to play a role, as have porins in the outer membrane of T. ferrooxidans

(Rodriguez et al., 1986; Mjoli & Kulpa, 1988). The structural gene for rusticyanin, a major component of the electron transport chain of T. ferrooxidans, could be part of an inducible operon (Kulpa et al., 1986), and T. ferrooxidans mutants which lack rusticyanin show slow growth on ferrous sulphate (Cox & Boxer, 1986).

Ferrous iron is probably oxidized extra-cytoplasmically by all iron-oxidizing bacteria, but components of the electron transfer chain differ in taxonomically unrelated bacteria as indicated by optical spectroscopy (Norris, 1990). L. ferrooxidans, a Gram negative mesophile has abundant levels of an acid stable cytochrome but no detectable rusticyanin (Blake et al., 1989, Barr et al., 1990) whilst the Gram positive moderate thermophile strain TH1, which lacks the typical periplasm of Gram negative bacteria, does not contain abundant levels of small, soluble electron-carriers; difference spectra suggest the presence of b-type cytochromes and possibly aa_3 cytochrome oxidases in strains TH1 and ALV (Barr et al., 1990).

1.4 Thermophiles.

The classification of thermophilic bacteria was for some time ill-defined and terminology has proliferated to describe bacteria which grow at higher temperatures than mesophiles. Mesophiles are organisms with an optimum growth temperature in the range 20–45°C and Brock (1986) has defined the 'thermophile boundary' as 50 to 60°C, above which only obligate or extreme thermophiles can grow. Bacteria growing with an optimum temperature of about 50°C are classified as facultative or moderate thermophiles.

A wide variety of genera in both the archaebacterial and eubacterial kingdoms include thermophilic representatives with optimum growth temperatures above 45°C and these have been isolated from numerous natural geothermal habitats (see Brock, 1986). Amongst the Gram positive bacteria, a range of organisms have been identified (see Brock, 1986; Langworthy & Pond, 1986):

i) Extremely thermophilic bacilli e.g. Bacillus caldolyticus and Bacillus caldovelox with growth between 75 and 85°C.
ii) Moderately thermophilic bacilli e.g. Bacillus stearothermophilus and Bacillus acidocaldarius with optimum growth at about 50-60°C and 60 to 65°C respectively.
iii) Extremely thermophilic clostridia e.g. Clostridium thermohydrosulfuricum with optimum growth at 68°C.
iv) Moderately thermophilic clostridia e.g. Clostridium thermocellum with optimum growth at about 50-60°C.

1.4.1 Aspects of the Physiology and Genetics of Thermophiles.

By 1973 several mechanisms had been proposed to explain how bacteria could grow at elevated temperatures. These theories broadly centred around the following concepts (see Singleton & Amelunxen, 1973):

i) Stabilization through lipid interaction
ii) Rapid resynthesis of heat-denatured cellular components
iii) The possession of macromolecular complexes with inherent heat stability.

It is now established that the macromolecular structures of thermophiles are inherently thermostable and subtle rather than gross differences in molecules lead to increased intramolecular bonding, e.g. hydrophobic interactions, hydrogen bonds, sulphur-sulphur bonds and ionic bonds (see Brock, 1986). Cellular factors such as polyamines also serve as intracellular stabilizing components and experiments in vitro indicate that the stability of some enzymes is enhanced by supplements to assays e.g. metal ions and components of the enzyme reaction (see Sundaram, 1986). In addition, the cell membranes of thermophiles contain more high-melting point fatty acids, e.g. longer chain and methyl-branched chains and carbohydrate-containing lipids, than do the membranes of mesophiles (see Langworthy & Pond, 1986).

The low protein turnover rate during thermophilic growth suggests that this typically thermolabile component of cells must be more stable in thermophiles than their mesophilic counterparts (Sundaram, 1986).

The DNA of some but not all thermophilic bacteria has a higher average mol% GC content compared to the mol% GC value of DNA from mesophiles (Stenesh, 1978); a higher G+C content may contribute to thermostability of DNA by extra bonding. The rRNA in thermophiles and mesophiles appears to have similar thermostability and RNA-protein interactions in the ribosomal complex seem to contribute to stability in ribosomes (see Sundaram, 1986). Protein synthesis at higher temperatures may be stabilized by polyamines; thermophiles contain a greater variety of polyamines and they may be required for *in vitro* protein synthesis at high temperatures when cell-free extracts are derived from thermophilic bacteria (see Oshima, 1986).

1.4.2 Factors for Consideration During Genetic Manipulations of Thermophiles.

The commercial advantages of thermostable compounds from thermophilic bacteria, e.g. enzymes, have been discussed elsewhere (see Kristjansson, 1989; Brock, 1986). However, there appear to have been relatively few reports of genetic studies of thermophiles and in particular, investigations of the genes, protein synthesis and related cellular components has centred on *B. stearothermophilus* or *Thermus* sp. (for reviews see Oshima, 1986; Imanaka & Aiba, 1986; Imanaka, 1983; Zhang *et al.*, 1988; Friedman, 1968). Plasmids have been isolated from these bacteria (Imanaka, 1983; Kroger *et al.*, 1988, Raven & Williams, 1989) and although restriction and modification systems were identified in *B. stearothermophilus* and *Thermus aquaticus* (Imanaka & Aiba, 1986; Oshima, 1986), both *B. stearothermophilus* and *Thermus thermophilus* have been transformed with DNA (see Imanaka & Aiba, 1986; Koyama *et al.*, 1990).

The use of plasmid vectors and the expression of some antibiotic

resistance genes has been studied in the moderate thermophile B. stearothermophilus. Factors of importance during the development of a host:vector system for B. stearothermophilus were plasmid stability and the stability of genes and their products at elevated temperatures. Vectors stable up to 65°C were required and indigenous plasmids were good candidates (Bingham et al., 1979; Imanaka, 1983). However, B. stearothermophilus CU21 was successfully transformed with the Staphylococcus aureus plasmid pUB110 (see Imanaka & Aiba, 1986). The frequency of transformation of B. stearothermophilus CU21 with plasmids isolated from Bacillus subtilis depended on the plasmid used because of a restriction system in the recipient bacterium (see Imanaka & Aiba, 1986). Other plasmids such as the S. aureus plasmid pC194 were unstable at 65°C and pTB19 (from a thermophilic bacillus) commonly formed deletion variants (see Imanaka & Aiba, 1986).

pUB110 (Kan^r) was maintained in B. stearothermophilus at 48°C and 55°C but was unstable at 60°C and 65°C although the replicon was still present after 20 generations at the latter temperatures (see Imanaka & Aiba, 1986). Results indicated that the protein product of the Kan^r gene was thermolabile. During a comparison of the kanamycin nucleotidyl-transferase gene of pUB110 and the same gene from a thermophilic source, a single base difference and the substitution of one amino acid in the protein product of the gene resulted in greater thermostability (see Imanaka & Aiba, 1986).

The nucleotide sequences of at least two genes from B. stearothermophilus have been determined (neutral protease and alpha-amylase). The G+C content was 58 mol% and in 72% of codons the third base was G or C (see Imanaka & Aiba, 1986); these factors could contribute to thermostability of the genes.

1.5 Acidophiles.

There are a variety of naturally acidic environments in which both prokaryotes and eukaryotes proliferate and these, together with their

microbial flora, have been reviewed elsewhere (Langworthy, 1978). The term acidophile is restricted to those organisms which grow optimally at pH 4 or lower and only a few truly acidophilic bacteria have been reported. Acidophilic bacteria fall into many genera including: Thiobacillus, Bacillus, Sulfolobus and Thermoplasma (see Cobley & Cox, 1983).

All acidophiles must maintain an intracellular environment far less acidic than that of the exterior environment and the proposed mechanisms or properties of acidophiles used to maintain an intracellular pH near neutrality have been reviewed (Booth, 1985; Krulwich & Guffanti, 1983; Matin, 1990). There are conflicting reports with respect to whether the transmembrane pH gradient is actively or passively maintained in acidophiles and three main theories appear to predominate:

i) The pH gradient is maintained by passive or impermeable properties of the cell.
ii) Active metabolism maintains the pH gradient.
iii) The pH gradient depends on a membrane function (antiporters ?) as well as an energy-dependent proton exclusion mechanism.

Bacteria which contain an electron transport chain with oxygen as the terminal electron acceptor consume protons during respiration, e.g. T. ferrooxidans (Ingledew, 1982), and such a respiratory mechanism could play an important role in establishing the transmembrane pH gradient. Also, as a result of electron flow, protons are translocated out of the cell as a consequence of the arrangement of the respiratory chain components within the membrane (see Cobley & Cox, 1983). The proton motive force drives protons back into the cell and in acidophiles this is almost entirely composed of osmotic (H^+) potential (but also partly the electrical potential difference). The return of protons into the cell through a reversible H^+-translocating ATPase drives the phosphorylation of ATP. In acidophiles with respiratory metabolism, uncouplers of oxidative phosphorylation do not seem to affect the transmembrane pH

gradient and maintenance of a neutral pH in the cells may be due to the impermeability of the membrane and the high buffering capacity of the cytoplasm (see Cobley & Cox, 1983). Uncouplers destroy the proton gradient across the membrane which consequently is no longer available to drive ATP synthesis and protons cease to enter through the ATPase (Ingledew, 1982).

As for thermophilic bacteria, it has been suggested that complex lipids confer on the membranes of some acidophiles a stability in acidic environments (see Cobley & Cox, 1983; Krulwich & Guffanti, 1983).

The development of genetic systems for acidophiles has received little attention except for the Gram negative bacteria T. ferrooxidans and Acidiphilium sp. Acidiphilium sp. are aerobic, acidophilic, heterotrophic bacteria found in association with T. ferrooxidans in acid mine drainages. A review by Holmes et al. (1986) discusses the isolation and cloning of Acidiphilium plasmids into E. coli and the production of auxotrophic mutants and spheroplasts of Acidiphilium organovorum. Ward et al. (1989) identified an endogenous bacteriophage from Acidophilium sp. and broad host range plasmids were mobilized from Escherichia coli and electroporated directly into Acidiphilium facilis (Roberto et al., 1989).

Attempts to develop a genetic system for T. ferrooxidans will be discussed separately because this work highlights some of the factors which must be considered whilst working with acidophilic bacteria.

1.5.1 Genetic Studies of T. ferrooxidans.

Before the initiation of the work described in this thesis, T. ferrooxidans was the only mineral-oxidizing bacterium for which there had been attempts to develop genetic systems, i.e. the identification and construction of cloning vectors and the transfer of DNA into the bacteria (reviewed by Woods et al., 1986; Rawlings, 1989).

Plasmids appear to be widespread among natural isolates of T. ferrooxidans (Martin et al., 1981; Sanchez et al., 1986) and in some cases these plasmids have been cloned into E. coli vectors, e.g. pTF1 and

pTF-FC2 (Holmes et al., 1984; Rawlings et al., 1984a), but all the plasmids studied so far appear to be cryptic (Rawlings, 1989).

Plasmid pTF-FC2 (12.4 kb) was isolated from T. ferrooxidans strain FC, cloned into the E. coli vector pBR325 and shown to be capable of replication in E. coli from an origin of replication located in pTF-FC2 (Rawlings et al., 1984a). The minimum region required for replication was further characterized and extensive similarity was found between the replication genes of pTF-FC2 and those of the E. coli IncQ plasmid RSF1010 (Dorrington & Rawlings, 1989, 1990). Additionally, pTF-FC2 replicates in all Gram negative bacteria tested so far (Dorrington & Rawlings, 1990). pTF-FC2 can be mobilized between E. coli strains by the IncP plasmid RP4 (Rawlings & Woods, 1985) and the 5.3 kb region responsible for mobilization has been located (Rawlings et al., 1986).

The nucleotide sequence of pTF1, isolated from T. ferrooxidans ATCC 33020 (Holmes et al., 1984), was determined, and two predicted mobilization proteins had about 45% similarity to the mobilization proteins of E. coli plasmids RSF1010 and pSC101 (Drolet et al., 1990).

The direct transfer of plasmids from E. coli to T. ferrooxidans has not been investigated because for conjugation an important requirement is a suitable medium on which mating can occur. A mutually compatible medium for both T. ferrooxidans and E. coli cannot be provided but, in an attempt to circumvent this problem, an indirect two-stage process involving an intermediary host (which grows at neutral or low pH in the presence of organic matter or autotrophically) was investigated. First stage mating by conjugation between E. coli and Thiobacillus novellus or Thiobacillus neapolitanus occurred using the broad host range plasmid RP1 (from Pseudomonas aeruginosa) but second stage mating from either of these two thiobacilli to T. ferrooxidans has not been demonstrated (Woods et al., 1986; Kulpa et al., 1983).

The development of a transformation protocol for T. ferrooxidans has also been unsuccessful but spheroplasts were produced and regenerated (Barros et al., 1985a). Regeneration is an essential step during protocols for the transformation of protoplasts or spheroplasts and a

rich organic medium is normally used which usually inhibits the growth of iron-oxidizing bacteria. Barros et al. (1985a) did however report that 93% of spheroplasts of a mixotrophic strain of T. ferrooxidans were regenerated whereas, in contrast, those of an autotrophic strain were not regenerated.

Successful cloning and expression in E. coli of the T. ferrooxidans chromosomal glutamine synthetase gene (Barros et al., 1985b), nitrogenase operon (Pretorius et al., 1986), recA gene (Ramesar et al., 1988) and mercury resistance operon (Kusano et al., 1990) have been demonstrated. The sequences of these genes are also available (Pretorius et al., 1987; Rawlings et al., 1987, 1988; Rawlings, 1988; Ramesar et al., 1989; Inoue et al., 1989, 1990). In addition, two families of repeated DNA sequences of about 1 kb in length have been identified in the genome of T. ferrooxidans ATCC 19859. These sequences are repeated about 20-30 times and one family is also found on T. ferrooxidans plasmids. Subsequent characterization of one of these sequences revealed a similarity to bacterial insertion sequence elements i.e. an inverted terminal repeat, target sequence duplication and the presence of open reading frames (ORFs) (Yates & Holmes, 1987; Yates et al., 1988).

To date, all attempts at conjugal transfer or transformation of plasmids into T. ferrooxidans have failed but with the discovery of broad host range mobilizable plasmids in T. ferrooxidans it may only be a matter of time before a suitable vector is obtained and mobilized into T. ferrooxidans using a suitable donor bacterium.

1.6 The Development of Host:Vector Systems for Gram Positive Bacteria.

There are two basic requirements for a host:vector system:

a) A suitable cloning vector
b) A means of introducing the vector and/or chimaeric constructs into the chosen host cell.

1.6.1 Vectors.

Vectors should preferably be capable of autonomous replication or integration into the chromosome and usually stable maintenance in the intended host cell is required. The most commonly used vectors in gene cloning experiments are small plasmids (<10 kb), these are easier to isolate than large plasmids, plus they are more likely to contain unique restriction sites to facilitate the cloning of DNA fragments. Restriction sites used during cloning should be in non-essential regions of the vector and although not absolutely essential, it is desirable to identify suitable transcriptional control signals that may be employed to elicit the expression of cloned genes. To enable the rapid identification of bacterial cells containing vector DNA, the vector should carry a reporter gene such as an antibiotic resistance gene.

Indigenous plasmids are probably more suitable for use as cloning vectors because these will replicate in their host cells. Alternatively, broad host range plasmids are more likely to fulfil this criterion than plasmids which have a limited host range. If the chosen plasmid does not contain a reporter gene, recombinant vectors must be constructed *in vitro* by insertion of a selectable phenotype. However, it should be noted that the insertion of foreign DNA into a plasmid may cause inactivation of essential replication functions. It follows that should any transformation procedure fail to produce transformants then it would be difficult to distinguish between the inability of vectors to replicate or express reporter genes and failure of the transformation procedure itself. A potential way around this dilemma would be the identification of the plasmid replicon (i.e. the essential features of the plasmid required for replication) and consequent confirmation that the replication machinery of the plasmid remained intact during construction of chimaeras.

At the initiation of this work, nothing was known about plasmids in the moderate thermophiles and for this reason and due to subsequent work

described in this thesis, current knowledge of other Gram positive plasmids will be reviewed extensively.

1.6.1.1 The Replication of ssDNA Plasmids from Gram Positive Bacteria.

Extensive studies have been applied to the identification and characterization of plasmids from E. coli and this has been reviewed elsewhere (Scott, 1984; Novick, 1987; Caro et al., 1984; Couturier et al., 1988; Kues & Stahl, 1989). During the last five years some of the mechanisms which contribute to the replication of small (<10 kb) plasmids from Gram positive bacteria, in particular S. aureus, have been elucidated. All the plasmids characterized to date are highly interrelated and replicate via single-stranded intermediates most likely by rolling-circle replication (RCR) in an analogous fashion to bacteriophages of E. coli (reviewed by Baas, 1985). The plasmids have thus been called single-stranded (ssDNA) plasmids and several recent reviews have concentrated on the properties and replication of these plasmids (Gruss & Ehrlich, 1989; Bron, 1990; Projan & Novick, 1988; Alonso, 1989; Novick, 1989). The information from these reviews will be summarized in the following sections and the reader will be directed to the appropriate review. Particular key source references and more recent information will also be cited.

A model for RCR of ssDNA plasmids is shown in Figure 1.3. The primary replication functions which define the minimal replicon are the replication protein (Rep) and the origin of plus strand synthesis, denoted ori^+ (see Bron, 1990).

The Rep proteins of ssDNA plasmids share homology and the ori^+ sequences are conserved. Depending on these functions, plasmids can be classified into at least four families, representative members of each family are: pT181, pC194/pUB110, pE194 and pSN2 (see Novick, 1989; Bron, 1990; Sozhamannan et al., 1990). Rep proteins initiate replication and behave as trans-active single-stranded endonucleases with a specific topoisomerase-like activity (nicking-ligating; see Alonso, 1989; de la

Campa et al., 1990; Koepsel et al., 1985a, 1985b). They vary in size from about 25 to 40 kD (Horinouchi & Weisblum, 1982; Novick et al., 1982; Villafane et al., 1987). The transcription of rep genes is usually in the same direction as replication but other genes on the plasmids may be transcribed co-directionally (see Novick, 1989).

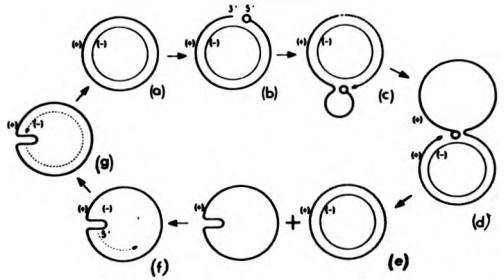


Figure 1.3 Model For Plasmid Rolling-Circle Replication.
Heavy lines indicate the plus strand (+); thin lines, minus strand (-); continuous lines, parental DNA; discontinuous lines, newly synthesized DNA; O indicates the replication protein (Rep) and ⊃, the minus origin of replication. The steps of replication are described in the text. (Figure taken from Bron, 1990).

The ori^{+} is usually located upstream of or within the 5' end of the rep gene and is composed of a highly conserved 25 to 45 base pair DNA sequence which can probably be divided into the 'nick site' and an adjacent 3' divergent region containing the plasmid-specific Rep protein recognition site (see Novick, 1989). In some cases a potential secondary

structure has been identified in the region of ori$^+$ and in the S. aureus plasmid pT181 a cruciform structure was demonstrated at the ori$^+$. The formation of this structure was enhanced by Rep binding and was dependent on superhelicity. Furthermore, a competing secondary structure occurred elsewhere in the plasmid (Noirot et al., 1990). The most extensively studied ssDNA plasmid is pT181 and the replication of this plasmid probably serves as a model for the other ssDNA plasmids isolated to date (Figure 1.3).

Replication of the leading strand is initiated only when the plasmid is supercoiled and in most cases Rep binds to and nicks at or near the ori$^+$ sequence (Figure 1.3 [b]). The Rep protein remains covalently attached to the 5' terminus of the plus strand via a phosphotyrosine linkage (Thomas et al., 1990) and the plus strand is displaced while unidirectional replication or elongation occurs from the 3' terminus of the 'nick site' using the minus strand as a template (Figure 1.3 [c]). After one full round of leading strand replication, Rep cleaves the regenerated ori$^+$ and closes the displaced strand (Figure 1.3 [d] & [e]); a 'termination enhancer' sequence may be present in the ori$^+$ (see Alonso, 1989).

Lagging strand synthesis (Figure 1.3, [f] & [g]) probably occurs as soon as the minus strand initiation signal (minus origin or MO) is exposed or may occur after leading strand synthesis terminates (see Alonso, 1989). The MO is about 200 nucleotides in length and contains imperfect palindromic sequences which are cis-acting and function only in one orientation (see Novick, 1989; Gruss & Ehrlich, 1989; Bron, 1990). At least three families of MO are known and these are non-essential for replication and function only in a limited number of hosts. In the absence of a functional MO, single-stranded plasmid DNA accumulates. This represents plus strand DNA. Bron (1990) has recently renamed each family of MO to indicate their functional similarity. The palA-type MO (formerly palA) is the most widespread and has been found on all small plasmids isolated from S. aureus except pUB110 (see Novick, 1989; Bron, 1990). The palU-type MO (formerly BA3-type) occurs in pUB110, pTB913 and pMV158

isolated from S. aureus, a thermophilic bacillus and Streptococcus agalactiae respectively and is the only MO to function in B. subtilis (see Bron, 1990). Finally the palT-type MO (formerly 'stab') is present on Bacillus plasmids pBAA1, pLS11 and pTA1060 (see Bron, 1990).

Initiation at the MO and elongation during lagging strand replication probably involves some host factors; inhibition by rifampicin has implicated RNA polymerase (see Novick, 1989). RNA primers are probably formed, elongated and then removed by host enzymes and DNA ligase and DNA gyrase probably complete the replication of the lagging strand (Alonso, 1989).

1.6.1.2 The Control of pT181 Replication.

Those factors implicated in the initiation of pT181 replication are the replication protein (RepC), secondary structures at and near the ori$^+$ and superhelicity of the plasmid (Noirot et al., 1990; de la Campa et al., 1990; see Novick, 1989). The levels of RepC protein in the cell are the primary determinants of the plasmid copy number and synthesis of the protein is controlled by a region 5' to the Rep encoding sequence. Two small (80 and 150 bases) inhibitory antisense RNAs (countertranscripts) are transcribed from the region specifying the 5' end of the untranslated repC mRNA leader (Figure 1.4).

The RNA countertrancripts of pT181 probably act together to interfere with translation of repC mRNA and a complicated series of secondary structures has been predicted which act in a similar way to the attenuation systems used for the regulation of amino acid biosynthesis. Formation of the two RNA countertranscripts results in the inhibition of synthesis of the rate limiting initiator protein RepC. (see Novick, 1989; Highlander & Novick, 1990; Novick et al., 1985). Similar copy control systems occur in ColE1 and IncF11-type plasmids of E. coli and in ColE1 a protein (Rom) potentiates the interaction between the RNA and its target but no such protein has been found in control of pT181 RepC synthesis (see Highlander & Novick, 1990). However, a small open reading frame in

the sequence immediately upstream of repC could encode a 46 amino acid peptide; translation of this reading frame would have a major effect on the secondary structure of the repC mRNA leader (see Projan & Novick, 1988). Small proteins (Cop) encoded by the cop region of pE194 and repA of pLS1 have been identified and appear to be negative regulators or repressors of both replication protein synthesis and their own synthesis (Byeon & Weisblum, 1990; del Solar et al., 1989).

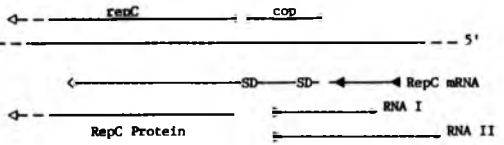


Figure 1.4 pT181 Regulatory Region.

The figure represents the 5' end of the repC gene and DNA immediately upstream. The dotted lines indicate the four transcripts produced by this region of pT181. SD = Shine-Dalgarno sequence and ◀ = promoter. At the top of the diagram the extent of the repC gene is indicated and the copy contol locus copA which has been implicated in the negative control of replication. Two RNA countertranscripts are transcribed from within the copA region and in the opposite direction to the repC transcript (figure taken from Novick et al., 1985).

Noirot et al. (1990) demonstrated that a secondary structure in copA competes with a secondary structure in ori$^+$ and affects the efficiency of RepC use. Also, a cis-acting locus of static DNA-bending (cmp) which is orientation dependent and about 1 kb away from the origin enhances binding of RepC at the ori$^+$. Plasmids which lack cmp have a normal copy number of about 22 copies/cell when maintained singly but if they are co-resident with a cmp$^+$ plasmid they suffer a marked reduction in copy number and stability (Gennaro & Novick, 1988).

1.6.1.3 Plasmid Incompatibility.

Plasmid incompatibility occurs when two plasmids are unable to co-exist in the same host so that after a few generations, daughter cells only contain one of the two types of plasmid.

For pT181, two forms of incompatibility have been described: IncA incompatibility is determined by the primary replication control region copA which expresses the two RNA countertranscripts. Secondly, the leading-strand replication origin is also an incompatibility determinant called incB and this incompatibility is due to competition for RepC. Plasmids which share either incA and/or incB incompatibility determinants exhibit segregational incompatibility (see Novick, 1987, 1989; Highlander & Novick, 1990). As discussed earlier the cmp locus may also have a role to play during this type of competition between plasmids and a weak incompatibility determinant of unknown mechanism occurs within the pre gene (see later) of pE194 (Novick, 1989).

1.6.1.4 Plasmid Cointegrate Formation.

Stable cointegrates of some ssDNA plasmids are formed by site-specific interplasmid recombination at one or two sites called RS_A and RS_B which are about 70 and 30 bp in size respectively and contain a highly conserved 'core' sequence. RS_B has been found on representatives of all four ssDNA plasmid families and requires plasmid co-transduction for cointegrate formation which suggests phage recombination functions are required. RS_A has been more extensively studied and is found on plasmids pT181, pUB110 and pE194 and in contrast to RS_B does not require plasmid transduction (see Gennaro et al., 1987).

Recombination at RS_A is recA independent and specifically mediated by a trans-acting plasmid recombination protein (Pre) which is about 400 amino acids long. Plasmid multimers accumulate as a result of the Pre-RS_A system for an unknown reason, but Pre-RS_A recombination does not appear

to result in multimer resolution (Gennaro et al., 1987). RS_B does however have slight homology with a portion of the par sequence of the E. coli plasmid pSC101 and may be a plasmid partitioning function (Novick, 1989; Novick et al., 1984b).

The Pre protein of pMV158 was required for conjugative mobilization (van der Lelie et al., 1990) and Bron (1990) has renamed the pre gene, mob (mobilization).

1.6.1.5 Plasmid Instability.

Mechanisms that contribute to the stable segregation of plasmids at cell divison have been reviewed (Nordstrom & Austin, 1989). There does not appear to be any strong evidence for segregation function(s) in the ssDNA plasmids. These plasmids are frequently highly unstable in their host due to both segregational instability and structural instability.

Maintaining the plasmid copy number is probably essential for ssDNA plasmids because random partitioning is assumed to occur at cell division and mutations which affect plasmid stability of pT181 all appear to affect replication functions and copy number control (Bron, 1990; Novick, 1989; Gruss & Ehrlich, 1989).

During the construction of recombinant vectors, inserted DNA may interfere with the efficiency of initiation of replication and the utilization of cmp; the distance between cmp and the ori^+ is important for cmp function. The absence of a functional MO also results in plasmid instability due to the generation of large quantities of ssDNA during replication and some of the ssDNA plasmids generate multimers of high molecular weight (HMW DNA) particularly if foreign DNA is inserted or if the MO is deleted. The mechanism for the production of HMW DNA probably involves inefficient termination of RCR but both phage infection and the DNA recombination functions of host cells have been implicated in the formation of concatemeric plasmids (Virat & Alonso, 1987; Bravo & Alonso, 1990; Bron, 1990). HMW DNA may serve as a substrate for transformation because this type of DNA is highly efficient during competent cell

transformation of B. subtilis (see Gruss & Ehrlich, 1989).

The structural instability displayed by ssDNA plasmids has been extensively reviewed elsewhere and many of the stages of RCR and direct repeats in the plasmid DNA sequences have been implicated in either the formation of deletions, illegitimate recombination or the formation of HMW DNA (see Bron, 1990; Gruss & Ehrlich, 1989). In addition deletions in plasmids can arise as a result of host factors such as the BsuM restriction system of B. subtilis and host recombination systems (see Bron, 1990).

1.6.1.6 Plasmid Host Range.

The ssDNA plasmids are widely distributed throughout the Gram positive eubacteria and have been isolated from Bacillus sp., Staphylococcus sp., Corynebacterium xerosis, Lactococcus sp., Clostridium sp. and probably Streptomyces lividans. In the latter case, tentative identification is based on the generation of ssDNA and a small amount of homology between the Rep protein of the plasmid with the active site of other ssDNA Rep proteins. In addition, there is evidence that ssDNA plasmids occur in Mycoplasma mycoides and Halobacterium sp. (see Gruss & Ehrlich, 1989; Bron, 1990).

Many of the ssDNA plasmids can replicate in a wide variety of Gram positive bacteria including staphylococci, streptococci and bacilli (see Novick, 1989). It has been proposed that these types of plasmid are composed of 'cassettes' of DNA because of similarities between plasmids and in some cases, identical regions of DNA sequence. A horizontal exchange mechanism of the cassettes might have occurred. Extensive regions of the plasmids are closely related whilst other segments are more distantly related with junctions which are abrupt; the sequence homology usually goes from 100% to zero across a single pair of nucleotides (Novick, 1989). Noteworthy is the fact that although pUB110 was isolated from S. aureus it has a higher copy number and is more stable in B. subtilis. also it is the only ssDNA plasmid from S. aureus

whose MO functions in B. subtilis; it is therefore likely that this plasmid was in fact native to B. subtilis (Novick, 1989). In all hosts, a plasmid lacking a functional MO is still viable but accumulates ssDNA e.g. pC194 produces about 20% of the total plasmid DNA as ssDNA in B. subtilis (see Bron, 1990).

1.6.1.7 Large Plasmids of Gram Positive Bacteria.

Workers have more recently focussed on the use of large (>25 kb) plasmids for cloning in Gram positive bacteria because of the instability of the small ssDNA plasmids. Vectors derived from these larger, broad host range plasmids allow efficient cloning and stable maintenance of long DNA segments of up to about 30 kb (Janniere et al., 1990, Bron, 1990). pAMβ1 (26.5 kb) from S. faecalis can transfer by conjugation to several Gram positive bacterial genera (see Section 1.6.2.3).

1.6.2 Vector Delivery Systems.

The most direct means of introducing a vector into a target cell is by transformation with the naked DNA but alternatively, the vector may be transferred from a donor cell during conjugation. Protoplast transformation and competent cell transformation methods are usually time consuming and tedious to develop and may be strain specific and conjugal mechanisms may be restricted by choice of a suitable donor bacterium, as discussed earlier for T. ferrooxidans.

1.6.2.1 Natural and Chemically Induced Transformation.

All transformation procedures depend on the preparation of cells 'competent' for the uptake of naked DNA and both competence and DNA uptake may be induced in a variety of ways.

In contrast to E. coli where competence is normally an artifically induced condition, Bacillus sp., Streptococcus sp. and members of other

diverse genera develop a natural physiological state when they are capable of absorbing DNA from their immediate environment (for reviews see Stewart & Carlson, 1986; Saunders et al., 1984; Chassy et al., 1988). It appears that genes may express 'competence factors' which allow the development of natural competence (see Stewart & Carlson, 1986). The mechanism of natural transformation has been most extensively studied in B. subtilis (see Dubnau, 1982). However, all bacterial transformation systems studied to date appear to follow a common sequence of events:

a) Development of competence
b) DNA binding at the cell surface
c) Uptake of DNA by the recipient
d) Intracellular processing of the DNA and the establishment of an autonomous plasmid or integration into the host chromosome (or into a resident replicon).

PEG-dependent transformation of intact whole cells has been reported for a limited number of Gram positive organisms, e.g. Bacillus brevis (Takahashi et al., 1983), Clostridium thermohydrosulphuricum (Soutschek-Bauer et al., 1985) and Streptococcus lactis (Sanders & Nicholson, 1987). In all cases, preconditioning of the cells in specific buffers was required and PEG treatment was absolutely essential for transformation.

The crucial observation that bacterial protoplasts obtained by digestion of the cell wall with lytic enzymes were able to incorporate DNA when incubated in the presence of PEG during the 1970's has led to the transformation of many Gram positive bacterial species e.g. B. subtilis (Chang & Cohen, 1979), Streptomyces sp. (Bibb et al., 1978), B. stearothermophilus (Imanaka et al., 1982), clostridia, (see review, Young et al., 1989), lactic acid bacteria (see reviews, de Vos, 1986; Kondo, 1989), Streptococcus thermophilus (see reviews, Mercenier & Lemoine, 1989; Mercenier, 1990), Corynebacterium glutamicum (Katsumata et al., 1984) and S. aureus (Gotz et al., 1981). Success in this kind of procedure relies on the ability to prepare protoplasts and subsequently regenerate intact cells, together with the provision of optimal conditions for the uptake of DNA. Unfortunately methods are often species

or strain dependent. The strategies employed have been reviewed elsewhere (Mercenier & Chassy, 1988). Efficiencies of transformation are usually in the range 10^4 to 10^6 transformants/μg DNA but methods are hampered by inconsistency and non-reproducibility of results, lengthy protoplast regeneration and the tedious nature of the technique.

1.6.2.2 Transformation by Electroporation.

Electroporation or electropermeabilization involves the application of a high-intensity electric field to bacterial cells to reversibly permeabilize the cell membrane, forming transient pores which permit the entry of macromolecules (see Knight & Scrutton, 1986). Electrotransformation of bacteria occurs when plasmids enter cells at these specific loci in the membrane. For each cell there will be a critical threshold voltage above which permeabilization or 'poration' occurs and a lethal voltage above which the cell membrane is irreversibly damaged by the electric field (for reviews, Chassy et al., 1988; Solioz & Bienz, 1990). Electroporation has only recently been applied for transformation of both Gram negative and Gram positive bacteria. It can be a quick and easy method to perform, particularly if frozen cell stocks are used (Dower et al., 1988; Mahillon et al., 1989; Masson et al., 1989; Kim & Blaschek, 1989). Many reports have appeared in the literature (see Table 1.2) and some strains previously thought to have been untransformable have been electrotransformed (Wirth et al., 1989; Chassy et al., 1988; Gilchrist & Smit, 1991) but some bacteria are recalcitrant to electrotransformation (Bone & Ellar, 1989; Wirth et al., 1989; Powell et al., 1988).

The basic steps in all electrotransformation methods are:

a) The production of a dense cell suspension in a low ionic strength buffer
b) Addition of DNA to a small aliquot of cells

Table 1.2 (a) Bacteria Transformed by Electroporation.

Gram negative Bacteria	Reference
Escherichia coli	Dower *et al.*, 1988
	Calvin & Hanawalt, 1988
	Fiedler & Wirth, 1988
	Willson & Gough, 1988
	Cymbalyuk *et al.*, 1988
	Heery *et al.*, 1989
	Wirth *et al.*, 1989
	Summers & Withers, 1990
	Jacobs *et al.*, 1990
Salmonella sp.	O'Callaghan & Charbit, 1990
Yersinia sp.	Conchas & Carniel, 1990
	Wirth *et al.*, 1989
Citrobacter freundii	"
Klebsiella pneumoniae	"
Enterobacter aerogenes	"
Erwinia carotovora	"
Serratia sp.	"
Hafnia alvei	"
Proteus sp.	"
Xanthomonas campestris	"
Pseudomonas sp.	Fiedler & Wirth, 1988
	Wirth *et al.*, 1989
	Smith & Iglewski, 1989
	Tevors & Starodub, 1990b
	Diver *et al.*, 1990
Rhodospirillum molischiarium	Wirth *et al.*, 1989
Acidiphilium facilis	Roberto *et al.*, 1989
Agrobacterium sp.	Wen-jun & Forde, 1989
	Wirth *et al.*, 1989
Bradyrhizobium sp.	Guerinot *et al.*, 1990
	Hatterman & Stacey, 1990
Bacteroides sp.	Thompson & Flint, 1989
Actinobacillus sp.	Lalonde *et al.*, 1989
Azotobacter vinelandii	Trevors & Starodub, 1990a
Vibrio sp.	Hamashima *et al.*, 1990
	Marcus *et al.*, 1990
Caulobacter sp.	Gilchrist & Smit, 1991
Bordetella pertussis	Zealey *et al.*, 1988

Table 1.2 (b) continued.

Gram positive Bacteria	Reference
Bacillus cereus	Luchansky et al., 1988
	Belliveau & Trevors, 1989
B. subtilis	Vehmaanpera, 1989
	Kusaoke et al., 1989
	Brigidi et al., 1990
B. amyloliquefaciens	Vehmaanpera, 1989
B. sphaericus	Taylor & Burke, 1990
B. brevis	Takagi et al., 1989
Clostridium perfringens	Allen & Blaschek, 1988
	Kim & Blaschek, 1989
	Scott & Rood, 1989
	Phillips-Jones, 1990
C. acetobutylicum	Oultram et al., 1988
Streptococcus lactis	Harlander, 1987
	Powell et al., 1988
	Muller et al., 1989
S. pyogenes	Suvorov et al., 1988
S. thermophilus	see Mercenier, 1990
S. sanguis	Suvorov et al., 1988
	Somkuti & Steinberg, 1989
S. cremoris	Powell et al., 1988
	van der Lelie et al., 1988
Enterococcus faecalis	Fiedler & Wirth, 1988
	Cruz-Rodz & Gilmore, 1990
Leuconostoc sp.	Luchansky et al., 1988
	David et al., 1989
Pediococcus sp.	Luchansky et al., 1988
Lactobacillus sp.	"
Lactococcus lactis	Holo & Nes, 1989
	McIntyre & Harlander, 1989a, 1989b
Staphylococcus sp.	Luchansky et al., 1988
	Augustin & Gotz, 1990
Listeria sp.	Luchansky et al., 1988
	Alexander et al., 1990
Streptomyces lividans	MacNeil, 1987
Corynebacterium sp.	Liebl et al., 1989
	Wolf et al., 1989
	Dunican & Shivnan, 1989
	Haynes & Britz, 1989, 1990
	Bonamy et al., 1990
Brevibacterium sp.	Dunican & Shivnan, 1989
	Haynes & Britz, 1989, 1990
	Bonamy et al., 1990
	Bonnassie et al., 1990
Rhodococcus fascians	Desomer et al., 1990

c) Application of the electric pulse to the cell/DNA mixture
d) Tranfer of electroporated mixture to growth medium
e) Selection of transformed cells
These details will be discussed at more length in Chapter 7.

Field strengths for the electrotransformation of bacteria typically fall in the range of 6 to 12 kV/cm, but each bacterium displays a unique optimum voltage and E. coli cells can withstand 12 kV/cm with almost no cell death (Dower et al., 1988). Maximal transformation efficiencies of 10^{10} transformants/µg DNA have been reported (Dower et al., 1988; O'Callaghan & Charbit, 1990) but the efficiency of electrotransformation is strain specific and efficiencies in the range 10^3 to 10^6 transformants/µg DNA are more usually reported (see references Table 1.2). When all electroporation parameters were identical but different bacterial strains were used, transformation efficiencies were markedly different (Bone & Ellar, 1989; Suvorov et al., 1988; Bonamy et al., 1990; Dower et al., 1988; van der Lelie et al., 1988; Taylor & Burke, 1990). The factors which contribute to the efficiency of electroporation are diverse and besides the electrical parameters they include:

a) The source and conformation of DNA
b) The host strain which may have extracellular or intracellular systems for restriction of DNA
c) The electroporation buffer
d) The cell size and chain length
e) The structure of the cell wall and membrane composition
f) Variables affecting plasmid establishment and maintenance.

Prior to applying the electric pulse, some bacterial cells have been protoplasted (Fiedler & Wirth, 1988; MacNeil, 1987; Kusaoke et al., 1989) or treated with cell wall 'weakening' agents such as glycine (Haynes & Britz, 1989, 1990; Holo & Nes, 1989) or lysozyme (Wolf et al., 1989) and following electroporation, osmotically sensitive cells were regenerated and/or protected in suitable media. These procedures however introduce additional steps in methodology and extend the protocol, circumventing some of the advantages of electroporation. In most cases 'weakening' of

bacterial cells is not required and field strengths within the range available (up to 12.5 kV/cm with the Biorad apparatus commonly used) are probably sufficient for electrotransformation, although in some cases higher efficiencies might be obtained if a field strength of over 12.5 kV/cm was used (Cruz-Rodz & Gilmore, 1990).

1.6.2.3 Conjugation and Mobilization.

Conjugative plasmids are usually large (about 20 kb) and possess transfer (tra) functions which enable them to transfer directly from one bacterium to another. Such plasmids occur in many Gram positive bacteria e.g. Lactococcus sp., Streptococcus sp. and Clostridium sp. (see Gasson, 1990; Steele & McKay, 1989; Mercenier, 1990; Dunny et al., 1987; Young et al., 1989).

Non-conjugative plasmids which are themselves incapable of forming effective contact between cells can be transferred during conjugation if they possess mobilization functions; the mobilization of plasmids between Gram positive bacteria has been reported (Schaberg et al., 1982; van der Lelie et al., 1990; Hayes et al., 1990a).

The conjugative plasmids pAMβ1 (26.5 kb) and pIP501 (30.2 kb) were originally isolated from Streptococcus faecalis (see Clewell, 1981) and both mediate resistance to the macrolide, lincosamide and streptogramin B (MLS) group of antibiotics and share considerable DNA sequence similarity as determined by hybridization studies (see Horaud et al., 1985). Conjugal transfer of both pAMβ1 and pIP501, intra- and intergenerically, occurs amongst a wide variety of Gram positive bacteria (reviewed by Horaud et al., 1985) and both plasmids can also mobilize non-conjugative plasmids to many Gram positive bacteria (Romero et al., 1987, Yu & Pearce, 1986). In addition, plasmid transfer by conjugative mobilization from E. coli to various Gram positive bacteria has been demonstrated using recombinant vectors which contained the origin of transfer (oriT) of RK2 (Mob^+) and the replication region of pAMβ1; the recombinant vectors were mobilized by the conjugation system encoded by the broad host range IncP plasmids such as RK2 (Trieu-Cuot et al., 1987; Williams et al., 1990). Some plasmids have also been transferred by conjugation as 'passengers' on conjugative plasmids following the formation of a

cointegrate and in some cases after transfer, the cointegrate was resolved and the 'passenger' plasmid released in the recipient cell (see Romero et al., 1987).

The S. faecalis conjugative transposon Tn916 (16.4 kb, Tc^R) has been used to co-transfer several Gram positive plasmids (Naglich & Andrews, 1988; Clewell & Gawron-Burke, 1986; Clewell et al., 1988) and was found to transfer naturally between a variety of Gram positive and Gram negative eubacteria (Bertram et al., 1991).

1.7 The Expression of Genes in Gram positive Bacteria.

Prior to this project, no genetic studies of the moderately thermophilic iron-oxidizing bacteria had taken place. This, and the fact that they are Gram positive influenced the selection of reporter genes which could be considered for use on cloning vectors. In addition, as nucleotide sequence data are presented in this thesis it is appropriate to review briefly gene expression in other Gram positive bacteria and, in particular in B. subtilis, which has been extensively studied. Gene expression in prokaryotes is, in most cases, regulated through control of transcription initiation (for reviews see McClure, 1985; Ishihama, 1988; Rosenberg & Court, 1979; Raibaud & Schwartz, 1984; Chamberlin, 1974; Kozak, 1983).

1.7.1 Promoters.

Promoters are sites on DNA at which RNA polymerase binds and initiates transcription of DNA to mRNA. DNA protection studies have indicated that RNA polymerase actually covers the promoter region and protects it from nuclease digestion for about 60 bp upstream of the mRNA initiation site (see Rosenberg & Court, 1979). Although RNA polymerase binds to a significant stretch of DNA, upstream and downstream from the transcription start point, promoter recognition is principally governed by two hexanucleotide sequences centred about 35 and 10 bp upstream (5') of the transcription start point. Consequently, these hexanucleotide sequences are called the '-35' region, characterized by the sequence (5') TTGACA (3'), and the Pribnow box or '-10' region, characterized

by the sequence (5') TATAAT (3'). RNA polymerase is believed to interact directly with bases in both regions because these hexanucleotide sequences are conserved in a large number of promoters from Gram negative and Gram positive organisms and because mutations within the hexamers impair promoter function.

A compilation and analysis of 168 E. coli promoters (Hawley & McClure, 1983; McClure, 1985) and 29 promoters derived from Gram positive bacteria (mostly B. subtilis, Graves & Rabinowitz, 1986) demonstrated additional conserved bases outside the classic -10 and -35 hexamers. In particular during the latter analysis an 'A' cluster at positions -41 to -45 had a greater than 50% conservation rate and two areas bordering the -10 region were also conserved. These observations suggest broader limits to the promoter elements of Gram positive bacteria and Graves & Rabinowitz (1986) have proposed the existence of an 'extended promoter'.

The specific recognition of a promoter by RNA polymerase is mediated by its sigma subunit and sigma factors bind to the core enzyme to form the holoenzyme. The discussion thus far has concentrated on the promoters recognized by sigma^{43} (formerly sigma^{55}) containing RNA polymerase holoenzyme (E-sigma^{43}) of B. subtilis and E. coli E-sigma^{70}. These are the principal sigma subunits found in the vegetative cells of these bacteria and are required for the majority of cellular transcription. However, multiple RNA polymerase forms which contain alternative sigma factors are used particularly for the transcription of coordinately regulated 'sets' of genes in bacteria that display a morphologically complex programme of differentiation e.g. B. subtilis and Streptomyces sp. (see Doi, 1982; Losick et al., 1986; Losick & Pero, 1981; Halmann & Chamberlin, 1988; Buttner et al., 1988). In addition, the transcription of the heat shock genes of E. coli involves a minor sigma species (sigma^{32}; Cowing et al., 1985) and the transcription of genes controlled by nitrogen availability involves the ntrA gene product sigma^{54} (now known as RpoN, see Halmann & Chamberlin, 1988). The promoters recognized by alternative forms of RNA polymerase can differ significantly from the -10 and -35 regions recognized by E-sigma^{43} and E-sigma^{70} as indicated in Table 1.3.

Table 1.3 Bacterial Sigma Factors and Related Proteins.
(Taken from Helmann & Chamberlin, 1988).

Factor	Gene	Function	Consensus sequence	
			−35	−10
Bacillus subtilis				
σ^{A}	rpoD, sigA	housekeeping functions	TTGACA	TATAAT
σ^{D}	sigD	flagellar synthesis/chemotaxis	CTAAA	CCGATAT
σ^{E}	spoIIGB, sigE	sporulation genes	TT-AAA	CATATT
σ^{H}	spoOH, sigH	sporulation genes	unknown	
σ^{C}	sigC	unknown	AAATC	TA-TG-TT-TA
σ^{B}	sigB	unknown	AGG-TT	GG-ATTG-T
$\sigma^{spoIIAC}$	spoIIAC	sporulation genes	unknown	
?	spoIIIC	sporulation genes	unknown	
σ^{28}	SPO1 28	phage middle genes	T-AGGAGA—A	TTT-TTT
$\sigma^{29,34}$	SPO1 33,34	phage late genes	CGTTAGA	GATATT
Escherichia coli and Related Bacteria				
σ^{70}	rpoD	housekeeping functions	TTGACA	TATAAT
σ^{54}	glnF, ntrA, rpoN	nitrogen-regulated genes (+ C_4-dicarboxylate transport in *R. meliloti*)	CTGGCAC N_5 TTGCA	
σ^{32}	htpR, rpoH	heat-shock genes	CTTGAA	CCCCAT-TA
?	flhB + flaI	flagellar synthesis/chemotaxis	TAAA	GCCGATAA
σ^{28}	T4 gene 55	phage late genes	none	TATAAATA
?	unknown	flagellar genes (*C. crescentus*)	TGGC-C N_5 TTGC	

The names of sigma factors were originally derived from their apparent molecular weight on SDS-polyacrylamide gels, and more recently from their molecular weight as deduced from gene sequences. However, alternative nomenclature has been suggested (see Helmann & Chamberlin, 1988; Losick et al., 1986).

1.7.2 The Regulation of Initiation and Termination of Transcription.

Several mechanisms which control the initiation of transcription in prokaryotes have been identified and reviewed elsewhere (McClure, 1985; Raibaud & Schwartz, 1984). In summary, repressors of transcription act by binding to specific sites (operators) near the promoter and activators bind near or upstream of the -35 region, but many repressors have a bifunctional role and also behave as activators. Evidence suggests that regulation can also occur as a result of structural and conformational

changes in the DNA template and methylation of DNA.

Termination of transcription can occur at simple terminators (rho-independent, characterized by sequence features including a G+C-rich self-complementary DNA sequence followed by a 4 to 8 thymidine residues that give rise to a stem-loop structure in mRNA) and at complex terminators which require additional factors such as rho. In addition, the mRNA of several biosynthetic genes contains an untranslated leader sequence which can form secondary structures. One structure might allow the RNA polymerase to continue transcription whilst another might cause termination of the transcript prior to the coding region of the DNA. The formation of these secondary structures is determined by interaction with the translational apparatus. This type of control is called attenuation (see Platt, 1986; Rosenberg & Court, 1979; d'Aubenton et al., 1990).

1.7.3 Translational Control of Gene Expression.

Translational control of gene expression is probably effected primarily at the initiation of translation but also during elongation of nascent polypeptides. Apart from the transcriptional differences between the promoters and sigma factors of B. subtilis and E. coli already discussed, it appears that the expression of heterologous genes in B. subtilis is considerably more restricted at the translational level of expression and, in general, the ribosomes of Gram positive bacteria discriminate against and are unable to translate mRNAs from Gram negative bacteria (McLaughlin et al., 1981a).

1.7.3.1 The Initiation of Translation.

The initiation of protein synthesis by prokaryotic ribosomes involves identification of a ribosome binding site (RBS) on the mRNA by the 30S subunit of the ribosome (for reviews see Gold et al., 1981; Kozak, 1983). A RBS consists of an initiation codon and a Shine-Dalgarno sequence which is complementary to the 3' end of 16S rRNA. In addition, an appropriate spacing or 'window' between these two elements is necessary and translation efficiency is reduced if the 'window' is less than about 5 or greater than about 9 nucleotides (McLaughlin et al.,

1981a). Translation initiation may also be stimulated by initiation factors (see Kozak, 1983).

Analysis of over 40 translation initiation sites from genes of Gram positive bacteria indicated that they all had a 'strong' Shine-Dalgarno sequence (Hager & Rabinowitz, 1985a) and compared to the initiation of translation in E. coli, there is probably a requirement for more extensive complementarity between the rRNA and the RBS in B. subtilis. The free energy of interaction can be used to compare the degree of base-pairing exhibited between two RNA sequences (Tinoco et al., 1973) and the comparison can be extended to DNA-DNA and RNA-DNA pairing with each complementary base-pair contributing to the negative value of free energy of interaction. The free energies of interaction for B. subtilis RBS's are about -16 kcal/mol as compared to about -10 kcal/mol for E. coli RBS's (Moran et al., 1982, Hager & Rabinowitz, 1985b). The requirement for more extensive Shine-Dalgarno sequences is consistent with the reduced dependence for initiation factors during the initiation of translation in Gram positive bacteria compared to Gram negative bacteria (McLaughlin et al., 1981a, 1981b, 1981c; Hager & Rabinowitz, 1985a).

An atypical ribosome binding site has recently been described for two genes encoding DNA methylases (dpnM and dpnA) of Streptococcus pneumoniae (de la Campa et al., 1987) and for the repB gene of pLS1 (a derivative of pMV158, isolated from S. agalactiae. Lacks et al., 1986; de la Campa et al., 1990). Analysis of the N-terminal amino acids of the proteins confirmed the translation initiation codons predicted from the DNA sequence. The novel RBS was used by both S. pneumoniae and E. coli but had no complementarity with either the 16S or 23S rRNA of the bacteria; the authors suggested that a specific initiation factor might be required for recognition of the sequence (de la Campa et al., 1987). Several genes have been identified which appear to lack Shine-Dalgarno sequences (Lopez et al., 1989; Ptashne et al., 1976).

AUG is the usual but not the only initiator codon and other codons are used in both E. coli and B. subtilis. Analysis of mRNAs derived from Gram positive organisms has indicated that non-AUG initiation codons occur more frequently in B. subtilis mRNA than in mRNA of Gram negative sources; about 30% of identified initiation codons were GUG or UUG in

B. subtilis (Hager & Rabinowitz, 1985a). The sequence context around initiator codons and upstream of the Shine-Dalgarno sequence may also be important for the recognition of the RBS and efficiency of initiation or frequency of translation (see Kozak, 1983; McLaughlin et al., 1981a; Gold et al., 1981; Hager & Rabinowitz, 1985a; Kastelein et al., 1983).

Many mRNAs have the potential to form stem-loop structures in the RBS region and typically the Shine-Dalgarno sequence is found in the 'loop' with the initiation codon in the 'stem' of the structure. These mRNA secondary structures are believed to restrict translation until the stem is disrupted by the formation of alternative structures or by translation read-through (Stanssens et al., 1985; Kastelein et al., 1983).

1.7.3.2 Translation Elongation.

The translation specificity of Gram positive bacteria might also be explained by the failure to make the transition from initiation of translation to elongation.

Some bacteria appear to exhibit a preferential codon usage with the concomitant presence of varying amounts of iso-accepting tRNA species (Ikemura, 1981). In 25 genes of E. coli for example, CUG represents 70% of the six possible codons for leucine (Konisberg & Godson, 1983) and it has been suggested that highly expressed genes are comprised of codons corresponding to abundant tRNA (Gouy & Gautier, 1982; Grosjean & Fiers, 1982). As the rate of elongation may be determined by the abundance of tRNA species, codon usage may be a modulator of gene expression.

In contrast to E. coli, B. subtilis does not seem to have such a marked codon bias and differing tRNA species profiles have been found in vegetative cells and spores (Vold, 1973). Whilst codon usage is more evenly distributed in B. subtilis genes, it appears that coding and non-coding reading frames of Gram positive bacterial mRNAs have similar codon distributions (which is not the case in E. coli; see Hager & Rabinowitz, 1985a) but rare codons are present in some B. subtilis genes (Ogasawara, 1985).

It would appear that codon usage is probably not an important factor in the failure of B. subtilis to express genes from Gram negative

bacterial sources because several E. coli genes have been expressed in B. subtilis when translation initiation sites from B. subtilis were cloned in front of the genes (see Hager & Rabinowitz, 1985a).

1.7.3.3 Heterologous Gene Expression.

Gene cloning experiments have shown that E. coli is promiscuous in its ability to recognize the transcription and translation signals from a wide variety of microorganisms and many genes from Gram positive sources have been cloned and expressed in E. coli; examples include genes from Bacillus (Makaroff et al., 1983), Staphylococcus (Shuttleworth et al., 1987) Clostridium (Kadam et al., 1988), Streptococcus (Herman & McKay, 1986) and Lactococcus (de Vos & Gasson, 1989).

In general, studies with B. subtilis and clostridia have shown that in contrast to E. coli, these bacteria are very limited in their ability to express genes from Gram negative bacteria (see Graves & Rabinowitz, 1986; Murray & Rabinowitz, 1982). Some heterologous genes are expressed in B. subtilis but these are usually from Gram positive hosts e.g. drug resistance genes from Staphylococcus (Ehrlich, 1978; Kreft et al., 1978) and Streptococcus (Yagi et al., 1978). Similarly, preliminary experiments suggest that E. coli initiation signals are not recognized by S. thermophilus (Mercenier, 1990).

Heterologous gene expression in B. subtilis is probably restricted at both the transcriptional and translational levels. Promoters transcribed by E. coli RNA polymerase are not necessarily utilized efficiently by B. subtilis RNA polymerase e.g. lacUV5 (Lee et al., 1980), and B. subtilis ribosomes fail to translate effectively from E. coli mRNA (Legault-Demare & Chambliss, 1975). In addition, Wiggs et al., (1979) have shown that RNA polymerase from B. subtilis and Lactobacillus curvatus interact more weakly with T7 promoter sites than do RNA polymerases from Gram negative bacteria.

Moran et al. (1982) noted that the limited expression of E. coli genes in B. subtilis might be attributed to a requirement for more stringent and extended promoter sequences and more extensive complementarity between the B. subtilis ribosomes and mRNA. It is likely that these requirements are probably major factors which limit the range

of genes expressed by B. subtilis. A direct correlation was observed between the complementarity of the Shine-Dalgarno sequence and gene expression in B. subtilis by constructs which differed only in the sequence of the Shine-Dalgarno region. No such correlation was observed for E. coli and the range of expression in B. subtilis was presumed to be regulated at the level of translation because the plasmids all contained the same transcription signals (see Hager & Rabinowitz, 1985a).

Given the greater occurrence of non-AUG initiation codons in B. subtilis (and in other Gram positive bacteria) and 'strong' Shine-Dalgarno sequences, it has been postulated that efficient initiation depends more on the the Shine-Dalgarno sequence than the initiation codon, whilst the reverse is true for E. coli (Hager & Rabinowitz, 1985a), and the barriers to heterologous gene expression are translational specificities rather than transcriptional requirements. It follows that for the expression of genes in Gram positive bacteria success is more likely if genes derived from a Gram positive host are used or if appropriate signals are provided for transcription and translation.

1.8 The Aims of the Thesis.

The moderately thermophilic iron-oxidizing bacteria are potentially of commercial importance and have received more attention in recent years for the recovery of metals during mineral leaching.

Studies towards the development of genetic systems (host:vector systems) for the moderate thermophiles were proposed to facilitate strain improvement; for example, increased toxic metal resistance.

Prior to this thesis genetic studies of the moderate thermophiles had not occurred and cloning vectors derived from the moderate thermophiles had not been identified. The initial aim of genetic studies was the identification and characterization of a suitable plasmid indigenous to one of the moderate thermophile strains. This plasmid could then be used in the *in vitro* construction of a selectable *E. coli*/moderate thermophile shuttle vector and once developed, it was anticipated that this vector would be used to develope a reliable electrotransformation procedure for the moderate thermophile(s).

In addition, the moderately thermophilic strains grow poorly on solid media and some work was directed towards improving this form of growth and the development of filter disc assays to assess the sensitivity of strains to antibiotics and metals.

Chapter 2.
Materials and Methods.

2.1 Bacterial Strains and Plasmids.

The *E. coli* strains and plasmids used in this work are listed in Tables 2.1 and 2.2 respectively.

The bacteria used were available in the Department of Biological Sciences at Warwick University with the exception of *B. subtilis* 168 (*trp*$^-$) which was obtained from Dr. N. Minton, Porton Down, UK. The references describing the moderate thermophile strains TH1, BC1, LM1, LM2, TH3, and ALV are cited in the Introduction.

Bacterial strains containing plasmids were obtained as follows: pC194 and pCK1 from S. Cardy, Warwick University; pC221cop903 from Dr. I. Murray, Leicester University; pAT187 from Dr. P. Trieu-Cuot, Pasteur Institute, Paris, France; pMTL20C from Dr. N. Minton, Porton Down, UK. All other plasmids were obtained from laboratory strains or were constructed in the course of this work.

Bacteriophage vectors M13mp10 and mp11 were supplied by Amersham International, and M13mp18 and mp19 were obtained from Northumbria Biologicals Ltd (Yanisch-Perron *et al.*, 1985).

Table 2.1 Bacterial strains.

STRAIN	GENOTYPE	REFERENCE
E. coli DH1	F^-, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17* (r_K m_K), *supE44*, lambda$^-$.	Hanahan, 1983.
E. coli HB101	F^-, *hsdS20* (r_B, M_B), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5*, *mtl-1*, *supE44*, lambda$^-$.	Boyer & Roullaud-Dussoix, 1969.
E. coli TG1	(*pro-lac*)$^{\nabla}$, *supE*, *thi*, F', *traD*, *proAB*$^+$, *lacI*^{q}, *lacZ* ∇15	see Cardy, 1989a.

Table 2.2 Plasmids.

PLASMID	PHENOTYPE	HOST	REFERENCE
pC194	$+Cm^r$	B. subtilis	Horinouchi & Weisblum, 1982.
pC221cop903	$+Cm^r$	S. aureus	Projan et al., 1985.
pACYC177	Kan^r, Amp^r	E. coli	Chang & Cohen, 1978.
pBR325	Cm^r, Amp^r, Tc^r	E. coli	Bolivar, 1978.
pCK1	$+Cm^r$, $+Kan^r$	E. coli	Gasson & Anderson, 1985.
pAT187	$+Kan^r$	E. coli	Trieu-Cuot et al., 1987.
pMTL20C	$+Cm^r$, Amp^r, lacZ	E. coli	Swinfield et al., 1990.
pFEG7	Kan^r	E. coli	This Work
pFEG15	Kan^r	E. coli	This Work
pLZ5	Amp^r	E. coli	This Work
pLZ11	Amp^r	E. coli	This Work
pBRBC1	Amp^r, Cm^r	E. coli	This Work
pBRBC2	Amp^r, Cm^r	E. coli	This Work
pMTL20CK1	$+Cm^r$, Amp^r	E. coli	This Work
pMTL20CK2	$+Cm^r$, Amp^r	E. coli	This Work
pMTL20CH1	$+Cm^r$, Amp^r	E. coli	This Work
pMTL20CH2	$+Cm^r$, Amp^r	E. coli	This Work

Notes:

A + sign indicates the phenotype has been demonstrated in Gram positive and Gram negative bacteria.

2.2 Chemicals and Media.

2.2.1 Chemicals.

All chemicals unless otherwise stated were obtained from BDH, Sigma or Fisons and were of Analar grade. The following list indicates the source of specific materials:

Amersham International, UK: restriction endonucleases and buffers, T_4 DNA ligase, ^{35}S-methionine, ^{32}P-nucleotides, Hybond nitrocellulose, Prokaryotic DNA directed *in vitro* Transcription and Translation Kit, S1 nuclease, ^{14}C-methylated proteins (CFA.626). Bethesda Research Laboratories, Maryland, USA (BRL): restriction enzymes and buffers, DNA polymerase (Klenow), DNA kilobase ladder, Lambda DNA. Northumbria Biologicals Limited, UK (NBL): restriction enzyme *Lap*I (*Asu*II) and buffer, X-gal and IPTG. Lab M, Salford, UK: yeast extract. Diagen (Quisgen Inc.), California, USA: Quiagen Columns. Sigma, UK: agarose type II medium EEO, DNase. Pharmacia, UK: Sephadex G50, low molecular weight protein markers. E. I. Dupont de Nemours & Co Inc. Wilmington, Delaware, USA: Ludox HS40. Blagden Campbell Chemical, AMP House, Dingwall Rd., Croydon, Surrey: Pluronic polyol F127. Mast Labs, Liverpool, UK: antibiotic Filter Discs.

2.2.2 Media.

Media were sterilized by autoclaving at 15 psi for 15 minutes unless otherwise stated.

Salts Medium:

	g/l
$MgSO_4.7H_2O$	0.4
$(NH_4)_2SO_4$	0.2
KCl	0.1
K_2HPO_4	0.1

This was adjusted to the appropriate pH with H_2SO_4.

Low phosphate Medium:

As for Salts Medium but contains one tenth the concentration of K_2HPO_4.

Sulphur Medium.

Sulphur flowers (5 g/l) were added to the Salts Medium at pH 3.0, which was then autoclaved at 5 psi for 5 minutes on three consecutive days. Yeast extract was added prior to inoculation to a final concentration of 0.025% (w/v).

Pyrite Medium.

Pyrite (FeS_2) was added to Salts Medium at pH 2.0 and after sterilization yeast extract was added prior to inoculation to a final concentration of 0.025% (w/v).

L-broth:	g/l	
NaCl	10	L-broth and TYE-broth
yeast extract	5	were supplemented with
Bactotryptone	10	1.5% (w/v) Bacto agar to give solid media.

TYE broth:

	g/l
NaCl	8
Bactotryptone	16
yeast extract	10

M9 medium: see Maniatis et al. (1982).

Mannings Medium : Manning (1975).

D1 Salts Medium:

	g/l
$(NH_4)_2SO_4$	6
KCl	0.2
$MgSO_4.7H_2O$	1

Adjusted to the appropriate pH with H_2SO_4

Solid Medium For Growth Of The Moderate Thermophiles:

The following solutions were made and autoclaved:
472.5 ml 2x D1 Salts Medium at pH 2.6
5 g of agarose type II in 500 ml distilled water.
These solutions were allowed to cool to 45°C and mixed. The medium was then supplemented with 10 ml ferrous sulphate stock solution (1 M), 10 ml yeast extract (2.5% [w/v]), and mixed. For Fe/Ye/G medium 2.5 ml glucose (0.5 M) was also added.
Ye/G medium contained yeast extract and glucose (as above; but no ferrous sulphate).

For pour plates, 5 ml of culture grown chemolithoheterotrophically to mid-exponential growth phase were used to inoculate the cooled medium (as above). 20 ml of this were poured into a Petri dish. The plates were allowed to set at room temperature, and then inverted and incubated (at 45°C) in sealed sandwich boxes, containing silica gel, for 3-10 days.

For sloppy agarose tubes the medium was made as above except the final concentration of agarose was reduced to 0.3% (w/v). The medium was inoculated as for pour plates and then 3 ml poured into a 10 ml capped test tube.

For Ludox plates: Ludox HS40 (40% [w/v] silica) was autoclaved and mixed with Salts Medium pH 2.0 to give a final concentration of 1.5% (w/v) silica content (Praemer, 1957) and Pluronic polyol F127 was diluted to 20% (w/v) with Salts Medium pH 2.0 (Gardener & Jones, 1984).

2.2.3 Media Supplements.

Ferrous Sulphate.

1 M stock solutions of $FeSO_4$. H_2O were prepared. The pH was adjusted to 1.3 using H_2SO_4 and the solution autoclaved at 10 psi for 10 minutes.

For liquid media ferrous sulphate was used at a final concentration of 50 mM, and in solid media at 10 mM.

Yeast Extract

Stock solutions of 2.5% (w/v) Lab M yeast extract were prepared and sterilized by autoclaving.

In solid and liquid media for the moderate thermophiles yeast extract was used at a final concentration of 0.025% or 0.05% (w/v).

Glucose

Stock solutions of 0.5 M were prepared and sterilized by autoclaving. Glucose was used at a final concentration of 5 mM in media for the moderate thermophiles.

Antibiotics.

For the maintenance and selection of plasmid-containing strains, antibiotics were used where appropriate at the following concentrations:

Antibiotic	Stock Solution Conc. mg/ml	Final Conc. µg/ml
Ampicillin	50	50-100
Chloramphenicol	20	20
Kanamycin	50	50
Tetracycline	20	12.5

Antibiotic stock solutions were prepared as described in Maniatis et al. (1982).

In experiments with B. subtilis, chloramphenicol was used at a final concentration of 10 µg/ml following (where necessary), induction of the resistance phenotype with 0.5 µg chloramphenicol/ml for 1 hour.

Filter Disc Assays of the Moderate Thermophiles.

Pour plates of the moderate thermophile strains TH3, ALV and BC1 were made and allowed to set.

For metal resistances: 20 µl of metal solution was applied to a filter disc (6 mm diameter), and placed in a central position on top of the medium.

For antibiotic resistances: Mast Antibiotic discs were used and placed in a central position on top of pour plates.

Plates for assays were inverted and incubated at 45°C for 3-10 days.

2.2.4 Buffers.

TE buffer Tris-HCl (10 mM), EDTA (1 mM) at pH 8.0.

20x SSC 3 M NaCl, 0.3 M trisodium citrate.

10x TBE (g/l) Tris base 108 g, boric acid 55 g, EDTA 9.5 g.

2.3 Storage and Growth of Bacteria.

2.3.1 E. coli, B. subtilis and S. aureus.

Strains (except TG1) were routinely grown aerobically at 37°C either in L-broth or TYE broth. These strains were also cultivated on agar-solidified (1.5% [w/v]) broth media. Cultures were stored in the short term at 4°C on L-agar plates. Longer term storage was at -20°C, as broth cultures containing 50% (v/v) glycerol.

TG1 was grown in TYE broth but was maintained on M9 solidified agar (1.5% [w/v]) containing 0.1% (w/v) thiamine.

B. subtilis 168 was grown with a tryptophan (50 µg/ml) supplement.

2.3.2 Moderate Thermophiles.

Strains were grown at 45°C and maintained for several months at room temperature in Salts Medium containing pyrite (1% [w/v]) or sulphur (0.5% [w/v]), both media were supplemented with Lab M yeast extract (0.025% [w/v]).

For growth on yeast extract (heterotrophic growth), Salts Medium at pH 2.0 was supplemented with Lab M yeast extract (0.025% or 0.05% [w/v]).

For growth on iron/yeast extract (Fe/Ye; chemolithoheterotrophic growth), the Salts Medium at pH 1.7 was supplemented with Lab M yeast extract (0.025% [w/v]) and ferrous sulphate (50 mM).

A 10% (v/v) inoculum was routinely used and cultures grown aerobically, in an orbital shaker (120 rpm) at 45°C for 2-4 days.

For large quantities of strain BC1, 1 or 2 litres of freshly grown culture were used to inoculate 20 litres of the appropriate medium in a glass vessel which was supported over a heated magnetic stirrer. The

culture was aerated with filtered compressed air and incubated at 45-50°C for about 4 days.

2.3.3 Light Microscopy.

All cultures were examined by phase contrast microscopy, using an Olympus model stereoscopic microscope (x 1000 magnification).

2.3.4 Ferrous Iron Oxidation Assay.

The growth of the moderate thermophiles on iron plus yeast extract was monitored by an assay for the concentration of ferrous iron in solution. The validity of using ferrous iron oxidation as a measure of growth has been demonstrated previously (Marsh & Norris, 1983a).

1 ml culture samples were placed in 2 ml of 5% (v/v) H_2SO_4 and titrated against 0.005 M ceric sulphate using 1,10-phenanthroline ferrous sulphate complex as an indicator. The decrease in ferrous iron concentration with time was expressed in terms of percentage ferrous iron oxidized.

2.4 General Techniques for DNA Manipulation.

2.4.1 Phenol Extraction.

Proteins were removed from solutions containing DNA prior to ethanol precipitation by mixing with an equal volume of TE saturated phenol/ chloroform/ isoamylalcohol (25:24:1, prepared as in Maniatis et al., 1982). After mixing, the phases were separated by centrifugation in an MSE Microcentaur centrifuge for 5 minutes. The upper aqueous layer was removed and mixed with an equal volume of chloroform and centrifuged as before. The upper phase was removed and DNA isolated from this solution by ethanol precipitation.

2.4.2 Ethanol Precipitation.

To a DNA solution, one tenth volume of 3 M sodium acetate and two volumes of ethanol were added, mixed gently and unless otherwise stated chilled at -20°C overnight or in a dry ice ethanol bath for 1 h. The DNA was recovered by centrifugation in an MSE Microcentaur for 10 minutes at 4°C. The supernatant was removed and the pellet washed in 500 µl of 70% (v/v) ethanol, dried briefly under vacuum and then resuspended in TE buffer.

2.4.3 Restriction Endonuclease Digestion.

Restriction enzyme digestion of DNA was carried out according to the instructions of the enzyme manufacturers, although a two to ten-fold excess of enzyme was routinely used.

2.4.4 Dephosphorylation of DNA.

Calf Intestinal Alkaline Phosphatase (CIAP) was used to dephosphorylate the 5' terminus of DNA. Following digestion of the DNA with the appropriate restriction enzyme, CIAP was generally added directly to the reaction tube, at a concentration of 1-5 units of CIAP μg^{-1} of DNA, and incubated for a further 30 to 60 minutes at 37°C. Alternatively, to every 10 µl of restriction digestion solution, 1.5 µl glycine (0.5 M) pH 9.4, 1.5 µl $MgCl_2$ (10 mM), 1.5 µl $ZnCl_2$ (1 mM), 1.5 µl SDW and 0.5 µl CIAP were added, mixed and incubated at 37°C for 30 minutes. After dephosphorylation CIAP was removed by phenol extraction and the DNA subsequently ethanol precipitated.

2.4.5 Ligation of DNA.

Ligation of DNA was carried out according to the recommendation of Amersham the suppliers of the T_4 DNA ligase. A five fold excess of enzyme

was routinely used.

Vector and insert DNA's were mixed at the appropriate concentrations (Maniatis et al., 1982). Where blunt ends were to be ligated, a final concentration of 15% (v/v) PEG 6000 was added to the ligation mix to improve the efficiency. Ligations were carried out at 15°C for at least 16 h. In some instances, aliquots of the ligation sample were analysed on agarose minigels to detect the presence of new molecular species; this was an indication that in vitro ligation of the DNA fragments had occurred.

2.4.6 DNase-free RNase.

DNase-free RNase was used for the selective removal of RNA in DNA samples, particularly plasmid minipreparation samples. A stock solution of RNase A (20 mg/ml) in Tris-HCl (10 mM) at pH 7.5 was heated (in a boiling water bath) for 15 minutes, cooled slowly and then stored at -20°C. DNA samples were typically treated with RNase at a final concentration of 25 µg/ml by incubation at 37°C for 30 minutes. When necessary RNase was subsequently removed by phenol extraction.

2.4.7 Agarose Gel Electrophoresis.

Horizontal agarose slab gels (0.7% [w/v]) were routinely used, as described by Maniatis et al. (1982), using Tris-borate-EDTA buffer. Electrophoresis was carried out in a BRL Model H4 Horizontal Gel System at 60-150 volts for an appropriate length of time.

Where indicated, ethidium bromide (0.5 µg/ml) was added to the cooled gel matrix prior to pouring. When the gel contained no ethidium bromide, the DNA was stained with ethidium bromide as described by Maniatis et al. (1982). DNA was then visualized by transillumination with short wave UV light and photographed using Polaroid type 665 film.

Restriction digests were routinely checked by electrophoresis of samples in a Cambridge Bioscience 'mini-gel' apparatus.

2.4.8 Quantitation of DNA.

Two methods were used, as described by Maniatis et al. (1982): the minigel method using Lambda (digested with HindIII) DNA (60 ng/µl) as the standard, or the absorbance of the DNA solution at 260 nm was measured, and it was assumed 50 µg/ml double stranded DNA had an absorbance of 1.0.

2.4.9 Recovery Of DNA Fragments From Agarose Gels.

After resolution of the desired DNA fragment by agarose gel electrophoresis and ethidium bromide staining, the DNA band was localized with the aid of a UV transilluminator. The desired band was excised from the gel with a scalpel and placed in an appropriate length of ¼" or ½" dialysis tubing (prepared as in Maniatis et al., 1982) and submerged in 0.5x TBE buffer. The ends of the tubing were sealed and the dialysis bag placed lengthways at right angles to the current in a horizontal gel tank partially filled with 0.5x TBE buffer.

Electroelution of the DNA was carried out at 100 volts for about 1 h. After this time the current was reversed for 30 seconds to disengage the eluted DNA from the dialysis tubing. The TBE buffer containing the DNA was removed from the tubing and extracted once with an equal volume of TE saturated butan-1-ol, once with an equal volume of phenol, and once with an equal volume of water saturated ether. The DNA was then ethanol precipitated and later resuspended in an appropriate volume of TE buffer.

2.5 Isolation of Plasmid DNA.

2.5.1 Large Scale Preparation of Plasmid DNA from E. coli and B. subtilis.

The alkaline lysis method was used as described by Maniatis et al. (1982), except solution 2 (alkaline-SDS) was not placed on ice prior to use.

2.5.2 Caesium Chloride Density Gradient Centrifugation.

Samples were prepared for caesium chloride density gradient centrifugation by addition of caesium chloride and ethidium bromide (Maniatis et al., 1982) and filtered through a syringe containing glass wool into a Beckman opaque 'quickseal' tube. Plasmid DNA was separated from chromosomal DNA by centrifugation in a Beckman L8 ultracentrifuge and Vti50 rotor at 25°C, 45,000 rpm for 16 h and then in a Vti65 rotor at 25°C, 65,000 rpm for 4 h.

After centrifugation, the lower plasmid band was removed by piercing the side of the tube with an 18 gauge hypodermic needle and withdrawing the plasmid into a 5 ml syringe as described by Maniatis et al. (1982).

Ethidium bromide was removed by extracting the sample 3 to 4 times with an equal volume of TE saturated butan-1-ol. The plasmid DNA was directly precipitated (Davis et al., 1980), or was dialysed for 4 h at room temperature in 5 litres of TE buffer and then ethanol precipitated.

2.5.3 Large Scale Preparation of pBC1 Plasmid.

40 litres of BC1 culture were grown on yeast extract to an absorbance of 0.1-0.2 at 440 nm. Cells were concentrated in a continuous centrifuge (Westphalia, 10,000 rpm) and the resulting pellet collected, washed in 1 litre of Salts Medium at pH 2.0 and then in 1 litre of Tris-HCl (10 mM) at pH 8.0, centrifuging at 9,000 rpm for 10 minutes in a Beckman JA10 rotor between washes, and removing the supernatant with a pasteur pipette connected to a vacuum line. The resulting pellet was subjected to the large scale alkaline lysis plasmid preparation method as described in Section 2.5.1 (Maniatis et al., 1982).

Due to the small amount of plasmid obtained, it was purified as follows: DNA was RNase treated, loaded into a single large gel slot in a 0.8% (w/v) agarose gel, electroeluted and then phenol extracted. Alternatively, it was purified using a Quiagen column (Diagen) and the manufacturers conditions for purifying plasmid DNA.

2.5.4 Amplification of Plasmid DNA.

The method used for the large scale isolation of pBR325 and pACYC177 derived plasmids involved chloramphenicol amplification of plasmid DNA and gave better yields than growth of the plasmid containing cells in the absence of chloramphenicol, particularly for pACYC177 derived vectors.

A 2 litre baffled flask containing 1 litre of prewarmed L-broth and the appropriate antibiotic(s) for plasmid selection was inoculated with 4 ml of an overnight culture (10 ml) of the host bacteria and grown at 37°C with vigorous shaking for 5 h. 5 ml of chloramphenicol (34 mg/ml in ethanol) was added and the culture incubated at 37°C for 12-16 h.

The cells were then subjected to the large scale alkaline lysis preparation as described earlier for E. coli and plasmid DNA purified by caesium chloride density gradient centrifugation.

2.5.5 Isolation of Plasmid DNA from S. aureus.

This method is based on the large scale alkaline lysis method as described earlier (Maniatis et al., 1982), with an additional acetone sensitization step for S. aureus and also requires Lysostaphin instead of Lysozyme (pers. comm. Dr. I. Murray, Leicester University).

1 litre of S. aureus (grown overnight) was centrifuged (8,000 rpm, 10 minutes at 4°C), the resulting pellet resuspended in 100 ml of cold EDTA (10 mM) at pH 7.0 and centrifuged as before. Cells were resuspended in 80 ml of cold 1:1 (v/v) acetone:ethanol and incubated on ice for 20 minutes with occasional swirling. 320 ml of cold EDTA (10 mM) at pH 7.0 was added and the mixture left on ice for 20 minutes as before. The cells were centrifuged (8,000 rpm, 10 minutes at 4°C) and the pellet dried with compressed air to remove the acetone:ethanol.

The cell pellet was resuspended in 40 ml lysis buffer (50 mM sucrose, 10 mM EDTA at pH 8.0, 25 mM Tris-HCl at pH 8.0). Lysostaphin (Sigma) was added to a concentration of 50 µg/ml and incubated at 37°C for 30 minutes. The procedure then continued with the addition of 80 ml

of solution 2, 60 ml of solution 3 and isopropanol precipitation as described by Maniatis et al. (1982).

Following isopropanol precipitation the RNA-DNA-protein pellet was resuspended in 10 ml TE buffer, RNase was added to a concentration of 50 µg/ml, and incubated at 37°C for 30 minutes. Proteinase K (Sigma) was added to a concentration of 50 µg/ml, and incubated at 37°C for 30 minutes. The sample was then phenol extracted, the DNA ethanol precipitated, and resuspended in TE buffer. Plasmid DNA was purified by caesium chloride density gradient centrifugation as described in Section 2.5.2.

2.5.6 Plasmid Minipreparation Method.

The alkaline lysis method of Birnboim and Doly (1979) modified and described by Maniatis et al. (1982) was used for E. coli. B. subtilis and the moderate thermophiles.

For the moderate thermophiles, 40 ml of heterotrophically grown culture in late exponential growth phase was used. These cells were harvested at 10,000 rpm, for 5 minutes at 4°C. The cell pellet was washed in 1 ml Salts Medium at pH 2.0, and centrifuged for 3 minutes in an MSE Microcentaur (high speed), resuspended in 1 ml Tris-HCl (10 mM) at pH 8.0, centrifuged in an MSE Microcentaur as before, and treated as described by Maniatis et al. (1982).

When the cells had been grown in medium containing iron, they were centrifuged (10,000 rpm, for 15 minutes at 20°C) and the supernatant removed using a vacuum line. The resulting pellet was subjected to a series of washes and slow spins in a centrifuge to remove the precipitated ferric iron; the cells were centrifuged at slow speed for 5 seconds in an MSE Microcentaur centrifuge, the supernatant which contained cells was removed, leaving the ferric iron pellet behind. This was repeated several times with a progressively longer spin until most of the ferric iron was removed from the cells. The cells were pelleted and resuspended in 1 ml of Tris-HCl (10 mM) at pH 8.0 and the centrifugation

procedure repeated to remove additional ferric iron created by the change in pH. The cells were examined microscopically, to check that most of the ferric iron had been removed, and then pelleted and plasmid prepared following the alkaline lysis minipreparation method of Maniatis et al. (1982).

2.6 Transformation and Electroporation.

2.6.1 Transformation of E. coli.

Transformation was carried out using the procedure of Hanahan (1983) or following the method of electroporation.

2.6.2 Electroporation of E. coli.

The cell preparation and subsequent electrotransformation method of Dower et al. (1988) was used. The electroporation apparatus was a Biorad Gene Pulser coupled to a parallel resistance selector (Biorad Pulse Controller) and 0.2 cm plastic disposable electroporation cuvettes were used.

10 ml of E. coli was grown overnight in L-broth. This culture was used to inoculate 1 litre of prewarmed L-broth in a 2 litre baffled flask which was incubated shaking vigorously at 37°C for 3-4 h, until the absorbance at 600 nm was 0.5-1.0. The culture was then placed on ice for 10 minutes. Cells were harvested by centrifugation (at 4,000 rpm for 15 minutes at 4°C) and then the cell pellet was repeatedly resuspended and centrifuged (as before) in the following cold solutions; 1 litre SDW, 0.5 litre SDW, 20 ml glycerol (10% [v/v]) and finally resuspended in 2 ml of glycerol (10% [v/v]). The cells were frozen on dry ice prior to storage for up to 6 months at -70°C.

DNA from ligation mixtures was phenol extracted, ethanol precipitated and resuspended in 10 µl TE prior to electroporation.

A frozen aliquot of E. coli was allowed to thaw at room temperature

and then placed on ice. 2 µl of ligated DNA was mixed with 40 µl of E. coli cells and put into a chilled 0.2 cm cuvette. The sample was placed into the electroporation chamber and subjected to an electric pulse (25 µFD, 2.5 kV and 200 ohms). This typically gave a time constant of about 4.5 ms.

Immediately after the pulse, 1 ml of L-broth was added to the cuvette. The cells were transferred to an Eppendorf tube with a Pasteur pipette and incubated at 37°C for 1 h prior to plating on the appropriate selective media.

2.6.3 Electroporation of the Moderate Thermophile Strains ALV and BC1.

A standard protocol is given below but this method was modified on several occasions and will be discussed in Chapter 7.

Cells were prepared for electroporation as follows: a 100 ml culture of bacteria grown on iron plus yeast extract was used to inoculate duplicate 2 litre flasks containing 1 litre of iron plus yeast extract medium, prewarmed to 45°C. Typically a 4 ml inoculum was used. These flasks were incubated at 45°C overnight. The following day, growth was monitored by assaying the amount of ferrous iron oxidized. When about 30-40% of the ferrous iron had been oxidized the cultures were harvested at 4°C.

Cells were centrifuged at 5,000 rpm for 15 minutes and the supernatant removed using a Pasteur pipette connected to a vacuum line. The pellet was resuspended in 1 litre of Salts Medium at pH 1.7 and centrifuged as above. The supernatant was removed (as before) and the pellet resuspended in 80 ml SDW at pH 1.7 and centrifuged at 5,000 rpm for 10 minutes. The supernatant was removed (as before), the pellet resuspended in 5 ml of Tris-HCl (10 mM) at pH 8.0 and transferred to Eppendorf tubes. The cells were then repeatedly centrifuged as described in the plasmid minipreparation method (Section 2.5.6), to remove ferric iron, washed in 5 ml glycerol (10% [v/v]) and finally resuspended in 1.5 ml of glycerol (10% [v/v]) and stored on ice. This gave a cell suspension

of about 2 x 10^7 cfu/ml as counted using a haemocytometer. Cells were typically present as pairs although some longer chains were seen.

40 µl of cells and 5 µl of plasmid DNA (2-5 µg) were subjected to an electric pulse as described for electroporation of E. coli. However, various voltages and resistances were used which correspondingly gave different field strengths and time constants (Chapter 7).

Following electroporation, each sample was resuspended immediately in 5 ml of iron plus yeast extract medium at pH 1.7 and incubated at 30°C for 16 h, then at 45°C for 30 minutes. When induction of a cat gene was required chloramphenicol was added to a final concentration of 0.1 µg/ml.

To select for tranformants, 1 ml of the electroporated cells was used as an inoculum for flasks containing 100 ml of iron plus yeast extract medium at pH 1.7 and chloramphenicol added to a final concentration of 0, 1, 2 or 5 µg/ml. These flasks were then placed at 45°C and monitored for any growth. Growth was detected visually by the appearance of brown ferric iron or more accurately by assaying the amount of ferrous iron oxidized. Cells which grew in the presence of chloramphenicol were treated as for the plasmid minipreparation (Section 2.5.6).

2.6.4 DNase Treatment of Electroporated Cells.

Following a suggestion by Prof. D. E. Rawlings (University of Cape Town, South Africa), a method was developed herein to detect transfer of plasmid into the moderate thermophiles by electroporation. The method does not require plasmid replication in the cells or expression of antibiotic resistance by the moderate thermophiles but was intended to detect any plasmid which had entered the cells by electrotransformation.

Cells of the moderate thermophile strains ALV and BC1 were prepared for electroporation (Section 2.6.3). Following treatment of the cells and plasmid with an electric pulse, the cells were resuspended in 1 ml of glycerol (10% [v/v]). 100 µl of 10x DNase buffer (3 M Na acetate at pH 6.5, 1 M $MgCl_2$, 1 M $CaCl_2$) and 5 µl DNase (5 mg/ml) were added and

incubated at room temperature for 5 minutes. The cells were centrifuged (MSE Microcentaur, 3 minutes at high speed), the pellet resuspended in 1 ml of TE buffer and recentrifuged. The subsequent cell pellet was subjected to the plasmid minipreparation method described in Section 2.5.6 and the DNA finally resuspended in 20 µl of TE buffer.

5 µl of the plasmid sample were used for the electroporation of E. coli DH1 as in Section 2.6.2. and subsequently plated onto selective medium to detect transformants. The remainder of the plasmid minipreparation sample was analysed on an agarose minigel (0.7% [w/v]).

Following electroporation of the moderate thermophiles, plasmids contained within the cells were protected from the action of the DNase and plasmids outside the cells were digested by the DNase. Plasmids within the bacterial cells were subsequently isolated and the resulting DNA used to electroporate E. coli DH1. These cells were spread onto solid media and plasmids in E. coli detected by the expression of antibiotic resistance phenotypes. Thus, any plasmids successfully electroporated into the moderate thermophile strains were subsequently detected in E. coli.

2.6.5 Transformation of B. subtilis.

The method used is based on that of Anagnostopoulos and Spizizen (1961).

B. subtilis 168 was grown overnight at 37°C to give a lawn on L-agar. Four heavy loopfuls of these cells were used to inoculate 20 ml of competence medium in a 250 ml flask which was then vortexed to disperse the cells. This flask was incubated in a 37°C water bath, shaking vigorously to give good aeration for about 3-4 hours until the absorbance at 600 nm was 3-4. The culture was then diluted 10-fold into fresh transformation medium and mixed. 1 ml of the diluted culture was placed in a glass universal and up to 5 µg of DNA added and shaken vigorously at 37°C for 90 minutes. When induction of the cat gene was required, chloramphenicol was added to a final concentration of

0.5 µg/ml, 30 minutes into this 90 minute incubation. Cells were then plated onto the appropriate selective media.

	Competence Medium:	Transformation Medium:
SMM	20 ml	20 ml
Glucose (20% [w/v])	0.5 ml	0.5 ml
L-tryptophan (10 mg/ml)	100 µl	10 µl
Casein hydrolysate (10% [w/v])	40 µl	20 µl
$MgSO_4$ (0.5 M)	100 µl	200 µl
$CaCl_2$ (5 mM)	200 µl	-
$MnSO_4$ (0.5 mM)	20 µl	-

Spizizens Minimal Medium (SMM):	g/l
Ammonium sulphate	2
K_2HPO_4	14
KH_2PO_4	6
Na citrate.$2H_2O$	1
$MgSO_4.7H_2O$	0.2
at pH 7.0	

2.7 Southern Transfer of DNA to Nitrocellulose.

The procedure described by Maniatis et al. (1982) was used with the following modifications. Denaturation and neutralization of the DNA in the gel matrix were both carried out for 30 minutes at room temperature and 20x SSC was used instead of 10x SSC as the transfer buffer.

2.7.1 Nick-Translation of DNA.

The basic procedure of Maniatis et al. (1982) was used with the following modifications (V. Lees pers. comm.):

DNA in TE buffer (between 100 ng and 1 µg)	x µl
1 mM dATP	2 µl
1 mM dTTP	2 µl
1 mM dCTP	2 µl
^{32}P dGTP	2 µl
(Amersham, 3000 Ci/mmol, 10 µCi/µl)	
10x nick translation buffer	8 µl
(0.5 M Tris-HCl at pH 7.2, 0.1 M $MgSO_4$,	
1 mM DTT, 500 µg/ml BSA Pentax fraction 5)	
DNA polymerase 1	2 µl
DNase 1	2 µl
(diluted: 1 µl of 1 mg/ml stock into	
50 µl SDW, then 1 µl into 50 ml SDW)	
SDW	y µl

x and y adjusted to give a final volume of 40 µl.

The above constituents were placed in an Eppendorf tube and mixed, spun in an MSE Microcentaur briefly and incubated at 15°C for 3 hours. The reaction was stopped by the addition of 5 µl EDTA (0.5 M) at pH 8.0 and the nick-translated probe separated from unincorporated dNTP's using a Sephadex G50 column (Maniatis et al., 1982).

The labelled DNA was stored at -20°C until required when it was denatured by boiling for 15 minutes immediately prior to use.

2.7.2 Hybridization of Nick-Translated DNA to Southern Filter.

The procedure of Maniatis et al. (1982) was followed with the following modifications.

The dry nitrocellulose filter was placed in a heat-sealed bag containing 30 ml of prehybridization solution (6x SSC, 1x Denhardts solution, 10 mg sheared and heat-denatured Herring sperm DNA/ml).

After prehybridization in a water bath at 65°C for 2 hours, the prehybridization solution was replaced with 30 ml of fresh prehybridization solution supplemented with the ^{32}P labelled probe. Hybridization was carried out for a minimum of 18 hours at 65°C followed by a variety of stringency washes employing published guidelines (Marmur & Doty, 1962; Bonner et al., 1973) to estimate the percentage DNA homology required for hybridization.

2.7.3 Autoradiography.

Autoradiography was carried out at -70°C for ^{32}P-labelled material and at room temperature for ^{35}S-labelled material using Harmer film cassettes (with intensifying screens for ^{32}P) and Fuji RX X-ray film. Autoradiograms were developed in Kodak LX-24 developer and fixed in Kodak FX-40 according to the manufacturer's instructions.

2.8 DNA Sequencing.

The dideoxynucleotide chain terminating method of DNA sequencing was used (Sanger et al., 1977).

Defined DNA fragments were first cloned into the replicative form (RF) of the M13 cloning vectors mp10, mp11, mp18 and mp19. These vectors contain multiple cloning sites in the alpha-peptide of the beta-galactosidase gene. Single-stranded DNA template was prepared from the mature phage particles of the recombinants and was used directly in the sequencing reaction. Sequential extension by Klenow polymerase from a primer hybridized 3' to the insert DNA sequence took place. Termination of this extension occurred when dideoxynucleotide analogues were inserted into the newly synthesized and complementary DNA.

2.8.1 Subcloning into M13.

M13 vector and insert DNA were ligated, phenol extracted, ethanol precipitated and resuspended in 10 µl of TE buffer. 1-2 µl of this DNA was added to 40 µl of E. coli TG1 cells prepared for electroporation, and then electroporated as described in Section 2.6.2.

The electroporated cells were resuspended in 1 ml of TYE medium. A volume of 10-200 µl of these cells were added to a 5 ml test tube in a 45°C heating block containing: 3 ml molten TYE soft agar (0.7% [w/v]), 30 µl of X-gal (4% [w/v] in DMF), 30 µl IPTG (2.5% [w/v]) and 200 µl of an overnight culture of strain TG1. This mixture was vortexed and then poured onto a TYE agar plate, allowed to set, inverted and incubated at 37°C overnight. Recombinants were identified as colourless plaques resulting from the insertional inactivation of the functional beta-galactosidase gene.

2.8.2 Template Preparation.

Template preparation was essentially as described by Bankier et al. (1986), with the following modifications. A single, colourless plaque was picked with a cocktail stick and placed into a 5 ml test tube containing 1.5 ml of diluted E. coli TG1 (a 10 ml overnight culture was diluted 100-fold in TYE broth). After propagation of phage for 5 hours at 37°C (shaking vigorously), the culture was transferred to a 1.5 ml Eppendorf tube and centrifuged in an MSE Microcentaur (20 minutes, high speed at room temperature). After careful transfer of the supernatant to a fresh Eppendorf tube, 150 µl of PEG solution (g/l: PEG 6000 200 g, NaCl 146 g) was added, vortexed briefly, and left standing at room temperature for 10 minutes. The supernatant/PEG solution was centrifuged for 20 minutes and the supernatant discarded. Any residual PEG was removed after a 30 seconds respin. The resulting phage pellet was resuspended in 100 µl TE, allowed to stand at room temperature for 10 minutes and then phenol-extracted with a half volume phenol mixture (Section 2.4.1). 90 µl of the

resulting top aqueous layer were removed and placed into a fresh Eppendorf tube containing 45 µl ammonium acetate (7.5 M) and 100% ethanol (200 µl), vortexed, and placed at -20°C overnight. After centrifugation for 20 minutes the pellet was washed with 95% ethanol (1 ml), vacuum-dried briefly, and resuspended in 20 µl TE buffer and stored at -20°C.

2.8.3 Annealing of Primer to DNA Template and Sequencing Reactions.

Templates for sequencing first required annealing to the appropriate primer.

The following were mixed in a 0.75 ml Eppendorf tube:

Template DNA	3 µl
5x sequencing buffer	2 µl
M13 17 base Universal Primer	2 µl
SDW	3 µl

5x sequencing buffer:
Tris-HCl (200 mM) at pH 7.5
$MgCl_2$ (50 mM)
NaCl (250 mM)

The tube was briefly centrifuged and incubated at 55°C in an oven for 1 hour, and then briefly recentrifuged to ensure recovery of the contents to the bottom of the tube, ready for the sequencing reaction.

For each template, the following were added to the sides of the Eppendorf tube:

0.1 M DTT	1 µl
Extension Mix (7.5 mM dCTP, 7.5 mM dTTP, 7.5 mM dGTP) (diluted 5-fold prior to use)	2 µl
[alpha-^{35}S] dATP (Amersham code SJ1304)	0.8 µl
Klenow polymerase (BRL)	2.2 µl (=2 units)

The tube was spun briefly and left at room temperature for 5 minutes. 3.95 µl of each reaction mixture were dispensed onto the side of four wells of a microtitre plate. Each well contained 2 µl of one of the four different termination mixes (see below). The microtitre plate was spun for 30 seconds in an MSE Mistral 2000, and placed at 37°C for 17 minutes, then -20°C until use.

3 µl of formamide dye mix (see below) was added to each sample prior to use, incubated in an oven at 80°C for 15 minutes, and then loaded directly into the wells of a 6% (w/v) denaturing polyacrylamide sequencing gel.

Termination mixes (µM)

	T	C	G	A
dATP	250	250	250	25
dCTP	250	25	250	250
dGTP	250	250	25	250
dTTP	25	250	250	250
ddATP	-	-	-	300
ddCTP	-	100	-	-
ddGTP	-	-	150	-
ddTTP	500	-	-	-

Formamide Dye Mix:

Xylene cyanol	0.1 g	
Bromophenol blue	0.1 g	Made up to 100 ml
EDTA (0.5 M) at pH 8.0	2 ml	with deionised water.

2.8.4 Denaturing Polyacrylamide Sequencing Gels.

Base Runner Nucleic Acid Sequencer (IBI, Cambridge, UK) 60 cm vertical gel apparatus with wedged spacers was used, connected to an MBP 300E power supply (IBI). A 6% (w/v) polyacrylamide tris-borate-urea sequencing gel (made according to IBI instructions) was run at 55 watts typically for 3-7 hours. The use of tapered spacers (wedge) improved overall resolution and allowed more nucleotides to be read from a single loading (Mead & Kemper, 1986). The gel was then fixed with 10% (v/v) acetic acid and 10% (v/v) methanol for 30 minutes and dried (80^{o}C for 2 hours under vacuum). The gel was finally exposed to X-ray film at room temperature, typically overnight.

2.8.5 Sequence Analysis.

All DNA sequence derived from this work was recorded and analysed using the Microgenie sequence analysis program of Queen and Korn (1984), and an IBM PCat computer. Inverted repeats in the DNA sequence were identified and the hydrophobicity of proteins analysed (Kyte & Doolittle, 1982) using the Microgenie program.

The putative proteins encoded by open reading frames identified in the DNA sequence were screened using the NEWSWEEP program, against the OWL 10 database (SERC facility, Daresbury, Warrington) with the help of Dr. A. Morby.

A dendrogram detailing the phylogenic relationships of some proteins was constructed using the ROOT 66 program (J. Parkhill & D. A. Rouch unpublished, Birmingham University Computer Centre) with the help of

Dr. A. Morby.

Proteins were aligned for comparison using the CLUSTAL program (Higgins & Sharp, 1988) with the help of Dr. A. Easton.

2.8.6 Sequencing With Sequenase.

Regions of secondary structure in the DNA product of sequencing reactions resulted in abnormal banding on sequencing gels. The substitution of dITP (a nucleotide analogue) for dGTP eliminated these gel artefacts (Barns et al., 1983; Mizusawa et al., 1986).

The materials and methods of a Sequenase Version 2.0 Kit (United States Biochemical, Ohio, USA) were used to determine the DNA sequence of such regions.

2.8.7 Synthesis of Oligonucleotide Primers.

DNA primers for DNA sequencing were made by J. de Belin using solid phase oligonucleotide synthesis and an Applied Biosystems Model 380B DNA Synthesizer.

The concentration of oligonucleotide was measured spectrophotometrically. An absorbance at 260 nm of 1.0 was taken to represent a concentration of 20 µg/ml DNA (Maniatis et al., 1982). Samples were diluted in TE buffer and stored frozen at -20°C.

2.9 DNA Directed in vitro Transcription and Translation.

A prokaryotic DNA directed in vitro transcription and translation kit (N.380 Amersham) was used according to the manufacturers recommendations using ^{35}S-methionine as the labelled amino acid. This system is based on the method of Zubay (1973; see also Pratt, 1984).

For the in vitro transcription and translation of covalently closed circular plasmid or linear DNA, 3 µg or 6 µg of DNA were used respectively.

Plasmid encoded proteins were identified by fluorography of electrophoresed samples on 12% (w/v) SDS-polyacrylamide gels (Section 2.9.1).

L-[^{35}S]-methionine incorporation was measured using the TCA precipitation method described by the manufacturers of the kit.

2.9.1 SDS-Polyacrylamide Gel Electrophoresis.

The method was based on that of Laemmli (1970). A 10 or 12% (w/v) SDS-polyacrylamide gel with 5% (w/v) stacking gel was used in a BRL vertical gel electrophoresis system (20 x 20 cm gel plates), and run at a constant current of 15 mA at 4°C.

For the analysis of *in vitro* transcription and translation products, samples were diluted 1:1 with loading buffer (see kit instructions), and heated at 100°C for 15 minutes. A volume containing 20,000 cpm was loaded onto gels. Following electrophoresis the gel was fluorographed. ^{14}C molecular weight markers were supplied by Amersham (CFA.626) and non-radioactive low molecular weight protein markers were supplied by Pharmacia.

2.9.2 Fluorography of Polyacrylamide Gels.

Gels were fixed in 200 ml of glacial acetic acid for 5 minutes, washed in PPO (40 g in 190 ml of glacial acetic acid) for 90 minutes, and finally washed in water for 30 minutes. Gels were vacuum-dried in a gel drier at 60°C for 2 hours. The dried gels were then autoradiographed.

2.10 Detection of Single-Stranded DNA in BC1 Plasmid Samples.

This method involved the digestion of single-stranded (ss) DNA with S1 nuclease and was used for the detection of ss plasmid replication intermediates (te Riele *et al.*, 1986a).

BC1 was grown in iron plus yeast extract medium until about 40% of

the iron had been oxidized. 50 ml of culture was sujected to a plasmid minipreparation as in Section 2.5.6, and the DNA was finally resuspended in 48 µl of TE buffer. RNase (2 µl of 20 mg/ml stock) was added and the sample incubated at 37°C for 10 minutes. To 25 µl of the sample on ice, 3 µl of 10x S1 nuclease buffer (0.5 M Na acetate, 3 M NaCl, 0.05 mM $ZnSO_4$, at pH 4.7), and 2 µl S1 nuclease (20,000 u/ml) were added. The sample was then incubated at 37°C for 10 minutes and then placed on ice.

A 0.8% (w/v) agarose gel containing ethidium bromide was loaded with the 30 µl of S1 nuclease digested sample and 25 µl of the non-digested sample. After electrophoresis, the gel was sujected to Southern blotting, and the resulting filter hybridized with a nick-translated plasmid probe.

Following autoradiography of the filter, ss plasmid was visualized as a fast migrating DNA species present only in undigested (S1 nuclease) samples.

Chapter 3.
Growth of the Moderate Thermophiles on Solid Media.

3.1. Introduction.

Basic studies of the growth of strains TH3, ALV and BC1 on solid media were undertaken in an attempt to improve colony formation. Previously this was regarded as troublesome, requiring two to three weeks for colonies to appear. Good growth on solid media was considered necessary for many experimental protocols and in particular for the screening of bacteria following transformation experiments in order to allow the selection of recombinant clones.

Some of the most important factors likely to affect growth of the moderate thermophiles on solid media are pH, salt concentration, inoculum preparation, nutrient availability, temperature, oxygen concentration and the type of solidifying agent (for reviews see Codner, 1969; Harrison, 1984; Meyrath & Suchanek, 1972). Many bacteria, including acidophiles and thermophiles, exhibit slow and often limited growth on solid media (Codner, 1969) and additional factors may play a role at high temperature and low pH in inhibiting or limiting growth on conventional agar media.

The moderately thermophilic bacteria used in these studies grow at an optimum pH of about 2.0 and optimum temperature of 45-50^{o}C. Agar hydrolyses at high temperature and low pH producing byproducts which are inhibitory or toxic to the growth of some organisms (Harrison, 1984). Often highly purified agars or agarose must be used at low concentrations. After exposure to high incubation temperature and low pH, agar-based media lose gel strength and exhibit syneresis, with the presence of significant amounts of surface water escaping from the gel (Lin & Casida, 1984). Alternative inert gelling agents to agar include gelatin (Codner, 1969), silica compounds, e.g. Ludox (Pramer, 1957; Kingsbury & Barghoorn, 1954), Gelrite (Kang et al., 1982) and pluronic polyol F127 (Gardener & Jones, 1984). The preparation of silica gel is

time consuming and prepared batches produce inconsistent results (Codner, 1969). Ludox however, is a colloidal silica solution containing 30-40% hydrated silica (Ludox HS-30 and HS-40 are available from Dupont, USA). In 1950, Taylor described the method for preparing Ludox from sodium silicate using a cation-exchange resin. The silica sol can be heat sterilized and converted to a gel by adjusting the pH and adding ions, e.g. sodium chloride.

Pramer (1957) investigated the influence of physical and chemical factors on the preparation of silica gels. In particular at low pH values, i.e. below pH 4, and with a silica content of 1.5% (w/v), the time required for gelation at 28°C was over 3 days. As the temperature of gelation was increased (at pH 6.0), the time required for gelation decreased but resulted in more syneresis.

Gelrite (Kang et al., 1982; Shungu et al., 1983) or Gellan Gum (Lin & Casida, 1984), is an agar-like polysaccharide produced by Pseudomonas species and is used for the cultivation of some thermophiles (Lindstrom & Sehlin, 1989; Deming & Baross, 1986). Gelrite forms a clear gel (with the aid of a cation such as magnesium or calcium) after heating and cooling and has a holding temperature of 60°C below which solidification occurs (Shungu et al., 1983).

Pluronic polyol F127 (Gardener & Jones, 1984; Ko & Van Gundy, 1988) is a co-polymer of polypropylene oxide and ethylene oxide. A stable semi-solid gel is formed at temperatures above 10°C, the exact temperature depending on the concentration of the polyol used.

Some bacteria produce compounds such as acid which may lead to inhibition of growth. On solid media when bacteria are sensitive to their own acid production, colonies may be very small or absent on a densely seeded plate (Kuenen & Tuovinen, 1981). Also, the success of colony formation can depend on the constitution of the medium and the nature of the inoculum. Among media constituents, the phosphate concentration was shown to be critical for the growth of T. ferrooxidans (Johnson et al., 1987). The formation of colonies from individual microorganisms present in samples enables microorganisms to be enumerated without resort to

tedious and often difficult microscopic observation. Also, the reaction of metabolic products and compounds may have either stimulatory or antagonistic effects which can be directly observed and measured on solid media.

Studies of the growth of the moderate thermophile strains was centred on strain TH3. This strain was more amenable to growth on solid media than the other moderate thermophile strains. Most of the results in this chapter will be discussed for TH3 but a comparison between results with this organism, strain ALV and strain BC1 will also be made at the end of the chapter.

3.2 Results.

For all experiments the inoculum consisted of mid-exponential growth phase cells. These cells were grown in iron/yeast extract/glucose Salts Medium (Fe/Ye/G) or yeast extract/glucose Salts Medium (Ye/G; Methods 2.2.2 & 2.2.3) as appropriate for the solid medium used, i.e. for heterotrophic growth the latter medium was used and for chemolithoheterotrophic growth the former. All plates were incubated at 45^{o}C in sealed sandwich boxes containing silica gel and observed over a 10-14 day period.

3.2.1 Investigation of the Optimum pH for Growth.

Salts Medium was adjusted to pH 1.8-3.2 (Methods 2.2.2) and solidified with 0.6% (w/v) agarose. Strain TH3 was used to inoculate streak plates containing Fe/Ye/G or Ye/G media. The results following a 10 day incubation are shown in Figures 3.1 and 3.2 and clearly indicate that for both types of media the optimum pH for growth of strain TH3 was pH 2.0. Outside the optimum pH, growth was restricted to areas of heavy inoculation and single colonies failed to appear. The 'pin-head' sized single colonies observed on the media at pH 2.0 failed to increase in

Figure 3.1 Heterotrophic Growth of Strain TH3 on Solid Medium at pH 1.8-2.8.

Strain TH3 was streaked onto solid medium containing Salts Medium (pH as indicated), agarose type II (0.6% [w/v]), yeast extract (0.025% [w/v]) and glucose (5 mM).

The figure indicates the amount of growth and occurrence of single colonies after 10 days incubation at 45°C.

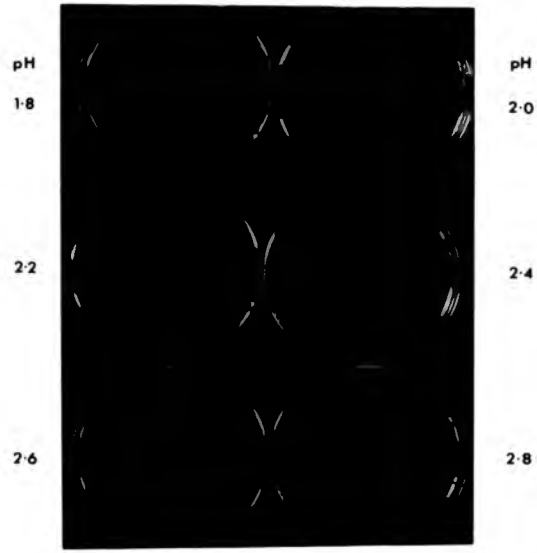
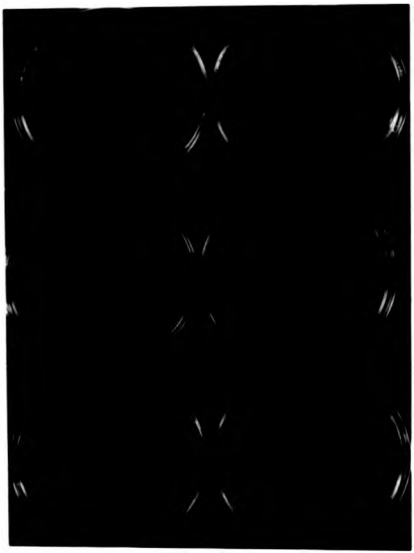

pH
1·8
pH
2·0
2·2
2·4
2·6
2·8

Figure 3.2 Chemolithoheterotrophic Growth of Strain TH3 on Solid Medium at pH 1.8-2.8.

Strain TH3 was streaked onto solid medium containing Salts Medium (pH as indicated), agarose type II (0.6% [w/v]), yeast extract (0.025% [w/v]), glucose (5 mM) and ferrous sulphate (10 mM).

The figure indicates the amount of growth and occurrence of single colonies after 10 days incubation at 45°C.

The ferric iron produced by the oxidation of ferrous iron by strain TH3 is clearly visible as a brown deposit around areas of bacterial growth.


pH
1·8
pH
2·0
2·2
2·4
2·6
2·8

size when the plates were incubated for longer periods of time. The pH of solid medium was also found to be critical for colony development of T. ferrooxidans (Tuovinen & Kelly, 1973; Johnson et al., 1987).

Ferrous iron (Fe^{2+}) was oxidized to ferric iron (Fe^{3+}) by strain TH3 and resulted in the depositition of hydrated ferric oxides around bacterial growth (Figure 3.2). pH and temperature also affected the rate of ferrous iron oxidation and darker brown colouration, indicating the presence of more ferric oxides, was seen at pH values above 2.2 (Figure 3.2).

3.2.2 Investigation of Solidifying Agents.

Different agars and solidifying agents were assessed in order to optimize growth of strain TH3. Solid medium was made as in Methods 2.2.2 using Salts Medium pH 2.0 and the following solidifying agents: Noble agar (0.6% [w/v]), Japanese agar (Davis Gelatin, no longer available, 0.6% [w/v]), Oxoid No. 1 agar (0.6% [w/v]), agarose type II (0.6% [w/v]), Ludox HS-40 (1.5% [w/v]) and pluronic polyol F127 (20% [w/v]). Streak plates were prepared using strain TH3.

Gelrite was not used as an alternative gelling agent as this has a holding temperature of 60°C (Shungu et al., 1983), which might inactivate moderate thermophiles in a pour plate technique (see Section 3.2.4).

Solidification of Ludox did not occur within one day, under the conditions employed (Methods 2.2.2). No growth was seen on media containing pluronic polyol F127, Noble and Japanese agars, during a two week period of observation. Of the remaining solidifying agents, comparable growth occurred on Oxoid No. 1 and agarose type II after a 10 day incubation period. However, the latter produced a firmer gel and streaking of the inoculum onto the medium surface was more easily accomplished.

Compounds toxic to some bacteria may be released by gelling agents during incubation at low pH (Kuenen & Tuovinen, 1981; Manning, 1975; Ko &

Van Gundy, 1988) and agarose type II was the gelling agent of choice following this experiment.

3.2.3 Comparing Different Salts Media.

Various salts based media were investigated in an attempt to optimize growth and particularly to enable the cultivation of single colonies of the moderate thermophiles.

The following media were prepared : Salts, Mannings, DI and Low Phosphate at pH 2.0 (Methods 2.2.2). These were supplemented with Fe/Ye/G or Ye/G (Methods 2.2.3) and 0.6% (w/v) agarose.

An appropriate culture of strain TH3 (see Section 3.2) was diluted in Salts Medium at pH 2.0, 10^{-1} to 10^{-6} and 100 µl of each dilution spread onto the agarose plates. The results of colony formation by strain TH3 are shown in Table 3.1. The growth of single colonies of strain TH3 on Fe/Ye/G medium was obtained using Mannings and DI salts whereas low phosphate medium was optimal for heterotrophic growth of single colonies. No single colonies were observed using the other types of salts media. DI and Mannings differ only in that the latter contains $Ca(NO_3)_2$ and the Low Phosphate Medium contains a concentration of phosphate one tenth that of Salts Medium (Methods 2.2.2).

Many of the single colonies failed to increase in size, remaining 'pin-head' size but colonies arising from the more dilute inoculum approximately doubled in size during further incubation. The time required for colony development was greater for heterotrophic growth (3 weeks) than for chemolithoheterotrophic growth (10 days).

3.2.4 Use of the Pour Plate Method.

The spread plate method was not an ideal method for inoculating solid media with the moderate thermophiles. Agarose at 0.6% (w/v) produced a fairly soft gel which was easily disturbed when spreading an inoculum over the surface. Previous results also indicated that the

Table 3.1. Growth of Strain TH3 Using Different Salts Media.

Fe/Ye/G Media.

Dilution	Mannings	D1	Salts	Low PO_4^-
10^{-1}	C	C	C	C
10^{-2}	C	C	C	C
10^{-3}	TNTC	TNTC	-	-
10^{-4}	TNTC	TNTC	-	-
10^{-5}	79	96	-	-
10^{-6}	4	-	-	-

Ye/G Media.

Dilution	Mannings	D1	Salts	Low PO_4^-
10^{-1}	-	-	C	C
10^{-2}	-	-	C	TNTC
10^{-3}	-	-	-	TNTC
10^{-4}	-	-	-	140
10^{-5}	-	-	-	-
10^{-6}	-	-	-	-

These results indicate the number of colonies produced by an inoculum of TH3 which was serially diluted 10^{-1} to 10^{-6}.

C = Confluent

TNTC = Too numerous to count

\- = No growth

The media consisted of different salt solutions (pH 2.0) as indicated. Low PO_4^- indicates Low Phosphate Medium. These solutions were supplemented with Fe/Ye/G or Ye/G and agarose (0.6% [w/v]). Plates were incubated at 45°C for 10 days (Fe/Ye/G) and 3 weeks (Ye/G).

number of single colonies produced following this method of inoculation were considerably less than the number of cells in the inoculum (as counted microscopically).

Preliminary experiments using D1 salts in Fe/Ye/G medium indicated that using the pour plate method of inoculation a medium pH of 2.6 produced optimal growth.

Pour plates were prepared (Methods 2.2.2) using 0.5% (w/v) agarose as the gelling agent and D1 salts pH 2.6 for Fe/Ye/G medium. The inoculum was diluted in D1 salts solution at pH 2.6 before addition to the molten medium (45°C).

The results of this experiment are shown in Figure 3.3A and 3.3B. Clear single colonies were observed at inoculum dilutions of 10^{-5} and 10^{-6} and several features of growth became apparent. 'Pin-prick' size colonies produced at a dilution of 10^{-3} remained this size, even after a longer incubation, and a ten-fold drop in dilution of the inoculum resulted in a greater than ten-fold drop in colony numbers, clearly visible in Figure 3.3B.

A variety of colonies were seen differing in colour, particularly at the lower dilutions, e.g. 10^{-5} (Figure 3.3B). This seemed to be due to the amount of ferrous iron oxidized and those cells on the suface of the medium were darker brown because the deposition of ferric oxides was accelerated due to exposure to air.

The size of the colonies produced appeared to reflect the distance between colonies. This may be due differences in substrate concentration in the medium as a result of colony growth, also, ferrous iron was oxidized to ferric iron by the bacteria and ferric iron inhibits this reaction probably resulting in small colonies.

3.2.5 Investigation of Microaerophily.

Test tubes containing sloppy medium inoculated throughout with bacteria are often used to investigate the oxygen tolerance of bacteria

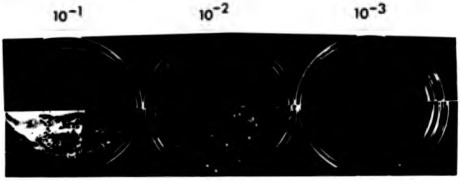
Figure 3.3 Pour Plates and Sloppy Agarose Tubes of Strain TH3.

A) and B) Chemolithoheterotrophic growth produced by strain TH3 in pour plates. The inoculum for these plates was grown in liquid Salts Medium pH 1.7 supplemented with Fe/Ye/G (50 mM/ 0.025% [w/v]/ 5 mM) to mid-exponential growth phase, and then diluted 10^{-1} to 10^{-6} as indicated in the figures. The inoculum was added to the molten medium (45°C) prior to pouring. The solid medium was prepared as in Methods 2.2.2. Plates were incubated at 45°C for 10 days.

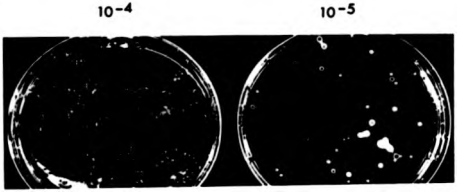
The colonies in the 10^{-5} dilution in B) exhibit different amounts of ferric oxide deposition depending on their depth in the medium i.e. those at the surface of the medium are darker brown due to ferric iron oxidation enhanced by exposure to air.

C) Bands of growth (indicated by arrows) of strain TH3 in sloppy agarose (0.3% [w/v]) tubes. The medium was made as in Methods 2.2.2 and contained Ye/G (1) or Fe/Ye/G (2) and was inoculated throughout. Tubes were incubated at 45°C for 3 days.

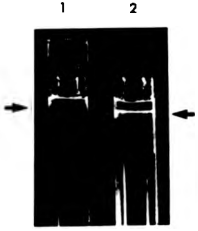
A)



B)



C)



(Krieg & Hoffman, 1986). Aerobic bacteria grown in this type of medium are usually most visible towards the top of the tube. Anaerobic bacteria however, grow at a lower position because growth is inhibited by oxygen or its derivatives e.g. $H_2O_2^-$ and O_2^-. Microaerophilic bacteria (for review see Krieg & Hoffman, 1986) may use oxygen during respiration but the concentration of oxygen in the air (21% [v/v]) may also be deleterious to growth. As a result, these bacteria produce a band of growth in sloppy medium and at this position in the medium, the concentration of oxygen is optimal for growth. Preliminary investigations revealed that bands of growth were produced by strain TH3 after only 3 days incubation (Figure 3.3C).

A batch of sloppy Salts Medium was prepared (Fe/Ye/G; Methods 2.2.2) containing 0.3% (w/v) agarose and inoculated with strains TH3, ALV or BC1 before pouring into test tubes fitted with loose tops. When the medium had set the tubes were incubated at 45°C.

After a three day incubation, bands of bacterial growth were clearly visible in all tubes. The position of the band of growth was measured from the meniscus of the medium to the band. Strain TH3 produced a band of growth at 7 mm down, strain ALV at 5 mm and strain BC1 at 12 mm down. This distance depended on the amount of air in the medium which varied with different batches of media. The bacteria investigated are clearly microaerophilic and each strain had a different tolerance to oxygen.

The microaerophilic growth of strain TH3 was investigated using sloppy agarose tubes containing Fe/Ye/G Salts Medium (pH 2.0) and Ye/G Salts medium (pH 2.0; Figure 3.3C). The position of the band of bacterial growth was lower in medium containing iron where the mode of nutrition of the bacteria was also different i.e. chemolithoheterotrophic as opposed to heterotrophic. This suggests that either the oxygen tolerance of the bacteria depends on their mode of nutrition or that Fe^{2+} in the medium affected the oxygen availability or degree of toxicity.

In a further experiment using strain TH3 the air space at the top of some of the tubes was flushed with oxygen, nitrogen or treated as indicated in Table 3.2. The results (Table 3.2) confirmed that strain TH3

Table 3.2 Microaerophilic Growth of Strain TH3.

Treatment of Tube	Medium and depth (mm). of band of growth.	
	Fe/Ye/G	Ye/G
Tube with loose top (21% [v/v] oxygen)	4	1
Tightly sealed tube	4	1
Tube flushed with 100% (v/v) N_2	-	-
Tube flushed with 100% (v/v) O_2	31	29
Level of O_2 in air space adjusted to 2.5% (v/v)	1	0

Sloppy agarose (0.3% [w/v]) tubes inoculated throughout with strain TH3 were subjected to treatments as described in the table. A 2.5% (v/v) concentration of oxygen was achieved by flushing a suba sealed tube with 100% (v/v) nitrogen and then adding a specific quantity of 100% (v/v) oxygen determined by calculating the volume of the air space at the top of the tubes.

The figures indicate the distance between the band of growth and the meniscus of the media after 3 days incubation at 45°C. - indicates no growth. Air contains 21% (v/v) oxygen (Krieg & Hoffman, 1986). The medium used contained Salts Medium pH 2.0 supplemented as indicated (Methods 2.2.2).

is a microaerophilic bacterium. When the bacteria were subjected to an anaerobic environment (100% [v/v] N_2), no growth occurred. When the concentration of oxygen in the air space at the top of the tubes was increased (above that in air) to 100% (v/v) O_2, the band of bacterial growth developed at a lower position, presumably reflecting a greater concentration gradient of oxygen through the medium. Finally, when the oxygen concentration in the tube was reduced from that found in air to 2.5% (v/v), the band of bacterial growth occurred at a higher position in the tube and the bands of bacterial growth were at or just below the media surfaces. It is therefore probable that the bacteria are tolerant to about 2.5% (v/v) oxygen when grown heterotrophically and slightly less than this concentration when grown chemolithoheterotrophically. Thus, the position of bands of growth depended on the concentration of oxygen and also the medium used.

3.2.6 A Comparison of the Growth of Strain TH3 with Strain ALV and Strain BC1.

Most of the results discussed so far have centred around the moderate thermophile strain TH3. This bacterium grew better than strain BC1 in all experiments on media containing iron. Strain BC1 grew poorly, often requiring a longer incubation time, producing most growth where heavy inocula were used and this bacterium was the most sensitive to oxygen or its derivatives as determined using sloppy agarose tubes (Section 3.2.5).

Single colonies of strain ALV were produced by chemolithoheterotrophic growth and these usually grew to a larger size than those of strain TH3. Strain ALV on the whole required a slightly shorter incubation time (about one week) than strain TH3 and was the least sensitive to oxygen or its derivatives in sloppy agarose tubes.

Heterotrophic growth of strains ALV and TH3 on solid media was poor and occurred only where heavy inocula were used. Heterotrophic growth of strain BC1 on/in solid media was never observed.

3.3 Discussion.

The difficulty in achieving growth of the moderate thermophile strains on solid media appeared to result from a complex combination of factors comprising:

A) The possible production of substances by the cells.
B) The aerotolerance of the cells.
C) The requirement for acidic conditions.
D) The requirement for a high temperature.
E) The inhibition of ferrous iron oxidation by ferric iron.

A limited amount of work was done to try and improve growth on solid media. Improved growth, particularly of single colonies, was achieved by using pour plates, Dl salts medium for chemolithoheterotrophic growth, low phosphate media for heterotrophic growth and a low concentration of agarose as the solidifying agent. Johnson et al. (1987) also noted that agarose was the preferred gelling agent for the iron-oxidizing acidophile T. ferrooxidans.

Kuanen & Tuovinen (1981) noted that Thiomicrospira sp. were sensitive to their own acid production, colonies only developing with a more dilute inoculum and some workers have noted a similar effect during the growth of extremophiles or that growth of bacteria depends on a heavy inoculum to establish growth and single colonies may not appear (Harrison, 1984). Lin & Casida (1984) noted that B. stearothermophilus seemed to require a threshold number of colony forming units on plates if growth was to ensue.

The growth of the moderate thermophiles as single colonies did occur but the results imply that a very specific set of growth conditions must be met for success. It is highly likely that during the incubation period conditions change, resulting in reduced growth rates or lysis of the cells. Small single colonies produced were examined microscopically and the cells had lysed.

The fact that the moderate thermophiles were originally purified via isolation of single colonies but have since been grown in liquid media

might suggest that they have adapted to the requirements for growth in liquid media, particularly on ferrous iron, and have lost the ability to establish or maintain growth outside 'laboratory' optimal conditions. The adaptation of bacteria to growth under specific conditions is common and 'laboratory strains' occur. All of the moderate thermophile strains have been maintained in liquid media for several years. Mishra et al. (1983) suggested that preadaptation of T. ferrooxidans might be required for the growth of this bacterium on solid media and, some workers have obtained cultures which when maintained on solid media exhibit improved growth following several subcultures.

A low concentration of phosphate is an important factor for heterotrophic growth of strain TH3. Johnson et al. (1987) also found that the concentration of potassium phosphate was critical for the growth of T. ferrooxidans on solid media. High phosphate levels have been implicated in the decreased aerotolerance of Spirillum volutans and Azospirillum brasilense (Krieg & Hoffman, 1986).

Some of the moderate thermophile strains are microaerophilic and it appears this property may result from the effect of toxic forms of oxygen on the metabolism of the bacteria. The position of bands of growth of strain TH3 in sloppy agarose tubes depended on the concentration of oxygen and also the medium used. The media differed only in ferrous iron concentration. Culture media exposed to illumination contain higher levels of toxic forms of oxygen (e.g. H_2O_2 and O_2^-) than similar media incubated in the dark. Toxic forms of oxygen have also been indirectly detected in non-illuminated culture media where enzymes such as catalase were found to increase the aerotolerance of bacteria (see Krieg & Hoffman, 1986). Ferric and ferrous iron have both been found to quench toxic forms of oxygen and increase the aerotolerance of Campylobacter sp. (Bowdre et al., 1976). However in this work, bacteria in the medium containing iron exhibited a lower aerotolerance and both types of media were incubated in the dark.

It seems likely that the differences in aerotolerance are due to the mode of nutrition of strain TH3 and toxic forms of oxygen perhaps

affecting the respiratory chain, particularly oxidases and cytochromes required for the oxidation of ferrous iron. Components of bacterial respiratory chains are affected by oxygen and are often oxygen labile (see Krieg & Hoffman, 1986). Alternatively, toxic forms of oxygen may be generated by the bacteria particularly during chemolithoheterotrophic growth.

Conditions for the growth of the moderate thermophile strains on solid media are much more exacting than the conditions required for many other bacteria. Outside this narrow set of conditions, growth only appeared where a heavy inoculum was used and to produce single colonies a more restricted set of conditions applied and the concentration of oxygen was probably an important factor. Jannasch (1977), while investigating marine bacteria found that populations of high density bacteria were able to overcome the toxicity of oxygenated media, whereas populations of low density were unable to do so unless aided by artificial means such as addition of 50% (v/v) nitrogen to the aeration gas. Other workers have noted that it is more difficult to grow colonies of microaerophiles from individual cells on the surface of solid media than it is to grow them in liquid media from an inoculum consisting of many cells (Padgett et al., 1982, George et al., 1978).

Overall, single colonies of strains ALV, TH3 and BC1 were produced on media containing ferrous iron, the growth of the latter strain was poor. The number of cells in cultures of the moderate thermophiles cannot be accurately enumerated by growth on agarose media because only a proportion of cells appears to initiate and/or sustain growth producing colonies of a visible size. Culture conditions need to be further refined for growth on solid media and important parameters probably include those already investigated.

Chapter 4.
Growth of the Moderate Thermophiles in the Presence of Antibiotics and Metals.

4.1 Introduction.

Antibiotic and metal sensitivity testing frequently employs the use of disc diffusion methods, providing a means of testing many bacteria and many antimicrobial substances. During the incubation of assay plates the test substance may diffuse out into the surrounding agar producing a concentration gradient, and zones of growth inhibition occur around those discs which contain substances to which the organism is sensitive. The size of the zone of growth inhibition and test result can be influenced by many factors such as:

A) The growth phase of the organism.
B) The composition of the solid medium.
C) The incubation conditions.
D) The incubation time.
E) The quantity of solid medium.
F) The diffusion of the substance.

Most of these factors can be standardized during tests, so that comparable results are obtained. Inocula for pour plates of moderate thermophile strains TH3, ALV and BC1 were prepared from exponentially growing cultures and the solid medium consisted of D1 salts at pH 2.6 and agarose type II (0.5% [w/v]), supplemented with Fe/Ye/G (Methods 2.2.2). 20 ml of medium per plate were used and the plates were allowed to set on a level surface. The components of this medium would probably affect some of the test substances or affect their activity e.g. the activity of tetracycline is affected by Mg^{2+} and acidic conditions (Maniatis et al., 1982). Yeast extract contains organic substances likely to alter the activity of antibiotics. Therefore, the results obtained have only a limited use, but can be used to compare the three moderate thermophile

strains and form the basis for further genetic studies.

Growth of one particular strain in the presence of a substance did not imply that the bacterium was resistant to that substance because the compound might have been inactivated by, for example, the low pH. However, it might imply that the strain was more resistant than the other moderate thermophile strains. Thus, the results are only comparable between the strains and under the conditions employed.

The incubation time for each strain was slightly different depending on the organisms rate of growth on the solid medium. The results given were recorded as soon as visible zones of growth inhibition occurred and if a substance lost the ability to inhibit growth, bacteria were not allowed the time to grow within the original zone of growth inhibition.

The diffusion of the substances used in the experiments is perhaps the most difficult parameter to standardize. The rate of diffusion of a substance in this type of test is influenced by many factors including, concentration, molecule sizes, surface tension, temperature and molecular interactions. The low concentration of agarose used in the medium was likely to produce less 'resistance' to the diffusion of molecules than the concentrations of agars normally employed in bacterial media. Subsequently, large zones of growth inhibition were observed in some cases. The higher temperature required for growth of the moderate thermophile strains would inactivate some substances and also increase the rate of diffusion.

The surface tension of the medium is important particularly when pour plates are used, because a substance will probably diffuse at a faster rate over the surface of the medium than the rate at which it diffuses through the medium. Cells on the surface of the medium would come into contact with the substance earlier than those cells embedded in the medium and this can be a cause of 'secondary' zones of growth inhibition.

During many of the disc assays, two zones of growth inhibition occurred. Primary zones (where no growth occurred) and secondary zones (where the amount of growth or iron oxidation which occurred, was less

than that seen over the periphery of the plate).

Secondary zones are commonly seen when bacteria have inducible resistance to an antibiotic, e.g. S. aureus and penicillinase production. Cells close to the source of the antibiotic are exposed immediately to high concentrations of antibiotic and killed before adequate amounts of penicillinase can be synthesized. Cells at the periphery of the zone are exposed to gradually increasing concentrations of the outwardly-diffusing antibiotic; such cells are able to synthesize adequate amounts of penicillinase and give rise to full-sized colonies.

Although disc assays of bacteria are quick and easy to do experimentally, the results may be very hard to interpret. As discussed, disc assays of the moderate thermophiles are complicated by factors not normally arising in this type of experiment and one might assume that little useful information would be obtained. However, these experiments provided information about the comparative responses of the three strains used and the stability of substances under the conditions employed.

Future work during the development of genetic systems for the moderate thermophiles will involve targeting substances, particularly antibiotics, for which reporter genes are available and these reporter genes can be used for the construction of recombinant vectors. The expression of such genes could be assessed using the disc assay technique.

Conventionally the diameter of zones of growth inhibition are measured, or the radii are measured from the centre of the disc. Due to the occurrence of many secondary zones of growth inhibition a clearer picture was given graphically if the zones were measured as follows: the primary zone of growth inhibition was measured from the edge of the disc to the circumference of the zone. The secondary zone of growth inhibition was measured from the circumference of the primary zone to the edge of the secondary zone. When a secondary zone occurred without a primary zone, this was measured from the edge of the disc to the circumference of the secondary zone.

4.1.1 Antibiotics.

Antibiotics are usually characterized as bacteriostatic or bactericidal. The former inhibit growth or replication of bacteria but do not kill the cells whilst the latter kill or lyse the bacteria. The division, however, is not clear cut, as bacteriostatic agents may be bactericidal at higher concentrations and vice versa. Bacteriostatic substances which are inactivated over a period of time can also produce secondary zones of growth inhibition.

Tetracycline and chloramphenicol have been used to select for Acidiphilium sp. transformants (at pH 3.5) following electroporation (Roberto et al., 1989) and kanamycin was used in a similar experiment at a concentration of 1 mg/ml (F. F. Roberto, pers. comm.).

Antibiotics inhibit cell wall formation, disrupt cytoplasmic membrane function, prevent DNA synthesis, interfere with protein synthesis, and halt folate synthesis (Hammond & Lambert, 1978). Resistance to antibiotics is a result of three major mechanisms: prevention of the antibacterial agent from reaching its receptor site, production of altered targets, and destruction or modification of the agents (for review see Neu, 1989).

The antibiotics used during the course of this work are detailed in Table 4.1 and an indication of the stability of these antibiotics at pH 2.0 and 45°C is given in Table 4.2.

4.1.2 Metals.

Although trace amounts of certain metal ions are essential for the growth of most microorganisms, higher concentrations may exhibit antimicrobial activity and in general, microorganisms are sensitive to high concentrations of heavy metal salts. It has also been recognized that microorganisms can develop resistance against high concentrations of many metal ions when adapted in the presence of increasingly higher concentrations. In addition, organisms can also be isolated that exhibit

Table 4.1 Characteristics of Antibiotics.

The characteristics of the antibiotics used in the antibiotic disc assays are detailed.

The classes of the antibiotics are as follows:

A = Aminoglycoside
Pm = Polymyxin
P = Penicillin
C = Cephalosporin
C* = Structurally related to C
S = Sulphonamide
T = Tetracycline
M = Macrolide
SC = Substituted coumarin
L = Lincomycin

Antibiotics differ in their spectrum of activity. Activity against Gram positive bacteria (G+) or Gram negative bacteria (G-) is indicated, where one of these is underlined this indicates that the antibiotic is more active against this type of bacterium.

Antibiotic action may be bacteriostatic ('static) or bactericidal ('cidal).
(1) Erythromycin has bacteriostatic activity at low concentration and bactericidal activity at high concentration.

The sites of action of the antibiotics in the bacterial cell, or the metabolic pathways affected, are indicated.

Antibiotic	Class	Range of Activity	Effect	Site of Action
Amikacin	A	G+/G-	'cidal	Protein Synthesis
Ampicillin	P	G+/G-	'cidal	Peptidoglycan Synthesis
Cephalexin	C		'cidal	"
Cephaloridine	C	G+/G-	'cidal	"
Chloramphenicol		G+/G-	'static	Protein Synthesis
Clindamycin	L	G+	'cidal	"
Cloxacillin	P	G+/G-	'cidal	Peptidoglycan Synthesis
Colistin sulphate	Pm	G-	'cidal	Cell Membrane
Erythromycin	M	G+/G-	(1)	Protein Synthesis
Fusidic acid	C*	G+	'cidal	"
Gentamicin	A	G+/G-	'cidal	"
Kanamycin	A	G+/G-	'cidal	Cell Membrane & Protein Synthesis
Lincomycin	L	G+	'cidal	Protein Synthesis
Methicillin	P	G+/G-	'cidal	Peptidoglycan Synthesis
Mezlocillin	P	G+/G-	'cidal	"
Novobiocin	SC	G+/G-	'static	Cell wall & Protein Synthesis
Penicillin G	P	G+/G-	'cidal	Peptidoglycan Synthesis
Streptomycin	A	G+/G-	'cidal	Protein Synthesis
Sulphamethoxazole	S	G+/G-	'static	Folic Acid Metabolism
Sulphatriad	S	G+/G-	'static	"
Tetracycline	T	G+/G-	'static	Protein Synthesis
Ticarcillin	P	G+/G-	'cidal	Peptidoglycan Synthesis
Tobramycin	A	G+/G-	'cidal	Protein Synthesis
Trimethroprim	S	G+/G-	'static	Folic acid Metabolism

Table 4.2 The Stability of Antibiotics

Name/Type of Antibiotic	Heat	Acid
Aminoglycoside	+	+
Penicillin	-	-
Cephalosporin	-	-
Chloramphenicol	+	+
Fusidic Acid	-	-
Sulphonamide	+	+
Tetracycline	+	+
Macrolide	+	-
Lincomycin	+	-

The table indicates the relative stabilities of some antibiotics when exposed to heat or acid conditions, as used in the disc assay experiments of the moderate thermophile strains. Data for this Table was kindly provided by Mr D. Eccleston, Mast Laboratories, Liverpool.

Notes:

The relative stabilities of the antibiotics are indicated as a + sign, and instability as - sign.

high levels of resistance against some metal ions in their natural state. Such resistant bacteria often harbour plasmids that specify the resistance (see Silver et al., 1989; Silver & Misra, 1988; Hughes & Poole, 1989).

The toxicity of a particular metal for a bacterium can depend on the growth substrate (e.g. Fe^{2+}), the growth medium and the design of the toxicity assay. The toxicity of metal ions towards T. ferrooxidans can be described in a series beginning with the most toxic (Norris, 1989):

silver > mercury > uranium > thallium > copper, cobalt, zinc
gold thorium nickel, cadmium

The binding of metals to organic materials, precipitation, complexation and ionic interactions are all important phenomena that must be considered carefully in laboratory studies. For example, the toxic effects of copper on Aerobacter aerogenes were prevented by the addition of yeast extract to the assay medium and the toxic effects of mercury for the protozoan Tetrahymena pyriformis were 40 times higher in a complex medium as opposed to a simple medium (see Gadd & Griffiths, 1978; Hughes & Poole, 1989). pH can have a considerable affect on the availability and toxicity of metals. In general, at an acid pH, metals are more likely to exist as free cations and anions are able to reduce metal toxicity by precipitation e.g. as phosphates and carbonates.

There have been very few studies of the effect of toxic metals on the moderate thermophile strains. Strain ALV and strain TH3 were more and less sensitive respectively than strain BC1 to inhibition by copper and, strain TH3 was more resistant than strain TH1 to uranium (Norris, 1989).

Norris et al. (1986b) investigated the effects of some metals on iron-oxidizing bacteria. Autotrophic exponentially-growing cells were moderately inhibited by the indicated concentrations of different metals (Table 4.3).

Table 4.3 The Concentrations of Some Metals that Gave Moderate Inhibition of Growing, Iron-oxidizing Acidophilic Bacteria.

Organism	Metal and concentration (mM)			(μM)	
	U	Cu	Mo	Ag	Hg
T. ferrooxidans	0.5	100	0.25	0.5	0.25
L. ferrooxidans	2.5	1	1	5	0.25
BC1	2.5	50	0.5	1	2.5
Sulfolobus (BC)	2.5	75	1	0.1	5

The metal compounds used were uranium sulphate, copper sulphate, sodium molybdate, silver nitrate and marcuric chloride. Data taken from Norris et al. (1986b) and Norris (1989). The charge of ions is not given because several alternatives may occur in the medium.

4.2 Results.

4.2.1 Antibiotic Disc Assays.

Zones of growth inhibition of strains TH3, ALV and BC1 were clearly observed for many of the antibiotics tested (examples are shown in Figure 4.1). For most antibiotics a secondary zone was also measured and the results are displayed graphically in Figure 4.2, where the sizes of primary and secondary zones can be compared directly.

The following antibiotics produced no zones of growth inhibition for all three strains of bacteria: Lincomycin (2 µg), Penicillin G (1 unit) and Clindamycin (2 µg). The aminoglycoside antibiotics produced relatively small zones of growth inhibition with all three bacterial strains and this was probably due to the instability of these antibiotics under the conditions employed. Rawlings et al. (1983) suggested that streptomycin, tobramycin and gentamicin were all inactivated in low pH media containing iron.

The largest zones of growth inhibition, in particular primary zones equal to or greater than 25 mm, were produced by cephalexin, fusidic acid and chloramphenicol. This implied that these antibiotics might be more stable, that they diffused further from the disc, or that the concentrations of these antibiotics were substantially higher. Antibiotics diffuse through agar gels at different rates, e.g. Penicillin G and chloramphenicol diffuse rapidly while polymyxin diffuses slowly, so that the zone sizes produced when testing different substances are not directly comparable and it cannot be assumed that one organism is more susceptible to one agent than to a different agent on the basis of a larger zone size (Hammond & Lambert, 1978).

Since many antibiotics were tested, an average or general assumption about the overall sensitivity or resistance of the bacteria can be made. Overall, BC1 appeared to be the most sensitive to the antibiotics used and strain TH3 the least sensitive. Particularly large secondary zones of growth inhibition occurred for strain TH3 in the presence of erythromycin

Figure 4.1 Antibiotic Disc Diffusion Assays.

The figure illustrates the primary (total inhibition) and secondary (partial inhibition) zones of growth inhibition produced by strains TH3 (A) and ALV (B), when exposed to paper discs impregnated with antibiotics:

(A) Fusidic Acid (10 mg; Fc)

(B) Streptomycin (10 mg; S) and chloramphenicol (25 mg; C).

A

B

Figure 4.2 Zones of Growth Inhibition of Strains TH3, ALV, and BC1 in Antibiotic Disc Diffusion Assays.

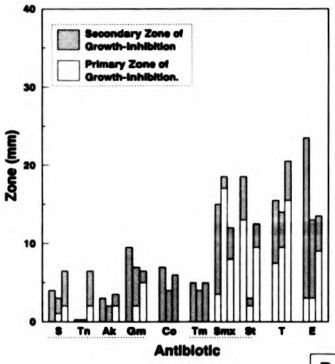
The graph illustates the sizes of zones of growth inhibition (mm) produced by antibiotic discs (Mast Laboratories, Liverpool).

Primary zones of growth inhibition (empty bar) were measured from the edge of the disc to the periphery of the zone. Secondary zones of growth inhibition (shaded bar) were measured from the edge of the primary zone to the periphery of the secondary zone.

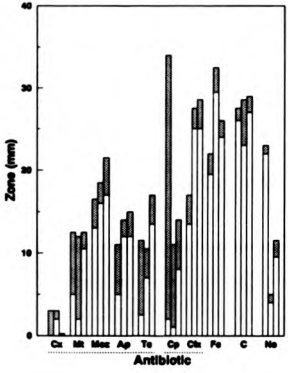
For each antibiotic, three bars are shown; the first represents strain TH3 after 4 days incubation, the second strain ALV after 5 days incubation and the third strain BC1 after 9 days incubation at 45°C. Antibiotics of the same class are indicated by dotted lines. The antibiotics used were as follows:

Antibiotic	Code	µg on disc
Amikacin	Ak	30
Ampicillin	Ap	10
Cephalexin	Cfx	30
Cephaloridine	Cp	5
Chloramphenicol	C	25
Cloxacillin	Cx	5
Colistin sulphate	Co	25
Erythromycin	E	5
Fusidic acid	Fc	10
Gentamicin	Gm	10
Methicillin	Mt	10
Mezlocillin	Mez	30
Novobiocin	No	5
Streptomycin	S	10
Sulphamethoxazole	Smx	25
Sulphatriad	St	200
Tetracycline	T	10
Ticarcillin	Tc	75
Tobramycin	Tn	10
Trimethroprim	Tm	1.25

Clindamycin (2 µg), Lincomycin (2 µg) and Penicillin G (1 unit) did not inhibit the growth of strains TH3, ALV, or BC1.



Bar 1 = Strain TH3
Bar 2 = Strain ALV
Bar 3 = Strain BC1



and cephaloridine.

In an experiment using similar conditions, Rawlings et al. (1983) studied the antibiotic sensitivity profile of T. ferrooxidans (grown at about pH 2.0 and 30°C on a medium containing Fe^{2+}). All strains tested were sensitive to rifampicin (12.5 μg/ml), chloramphenicol (75 μg/ml), cephaloridine (75 μg/ml), and ampicillin (75 μg/ml), but resistant to 100 μg/ml of gentamicin, kanamycin, streptomycin, tetracycline, vancomycin, tobramycin and erythromycin. The apparent resistance of T. ferrooxidans to the antibiotics was in all cases shown to be due to the instability of the antibiotics in the low pH and high Fe^{2+} medium.

In summary, chloramphenicol was targeted as a useful antibiotic which appeared relatively stable at low pH and high temperature.

4.2.2 The Effect of Chloramphenicol on Strains ALV and BC1.

Chloramphenicol was chosen as an antibiotic for use as a potential marker during gene transfer experiments of the moderate thermophile strains. A further consideration was the availability of a chloramphenicol resistance reporter gene. Several inducible chloramphenicol acetyltransferase (cat) genes are available which are derived from Gram positive bacterial sources. Strain BC1 contained a small plasmid which was investigated as a potential cloning vector (Results Chapter 6).

Strain BC1 was shown to grow poorly on solid media (Results Chapter 3) and strain ALV produced better growth. Strain ALV is closely related phylogenetically to strain BC1 (Lane & Harrison, 1989) and both strains were selected as potential hosts for recombinant vectors and their sensitivity to chloramphenicol (Cm) studied.

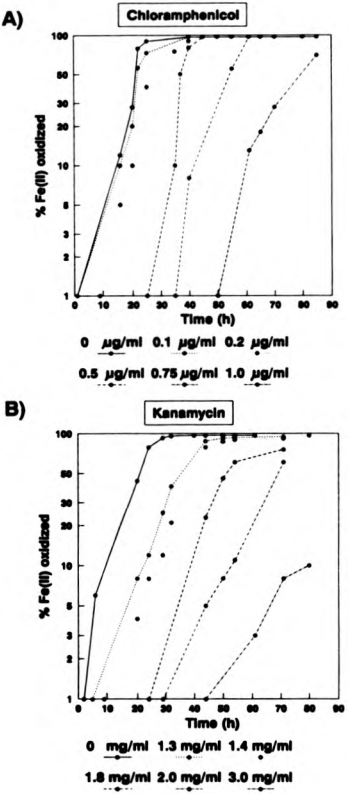
Various concentrations of chloramphenicol were added to Salts Medium at pH 1.7 and supplemented with Fe/Ye/G (Methods 2.2.2 & 2.2.3). Flasks were inoculated with an exponentially growing culture of strain ALV and incubated at 45°C. Growth was monitored by assaying iron oxidation (Methods 2.3.4; Figure 4.3A).

Figure 4.3 The Effect of Chloramphenicol and Kanamycin on Strain ALV.

The graphs show the growth of strain ALV in liquid medium containing various concentrations of:

(A) Chloramphenicol
(B) Kanamycin

A control flask which did not contain antibiotic is shown for comparison in each graph. Exponential growth phase cultures were used to inoculate the flasks containing antibiotic and medium for chemolithoheterotrophic growth and the growth of strain ALV was monitored as ferrous iron oxidized.


A)
Chloramphenicol
% Fe(II) oxidized
Time (h)
0 µg/ml 0.1 µg/ml 0.2 µg/ml
0.5 µg/ml 0.75 µg/ml 1.0 µg/ml
B)
Kanamycin
% Fe(II) oxidized
Time (h)
0 mg/ml 1.3 mg/ml 1.4 mg/ml
1.8 mg/ml 2.0 mg/ml 3.0 mg/ml

Strain ALV produced no growth when exposed to 2.0 and 5.0 μg Cm/ml during a 70 hour incubation. The minimum inhibitory concentration (MIC) of an antibiotic is usually described as the lowest concentration that will inhibit the growth of a specific organism (Hammond & Lambert, 1978) and the MIC of chloramphenicol for strain ALV was determined to be 2 μg Cm/ml. Concentrations of chloramphenicol below the MIC usually produced an extended lag phase followed by growth at a rate comparable with the control (containing no antibiotic; Figure 4.3A). This implied that the chloramphenicol was probably inactivated during the lag period and this was confirmed by incubating flasks as before but without bacteria. Following an incubation of 24 hours, a flask containing 0.5 μg Cm/ml was inoculated with strain ALV and resulted in growth comparable to a control containing no antibiotic.

The *cat* genes derived from Gram positive bacteria require induction with sub-inhibitory concentrations of chloramphenicol. A change in chloramphenicol concentration from 0.1 μg Cm/ml to 0.2 μg Cm/ml produced an increased lag phase of about 10 hours for strain ALV (Figure 4.3A). Thus, a sub-inhibitory chloramphenicol concentration of 0.1 μg Cm/ml should remain active under the conditions employed for about 10 hours, and enable induction of *cat* genes.

In a comparable experiment, the effect of chloramphenicol on strain BC1 was investigated and produced similar results (data not shown). The MIC of chloramphenicol for strain BC1 was determined to be 2.0 μg Cm/ml.

4.2.3 The Effect of Kanamycin on Strain ALV.

Vectors which specify resistance to kanamycin (Kan) have been transferred into Gram positive bacteria and recombinant bacteria have been selected by their ability to express the kanamycin resistance gene e.g. pAT157 (Trieu-Cuot *et al.*, 1987) and pCK1 (Gasson & Anderson, 1985). Kanamycin was investigated as an potential marker for recombinant plasmids.

Various concentrations of kanamycin were added to Salts Medium at

pH 1.7 and supplemented with Fe/Ye/G (Methods 2.2.2 & 2.2.3). Flasks were inoculated with an exponentially growing culture of strain ALV and incubated at 45°C. Growth was monitored by assaying iron oxidation (Methods 2.3.4; Figure 4.3B).

As discussed earlier the minimum inhibitory concentration (MIC) of an antibiotic is the lowest concentration that will inhibit the growth of a specific organism (Hammond & Lambert, 1978) but in the experiments described in this work the MIC increased relative to the length of incubation period because antibiotics were inactivated resulting in extended lag phases and then exponential growth occurred. For kanamycin, no MIC was determined because flasks containing concentrations up to 3 mg Kan/ml still grew after extended lag phases. Therefore, for kanamycin, the MIC was determined as the concentration which inhibited growth during the time taken for the control (with no antibiotic) to reach stationary growth phase and the MIC for strain ALV was 2 mg Kan/ml during a 30 hour incubation (Figure 4.3B).

Kanamycin was highly unstable under the conditions employed and even when high concentrations of antibiotic were required to inhibit growth one might envisage that during an incubation period as the antibiotic was inactivated, a concentration would be reached which would select for resistant bacteria. As long as the time at which sensitive bacteria grow is known (for a given concentration of antibiotic) then unstable antibiotics can be used to select for resistant bacteria in a mixed population.

In a similar experiment with strain BC1, comparable results were obtained (data not shown) and the MIC of kanamycin was determined to be 2 mg Kan/ml following a 30 hour incubation.

4.2.4 Metal Disc Assays.

20 µl of a metal solution was applied to a paper filter disc. The solutions used were copper sulphate (1, 100, 1000 mM), sodium arsenate (1, 10, 100 mM), mercuric chloride (0.1, 0.5, 1.0 mM), silver nitrate (1,

5, 10 mM) and uranyl sulphate (1, 10, 100 mM). The discs were placed on top of pour plates (Fe/Ye/G; Methods 2.2.2) inoculated with the moderate thermophile strains TH3, BC1 and ALV. Following incubation primary and secondary zones of growth inhibition occurred (Figure 4.4). The zones of growth inhibition were measured and used to construct graphs to illustrate the toxicity of the metals (Figure 4.5).

Mercuric chloride and silver nitrate produced the largest zones of growth inhibition for all three moderate thermophile strains and large secondary zones of growth inhibition of strains TH3 and ALV were produced by copper sulphate. Overall, the responses of the moderate thermophile strains indicated that strain BC1 was the most sensitive to inhibition by the metals with the exception of mercury. Inorganic salts and organic compounds of copper are strongly bacteriostatic and the large secondary zones of growth inhibition caused by copper could be due to the induction of copper resistance (Mellano & Cooksey, 1988) or the production of copper-binding proteins (Harwood-Sears & Gordon, 1990). The latter suggestion is supported to some extent by the appearance of many single colonies within the secondary zones of growth inhibition when using copper sulphate (Figure 4.4), as if only a proportion of cells overcame the toxicity exerted by the copper. This kind of response might also be due to accumulation of the metal in some cells, or because Cu^{2+} is thought to compete with Fe^{2+} for binding sites in some bacteria during the oxidation of iron, e.g. in T. ferrooxidans (see Tuovinen & Kelly, 1974c).

4.2.5 The Effect of Metals on Strain ALV in Liquid Culture.

Metal solutions were added to Salts Medium at pH 1.7 and supplemented with Fe/Ye/G (Methods 2.2.2 & 2.2.3). Flasks containing this medium were inoculated with an exponentially growing culture of strain ALV and incubated at 45°C.

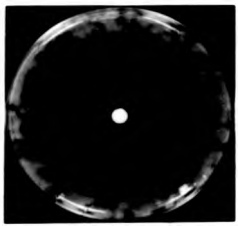
Growth of strain ALV was monitored by measuring the amount of iron oxidized over a 60 hour period (Figure 4.6). When the concentration of

Figure 4.4 Metal Disc Diffusion Assays.

The figure illustrates the primary (total inhibition) and secondary (partial inhibition) zones of growth inhibition produced by strains ALV (A) and TH3 (B) when exposed to paper discs impregnated with metal solutions:

(A) Copper sulphate (20 µl of 1 M solution)
(B) Mercuric chloride (20 µl of 0.5 mM solution)

(A)



(B)

Figure 4.5 Zones of Growth Inhibition of Strains TH3, ALV and BC1 in Metal Disc Diffusion Assays.

The graph illustates the sizes of zones of growth inhibition (mm) produced by discs impregnated with 20 µl of a metal solution. Primary zones of growth inhibition (empty bar) were measured from the edge of the disc to the periphery of the zone. Secondary zones of growth inhibition (shaded bar) were measured from the edge of the primary zone to the periphery of the secondary zone.

For each metal solution nine bars are shown; the first three bars represent strain TH3, the second three bars, strain ALV and the last three bars, strain BC1.

The metal solutions used were as follows:

Solution	Concentrations used (mM)
Copper sulphate	1000, 100, 1.
Mercuric chloride	1, 0.5, 0.1.
Silver nitrate	10, 5, 1.
Sodium arsenate	100, 10, 1.
Uranyl sulphate	100, 10, 1.

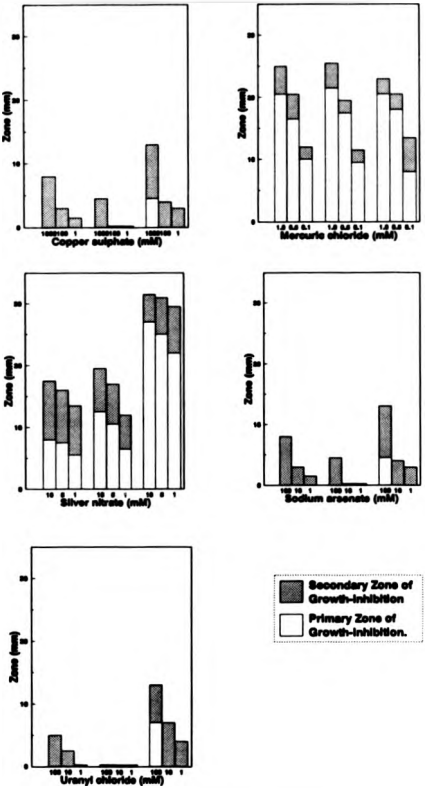

Zone (mm)
Copper sulphate (mM)
Mercuric chloride (mM)
Silver nitrate (mM)
Sodium arsenate (mM)
Uranyl chloride (mM)
Secondary Zone of Growth-Inhibition
Primary Zone of Growth-Inhibition.

Figure 4.6 The Effect of Metals on Strain ALV.

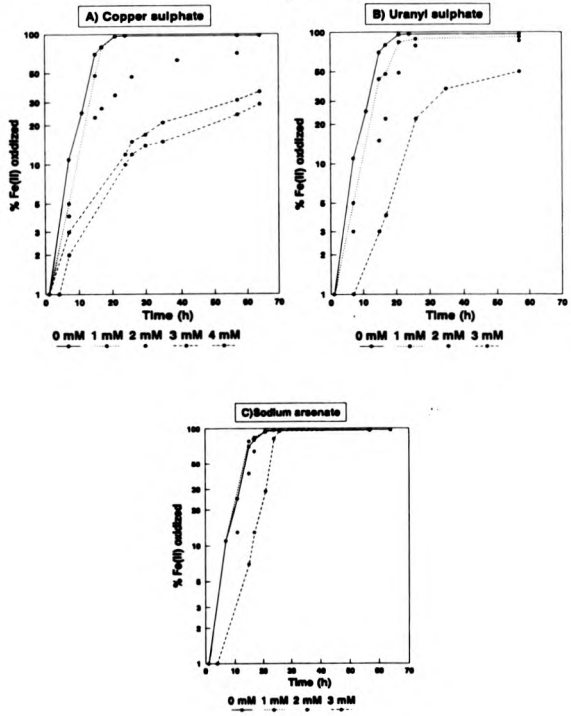
The two pages of graphs show the growth of strain ALV in liquid medium containing various concentrations of the indicated metal salts. A control flask which did not contain any added metal is shown for comparison in each graph.

Exponential growth phase cultures were used as inocula for chemolithoheterotrophic growth.

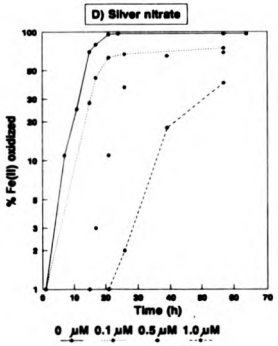
The growth of strain ALV was monitored as ferrous iron oxidized. The growth rates (iron oxidation rate) of the cultures were as follows:

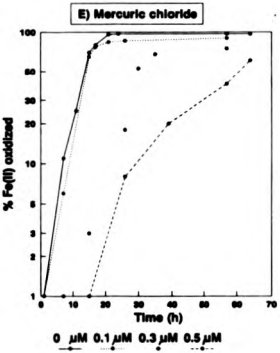
Metal	Conc. (mM)	Growth Rate (h^{-1})	Metal	Conc. (μM)	Growth Rate (h^{-1})
Copper sulphate	0	0.28	Silver nitrate	0.0	0.28
	1	0.23		0.1	0.23
	2	0.23		0.5	0.23
	3	0.09		1.0	0.14
	4	0.10			
Uranyl sulphate	0	0.28	Mercuric chloride	0.0	0.28
	1	0.23		0.1	0.23
	2	0.14		0.3	0.15
	3	0.14		0.5	0.10
Sodium arsenate	0	0.28			
	1	0.28			
	2	0.23			
	3	0.23			

1)


A) Copper sulphate
% Fe(II) oxidized
Time (h)
0 mM 1 mM 2 mM 3 mM 4 mM
B) Uranyl sulphate
% Fe(II) oxidized
Time (h)
0 mM 1 mM 2 mM 3 mM
C)Sodium arsenate
% Fe(II) oxidized
Time (h)
0 mM 1 mM 2 mM 3 mM

2)


D) Silver nitrate
% Fe(II) oxidized
Time (h)
0 µM 0.1 µM 0.5 µM 1.0 µM


E) Mercuric chloride
% Fe(II) oxidized
Time (h)
0 µM 0.1 µM 0.3 µM 0.5 µM

copper sulphate in the flask was increased, initial growth rates of strain ALV were similar but after a longer incubation the growth rates decreased with increased concentration of copper. This suggests that the bacteria gradually accumulated the metal ions. A similar response, although less obvious, was observed for strain ALV with different concentrations of uranium ions. If competition for Fe^{2+} binding sites were occurring a more immediate response might be expected.

Sodium arsenate up to a concentration of 3 mM had little effect on the oxidation of iron by strain ALV. The tolerance of bacteria to arsenate is important and has received increasing interest because of the microbial leaching of auriferous arsenopyrite for the recovery of gold.

The binding of silver ions to proteins is thought to account for the antibacterial action of silver but the increased lag phase of strain ALV cultures exposed to increasing concentrations of silver and mercury ions was probably due to precipitation or volatilization of the metals respectively. The observation that silver, copper, uranium and mercury all reduced the growth rate and prevented cultures from completely oxidizing all the Fe^{2+} in the medium was indicative of an accumulative or competitive effect exerted on the cells.

The initiation of growth of T. ferrooxidans on iron was found to be inhibited by 1 μM mercuric chloride but growth did occur after an extended lag phase (Norris & Kelly, 1978). An extended lag phase resulted when cultures of strain ALV were exposed to mercuric chloride (0.3 and 0.5 μM; Figure 4.6) and moderate inhibition of strain ALV by mercuric chloride was comparable to that of T. ferrooxidans (Figure 4.6; Table 4.3), and some strains of T. ferrooxidans contain a mercuric reductase gene (Inoue et al., 1989).

4.3 Discussion.

The growth of strains ALV, TH3 and BC1 was inhibited by antibiotics and metals during disc diffusion assays (Figures 4.2 & 4.5).

The information provided indicated that chloramphenicol would be a

useful antibiotic for the selection of moderately thermophilic and acidophilic bacteria containing recombinant vectors following gene transfer experiments.

The moderate thermophiles strains ALV and BC1 grow poorly on solid media so the selection of antibiotic resistant bacteria from a mixed population of resistant and wild type bacteria in liquid culture was proposed.

Chloramphenicol was the antibiotic of choice with a MIC of 2 µg Cm/ml for strains ALV and BC1. Several chloramphenicol resistance genes are available from Gram positive bacterial sources, and these genes usually require induction by a sub-inhibitory concentration of chloramphenicol. Where induction of a *cat* gene would be required, 0.1 µg Cm/ml would probably remain active for about 10 hours in the growth medium of the moderate thermophiles at low pH and 45°C.

Kanamycin was also investigated because several broad host range plasmids were available which had been transferred into Gram positive bacteria (Trieu-Cuot *et al.*, 1987; Gasson & Anderson, 1985) and kanamycin (1 mg/ml) had also been used to select for resistant *Acidiphilium* sp. at pH 3.5 following electroporation (F. F. Roberto, pers. comm.). Kanamycin resistance genes are usually expressed constitutively.

Kanamycin was more unstable than chloramphenicol under the growth conditions of the moderate thermophile strains and the MIC was 2 mg Kan/ml for strains ALV and BC1 during a 30 hour period of incubation.

The development of genetic systems for the moderate thermophiles would be useful for the production of strains more tolerant to metal ions. Metal resistance genes could be introduced into the genotype of the moderate thermophiles. Bacteria tailored to the requirements of a mineral leaching process could be produced so that a higher concentration of a particular metal or metals in the ore would be required to inhibit growth of the bacteria.

The growth of the moderate thermophile strains TH3, ALV, and BC1 was inhibited by metal ions (Figure 4.5) and overall, strain BC1 was least

tolerant to silver, arsenate, copper and uranium ions. Strain ALV was more or equally tolerant to uranium, silver and mercury ions than T. ferrooxidans (Figure 4.6 and Table 4.3). A similar observation was made for strain BC1 (Table 4.3).

Studies with yeast cells has suggested that the toxic action of Cu^{2+} resulted from its entering the cell cytoplasm and causing general cell disruption and, UO_2^{2+} and Fe^{2+} were proposed to bind at the same sites on the cell surface (Van Steveninck & Booij, 1964). A similar theory for the effect of UO_2^{2+} on the cells of T. ferrooxidans was described (Tuovinen & Kelly, 1974c, DiSpirito & Tuovinen, 1982) and it has been proposed that UO_2^{2+} specifically inhibited Fe^{2+} oxidation or disrupted K^+-transport in T. ferrooxidans (Tuovinen & Kelly, 1974a).

In T. ferrooxidans the action of silver was thought to be due to the penetration of the metal across the cell membrane (Sugio et al., 1981). The progressive accumulation of silver by T. ferrooxidans cells resulted in a rapid and massive loss of cell potassium, this response was not affected by Cu^{2+} (thought to compete with other ions for binding sites at the cell surface), and the uptake of silver by the cells probably occurs via an alternative route or mechanism to that of the accumulation of copper (Norris & Kelly, 1978).

Metal resistance genes, e.g. arsenate and mercury, have been used for the selection of resistant Gram positive bacterial clones (Silver et al., 1989). The concentration of some metals which might be required for the selection of resistant clones of the moderate thermophile strains ALV, BC1 and TH3 have been determined.

Chapter 5.
Plasmids in the Moderate Thermophiles.

5.1 Introduction.

The development of a gene cloning system for the moderate thermophiles required the identification of a potential cloning vector and the most commonly used vectors in gene cloning experiments are plasmids. Vectors and their hosts form integrated systems for constructing and maintaining recombinant DNA molecules and a major factor in the choice of vector may be the ability of the vector to be stably maintained in the host bacterium. For this reason, the moderate thermophiles were screened so that small indigenous plasmids (which would contain the replicative machinery for maintenance in the host bacterium) could be identified. Small plasmids are easier to isolate than large plasmids and will usually accept larger inserts of DNA during manipulation to form recombinant replicons. The availability of such a vector would facilitate the development of a transformation procedure.

The general procedure for isolating and characterizing plasmid DNA depends upon bacterial cell lysis, followed by a subsequent separation of the chromosomal DNA from the plasmid DNA. Some organisms are readily lysed using EDTA and lysozyme, the former disrupts the outer membrane integrity and the latter degrades the mucopeptide layer. Spheroplasts are formed which are stabilized by the presence of sucrose or glucose and the spheroplasts are lysed by a detergent. Cellular debris and chromosomal DNA are pelleted by centrifugation and the plasmid DNA remains in the supernatant fraction where it can be precipitated at $-20^{o}C$ with cold ethanol or isopropanol. Plasmid DNA can also be separated from the chromosome by caesium chloride-ethidium bromide centrifugation and numerous modifications of these methods have been described for plasmid isolation (see Table 5.1 for a summary of methods used for screening the moderate thermophiles for plasmids). Most methods of plasmid isolation

Table 5.1. Methods for Plasmid Isolation.

Features of Method	Reference
For small and large plasmid isolation. Alkaline-SDS lysis and elevated temperature to denature chromosomal DNA. Proteins and cellular debris removed using a phenol-chloroform extraction.	Kado & Liu, 1981.
EDTA-Lysozyme-SDS lysis and selective alkaline denaturation of high molecular weight chromosomal DNA. Followed by the addition of acidic sodium acetate to precipitate chromosomal DNA, protein-SDS complexes and high molecular weight RNA.	Birnboim & Doly, 1979.
EDTA-Lysozyme-SDS lysis in agarose gel wells. Particularly for the isolation of large plasmids.	Eckhardt, 1978.
Modified Birnboim & Doly method, developed to reduce damage to DNA by DNase released during cell lysis by low ratio of cells to lysis buffer and EDTA washing of cells.	Roberts *et al.*, 1986.
Antifoam-alkaline SDS lysis then direct loading into agarose gel. Particularly for large plasmid isolation.	Wheatcroft & Williams, 1981.

Features of the plasmid preparation methods used for the moderate thermophile strains are indicated.

The Eckhardt (1978) and Wheatcroft and Williams (1981) methods involve minimal manipulation of the sample and allow the isolation of large plasmids.

have some limitations therefore it was necessary to try several preliminary procedures (for reviews see Kieser, 1984; Grinsted & Bennett, 1984).

The sizes of plasmids isolated could not be accurately determined from the results because the markers used were linear DNA fragments and plasmid DNA identified probably represented one of several conformations of plasmid. However, plasmids are discussed as large, indicating that the plasmid was larger than the chromosomal DNA (this usually migrates in gels to a similar position as the 24 kb marker of Lambda digested with HindIII) and small, indicating that the plasmid was smaller than 24 kb. Where several plasmid bands were seen in a sample on an agarose gel, these probably represented different forms of the same plasmid i.e. covalently closed circular (CCC), open circular (OC), linear (L) and possibly multimers. When CCC plasmid is exposed to a pH of greater than 13.0, it gives rise to a denatured form which appears as a band running faster than CCC plasmid in agarose gels (Birnboim & Doly, 1979). Minor bands migrating in agarose gels may also represent single-stranded (ss) forms of plasmid DNA. Although ethidium bromide does not normally interchelate in ssDNA some fluorescence due to ethidium bromide may be observed in ssDNA samples (Birnboim & Doly, 1979; Kim et al., 1990)

5.2 Results.

5.2.1 Plasmid Screening.

The initial cell concentration was an important parameter in all methods. 40 ml of heterotrophically grown cells of the moderate thermophile strains were used with an absorbance at 440 nm of 0.1-0.15. Prior to the plasmid preparation the cell pellet was washed in 10 mM Tris-HCl at pH 8.0 so that the pH of the cell sample was raised. Without these conditions no results were obtained due to insufficient yield of plasmid, 'smeary' gel results or incorrect pH during the method.

Alkaline lysis of cells followed by neutralization and precipitation

with sodium acetate as in the method of Birnboim and Doly (1979) usually gave plasmid DNA with only small amounts of contaminating chromosomal DNA and some CCC plasmid remained intact (Figure 5.1). Small plasmids were clearly seen in DNA isolated from the moderate thermophile strains LM1, LM2 and BC1 (Figure 5.1A). The band which represented chromosomal DNA from strain NAL was positioned close to a second band (Figure 5.1A; track B) and, this result implied that a large plasmid was present in strain NAL. The two bands seen in samples from strain NAL were clearly differentiated in a second experiment (Figure 5.1B; track K) and, the chromosomal DNA of strain NAL migrated to a lower position in agarose gels than the chromosomal DNA of the other moderate thermophile strains (Figure 5.1B).

Three bands representing plasmids were seen in DNA isolated from strain BC1 (Figure 5.1B; track M) and strain TH1 (Figure 5.1B; track O). The similarity of the plasmid profiles of these two bacteria indicated that they contained plasmids of identical size. The brightest band (or middle plasmid band) in each sample probably represented CCC plasmid and, the band migrating slower (i.e. at a higher position in the agarose gel) than the CCC plasmid probably indicated the presence of OC plasmid. This form of plasmid commonly occurs in plasmid samples due to nicking of CCC plasmid during manipulations carried out as part of the preparation. The bands which migrated faster than those of the CCC plasmids from strains BC1 and TH1 probably represented single-stranded or denatured CCC plasmids. Small plasmids were also present in strain LM2 (Figure 5.1B; track N) and strain LM1 (Figure 5.1B; track P). Further investigations using the method of Birnboim & Doly (1979) revealed the presence of a large plasmid in strain LM2 (Figure 5.2A; track H) in addition to the small plasmid discussed earlier.

When plasmid preparations of the moderate thermophiles strains were analysed by agarose gel electrophoresis 'smeary' results were often obtained (Figure 5.2B; tracks A-H). This type of result is usually caused by contaminating nuclease activity and/or insufficient care taken during

Figure 5.1 Plasmid Minipreparations of the Moderate Thermophiles.

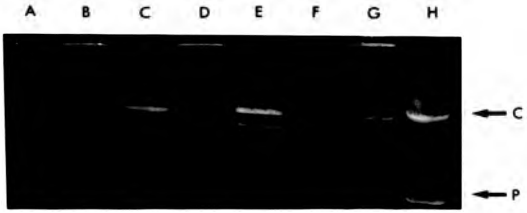
The figure illustrates the results of agarose gel electrophoresis following plasmid minipreparations (Birnboim & Doly, 1979) of the moderate thermophile strains. Agarose gels were run for about one hour at 80 volts, stained using ethidium bromide and photographed.
The samples are from the moderate thermophile strains as follows:

Figure A (A) LM1
(B) NAL
(C) LM2
(D) TH1
(E) Lambda DNA cut with HindIII
(F) TH3
(G) ALV
(H) BC1

Figure B (I) Lambda DNA cut with HindIII
(J) TH3
(K) NAL
(L) ALV
(M) BC1
(N) LM2
(O) TH1
(P) LM1

In the upper photograph (A), the positions of chromosomal DNA (C) and plasmid DNA (P) are indicated.
Lambda cut with HindIII was used as a marker and the sizes of the linear DNA fragments produced are indicated by arrows in the lower photograph (B).

(A)



(B)

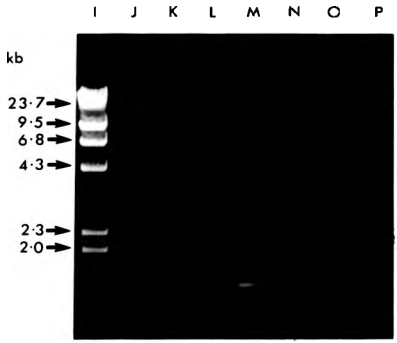


Figure 5.2 Plasmid Minipreparations of the Moderate Thermophiles.

The figure illustrates the results of agarose gel electrophoresis following plasmid minipreparations of the moderate thermophile strains. Agarose gels were run for about one hour at 80 volts, stained using ethidium bromide and photographed.
The samples are from the moderate thermophile strains as follows:

Figure A : Method of Birnboim and Doly (1979)

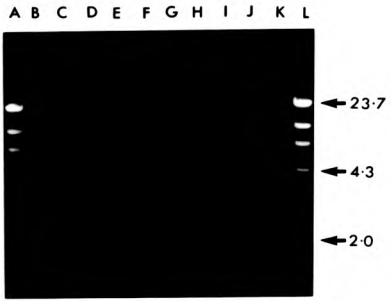
Track			
	(A) & (L) Lambda DNA cut with HindIII		
	(B) TH3	(F) ALV	(J) LM1
	(C) TH3	(G) ALV	(K) BC1
	(D) NAL	(H) LM2	
	(E) NAL	(I) TH1	

Figure B : Tracks A-H from the method of Birnboim and Doly (1979) and tracks J-Q from the method of Roberts et al. (1986).

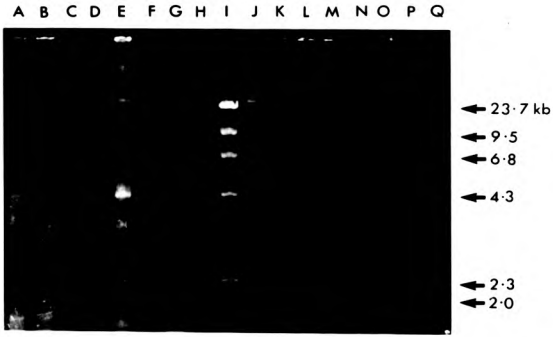
(A) BC1	(I) Lambda DNA cut with HindIII
(B) TH1	(J) TH3
(C) TH3	(K) T. acidophilus
(D) NAL	(L) ALV
(E) T. acidophilus	(M) BC1
(F) LM1	(N) NAL
(G) ALV	(O) TH1
(H) LM2	(P) LM2
	(Q) LM1

The arrows and numbers indicate the sizes (kb) of the linear DNA fragments produced when Lambda DNA was cut with HindIII.

(A)


A B C D E F G H I J K L
23·7
4·3
2·0

(B)


A B C D E F G H I J K L M N O P Q
23·7 kb
9·5
6·8
4·3
2·3
2·0

the experiment but smearing can also result from the activity of cell-associated and extracellular nucleases (Bron, 1990; Kieser, 1984; Roberts et al., 1986). DNase levels can be kept low by washing cells in EDTA-containing buffer and using exponentially growing cultures as in the method of Roberts et al. (1986). Less 'smeary' samples were obtained when the same cultures were processed according to the method of Roberts et al. (1986) than following the method of Birnboim & Doly (1979; Figure 5.2B). Although not shown, the method of Roberts et al. (1986) did indicate the presence of plasmids in some of the moderate thermophile strains. Overall, 'smeary' results were obtained on some occasions following the method of Birnboim & Doly (1979) but this method also gave more consistent results.

Faint bands which might represent large plasmids were seen in DNA samples from strains BC1 and LM1 and, from Thiobacillus acidophilus (Figure 5.2B; tracks A, F and E respectively). T. acidophilus contains several plasmids (Mao et al., 1980; Martin et al., 1981).

Small plasmids are easier to identify by plasmid minipreparation procedures because they are usually high copy number and form distinct bands on agarose gels following electrophoresis. Plasmids larger than the chromosome are usually present at lower copy numbers in cells and are not easily isolated without damage and, as a result, are harder to visualize on agarose gels. This is evident in Figure 5.3A, a plasmid preparation using the method of Kado and Liu (1981). In some samples smears were apparent in agarose gels above the chromosomal DNA which tentatively suggested the presence of large plasmids in strains TH1, ALV, LM1, BC1 and NAL (Figure 5.3A; tracks A, C, E, F and G respectively). A strongly fluorescent background indicated the presence of a contaminating substance(s), e.g. protein. Methods for large plasmid isolation involve minimal sample manipulation and produce preparations contaminated with cell debris making results harder to interpret but, for strains LM2 and NAL, large plasmids were isolated on several occasions. However, evidence for the presence of large plasmids in BC1, TH1, LM1 and ALV remained inconclusive.

Figure 5.3 Plasmid Minipreparations of the Moderate Thermophiles.

The figure illustrates the results of agarose gel electrophoresis following plasmid minipreparations of the moderate thermophile strains using the method of Kado & Liu (1981). Agarose gels were run for about one hour at 80 volts, stained using ethidium bromide and photographed.
The samples are from the moderate thermophile strains as follows:

Figure A

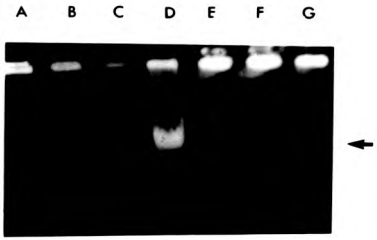
(A) TH1
(B) TH3 — The arrow indicates the position
(C) ALV — of chromosomal DNA.
(D) LM2
(E) LM1
(F) BC1
(G) NAL

Figure B

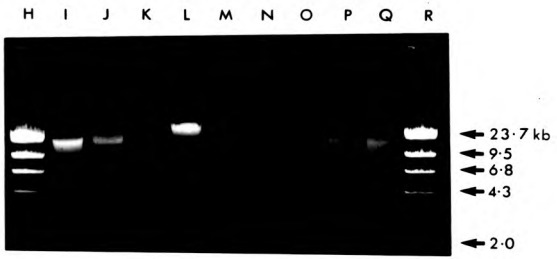
(H) Lambda DNA cut with HindIII
(I) BC1
(J) TH1
(K) LM1
(L) TH3
(M) -
(N) ALV
(O)-(Q) LM2
(R) Lambda DNA cut with HindIII

The arrows and numbers indicate the sizes (kb) of the linear DNA fragments produced when Lambda DNA was cut with HindIII.

A



B



A further attempt at the method of Kado and Liu (1981) produced samples which 'sucked' out of the wells of the agarose gel into the surrounding buffer during loading. Following agarose gel electrophoresis, the results were easier to interpret (Figure 5.3B) but obviously of little use because of partial sample loss. However, a small plasmid was isolated from strain LM2 (Figure 5.3B; track Q) and a faint band just below the well of the gel suggested the presence of a large plasmid in strain TH3 (Figure 5.3B; track L).

The Eckhardt (1978) and Wheatcroft and Williams (1981) plasmid preparations gave no useful results and, in many cases, samples did not remain in the wells of agarose gels but dispersed into the surrounding buffer. Frozen cell pellets were investigated as sources of plasmids. However, these resulted in smears of DNA throughout the agarose gel indicating excessive degradation of the DNA. The isolation of plasmids from cultures containing ferric iron initially proved unsuccessful so the plasmid minipreparation procedure was modified to remove most of the ferric iron and the method then proved successful (Data not shown, Methods 2.5.6).

5.2.2 Large Scale Plasmid Isolation From Strain BC1.

Strain BC1 gave the most reproducible results and contained a putative single plasmid of about 3 kb. The small plasmid from strain BC1 was purified by a larger scale procedure (Methods 2.5.3) and attempts were made to purify this plasmid by caesium chloride-ethidium bromide centrifugation (Methods 2.5.2). No plasmid band was visualized in the resulting gradient probably due to insufficient yield. A Streptomyces plasmid pSV1 could not be recovered from caesium chloride-ethidium bromide gradients (Aguilar & Hopwood, 1982). The procedure was therefore modified and plasmid purified by extraction from agarose gels or alternatively using Quiagen columns (Data not shown, Methods 2.5.3).

5.3 Discussion.

The methods of Roberts et al. (1986) and Birnboim and Doly (1979) gave comparable results with complete cell lysis and small plasmids (<6kb) were visible in strains LM1, BC1, TH1 and LM2. The latter method gave more consistent results, was less time consuming and was adopted for the isolation of small plasmids from the moderate thermophiles.

Most plasmid purification methods were originally developed for E. coli and the standard procedures for E. coli frequently give unsatisfactory results when applied to Gram positive bacteria (Bron, 1990). Plasmid yields may be low and the purity may be insufficient for subsequent manipulations, such as restriction, ligation and sequencing. Non-specific cleavage, resulting in 'smearing' of the fragments in agarose gels frequently occurs when Gram positive bacteria are used as sources of DNA. Endogenous DNases are present in members of the genus Clostridium (Roberts et al., 1986) and Kieser (1984) noted a DNA-degrading activity in Streptomyces lividans. Furthermore, Bacillus sp. secrete nucleases into growth media (see Bron, 1990). Attempts to purify such plasmid preparations, e.g. by phenol extraction or gel filtration, are usually unsuccessful and it appears that plasmid DNA extractions from Gram positive bacteria require in general more care than those from E. coli. This may be due to DNA-degrading substances or cell wall composition because incomplete lysis of cells results not only in low yields, but also in low quality plasmid DNA. However, this problem can usually be overcome by using low cell densities during the cell lysis step(s).

Extrachromosomal DNA has been shown to be abundant in lithoautotrophic bacteria. Its presence has been demonstrated in sulphur-, iron-, nitrite- and carbon monoxide-oxidizing strains (see Friedrich, 1989). Various strains of facultatively chemolithoautotrophic bacteria harbour high molecular weight plasmids of a size ranging from 400 to more than 700 kb (see Friedrich, 1989) and, it has been proposed that plasmids present in metal leaching acidophiles may encode heavy

metal resistances (Summers & Silver, 1978) but a causal relationship has not yet been established.

The Eckhardt (1978) and Wheatcroft and Williams (1981) plasmid preparations for the isolation of large plasmids gave no useful results but large plasmids were isolated from strains LM2 and NAL. The presence of large plasmids in the other strains of moderate thermophiles was not confirmed.

Whilst accurate size determination of plasmids (except the small plasmid in strain BC1; see next Chapter) was not attempted during the course of this work electron microscopy or restriction enzyme digestion can be employed for accurate sizing. In addition, different forms of plasmid DNA i.e. CCC, OC and linear may be distinguished by ultraviolet irradiation in the presence of ethidium bromide or by heat treatment (see Carlton & Gonzalez, 1985).

The small plasmid identified in strain BC1 was chosen for further study during attempts to develop a host:vector system for the moderate thermophiles because this plasmid was small and most consistently isolated.

Chapter 6
Characterization of pBC1

6.1 Introduction.

Following the identification of a small plasmid (pBC1) in the moderate thermophile strain BC1, the plasmid was further characterized and studies were made to investigate the host range of the plasmid. The information provided would be useful during construction of vectors for the development of a host:vector system for strain BC1.

Large scale preparation of pBC1 had proved difficult and it was decided that pBC1 would be cloned into an E. coli vector so that large quantities of pBC1-derived DNA could be produced. So far the plasmid was cryptic, i.e. no phenotypic character had been assigned to the plasmid and to use pBC1 as a cloning vector for the moderate thermophiles, it would be necessary to insert a marker or reporter gene into pBC1.

Phenotypic expression from plasmids is sometimes detected by curing of the plasmid from its host and then looking for changes in the host cell phenotype (see Carlton & Gonzalez, 1985). Curing of pBC1 from strain BC1 was not attempted in the course of this work.

Resistance to antibiotics is usually a fairly easy phenotypic character to assess but the growth conditions of the moderate thermophile strains would probably render most antibiotics inactive (as discussed in Chapter 4). Similarly, the detection of bacteriocin production would be somewhat difficult to assess using established bio-assays because the moderate thermophiles grow in acidic and thermophilic conditions and other test bacteria would not grow in the same media. Bacteriocins usually affect only a narrow spectrum of bacteria and it might therefore be more effective to try and detect substances in the growth supernatant of the bacteria using biochemical means e.g. high pressure liquid chromatography. These experiments were not carried out during this work.

The expression of genes from Gram positive bacteria has been

achieved in E. coli but in contrast to E. coli, B. subtilis is limited in its ability to express genes from other genera (see Chapter 1).

For the expression of genes in E. coli, several in vivo systems are available, e.g. minicells (Dougan & Kehoe, 1984) and maxicells (Sancar et al., 1981). An in vitro system based on that of Zubay (1973) is commonly employed and has been used to express genes from plasmids found in Gram positive bacteria (Pratt et al., 1981; Takiguchi et al., 1989). The bacterial cell-free coupled transcription and translation system allows the identification of the protein products of cloned DNA and also the mapping of the peptide products to defined DNA fragments (Pratt et al., 1981). These applications represent a major advantage over the use of in vivo procedures since the matching of cloned inserts with previously unidentified polypeptide products is difficult in systems such as minicells or maxicells.

Several groups of workers (Pratt, 1984; Pratt et al., 1981; Thompson et al., 1984) have reported the use of linear DNA templates during coupled in vitro transcription and translation studies. More specifically, these experiments used plasmids digested with restriction enzymes and the polypeptides produced could be mapped to positions within the plasmids. The overall efficiency of protein synthesis from linear molecules may be reduced 3-4 fold compared to the equivalent supercoiled template (Pratt, 1984) and large quantities of DNA (at least 5 µg per reaction) are therefore necessary.

6.2 Results.

6.2.1 Restriction Endonuclease Digestion of pBC1.

A large scale preparation of pBC1 (Methods 2.5.3) from 40 litres of strain BC1 had produced a small quantity of pBC1 (about 1 µg).

Restriction endonuclease digestions of pBC1 with the enzymes PstI, SalI, EcoRI, SmaI, HpaI, BamHI, ClaI and HincII were carried out in an

attempt to produce linear pBC1. The entire plasmid could then be cloned as one piece into an E. coli vector. The enzymes HincII, HpaI and SmaI produced an additional band to those found in uncut pBC1 when the digests were examined by agarose gel electrophoresis (data not shown). This additional band was above the band ascribed to covalently closed circular (CCC) plasmid and thus represented linear (L) plasmid. The size of linear pBC1 was estimated to be about 2.6 kb.

6.2.2 Cloning pBC1 into E. coli Vector pACYC177.

A single HincII site in the β-lactamase gene (Amp^R) of the E. coli vector pACYC177 (3.94 kb; Chang & Cohen, 1978) was exploited. This plasmid also carries a kanamycin resistance gene (Kan^r).

pBC1 and pACYC177 were linearized using the restriction enzyme HincII and ligated. The ligated DNA was used to transform E. coli HB101 and clones containing recombinant vector were identified by the expression of kanamycin resistance and their sensitivity to ampicillin, i.e. insertional inactivation of the β-lactamase gene. The recombinant vector was named pFEG7 (Figure 6.1). No recombinant vectors containing the pBC1 DNA in the opposite orientation to that of pFEG7 were identified. Further attempts to produce such clones were made.

pFEG7 was digested with HincII and the resulting two DNA fragments ligated and transformed into E. coli HB101. Subsequent analysis of the recombinant clones revealed a recombinant vector pFEG15 which contained the pBC1 DNA in the opposite orientation to that in pFEG7.

At a later stage in the work pBC1 DNA was also cloned into pACYC177 via a single HindIII site present in both plasmids. A single HindIII site was identified in pBC1 DNA during restriction mapping of pFEG7 (Section 6.2.3). It was important to obtain several different constructs containing the pBC1 DNA to increase the probability of retaining the functional replication machinery of pBC1.

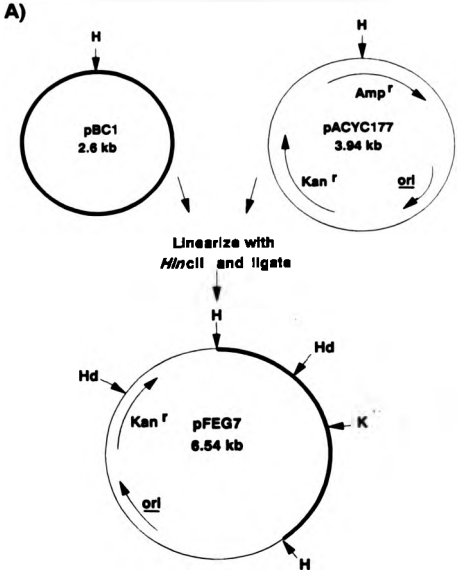
Figure 6.1 The Construction of pFEG7 and Restriction Map of pBC1.

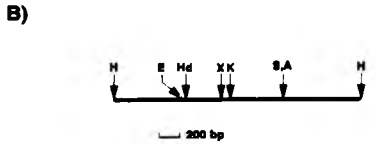
The vector pFEG7 (6.54 kb) was constructed from pACYC177 (3.94 kb; Chang & Cohen, 1978), an E. coli vector, and pBC1 (2.6 kb) from strain BC1. Both vectors were linearized using the restriction enzyme HincII, and then ligated as indicated in A). Amp^r indicates the ampicillin resistance determinant and Kan^r indicates the kanamycin resistance determinant of the pACYC177 DNA. The Amp^r determinant was insertionally inactivated during the construction of pFEG7. ori indicates the origin of replication of pACYC177. Thick lines represent pBC1 DNA and thin lines pACYC177 DNA.

A partial restriction map of pBC1 is shown in B).

Restriction enzymes are as follows:

A = AvaI
E = EcoRV
H = HincII
Hd = HindIII
K = KpnI
S = SmaI
X = XbaI


A)
H
pBC1
2.6 kb
H
Amp r
pACYC177
3.94 kb
Kan r
ori
Linearize with
HincII and ligate
H
Hd
Hd
Kan r
pFEG7
6.54 kb
K
ori
H


B)
H
E
Hd
X
K
S,A
H
200 bp

pFEG7 was digested with HincII and the 2.6 kb fragment representing pBC1 was isolated by agarose gel electrophoresis. This fragment was ligated to form circular pBC1 which was then digested with HindIII. pACYC177 contains a single HindIII site within the kanamycin resistance gene of the vector. pACYC177 was digested with HindIII. dephosphorylated (Methods 2.4.4) and then ligated to the 2.6 kb HindIII digested pBC1 DNA. The ligated DNA was transformed into E. coli DH1 using electroporation (Methods 2.6.2) and subsequently, clones containing the recombinant plasmids pLZ5 and pLZ11 isolated by their resistance to ampicillin and their sensitivity to kanamycin, i.e. insertional inactivation of the kanamycin resistance gene of pACYC177. pLZ5 and pLZ11 differ in the orientation of the pBC1 DNA insert (Figure 6.2).

6.2.3 Restriction Endonuclease Mapping of pBC1.

The pBC1 insert DNA in the vectors pFEG7 and pFEG15 was restriction mapped, see Figures 6.1 & 6.3. Figure 6.3 includes a sample of uncut pFEG7 (track 24). The majority of this plasmid existed as high molecular weight (HMW) multimers in E. coli HB101. Although pACYC177 formed some multimers in E. coli HB101 (Figure 6.3; track 23), the majority of this plasmid was present as monomer CCC DNA.

6.2.4 Comparison of the Small Plasmids in Strains TH1 and BC1.

The results of plasmid screening indicated that the plasmids in strains TH1 and BC1 were the same size (Chapter 5; Figure 5.1B).

Plasmids in some of the moderate thermophile strains were investigated for homology with pBC1. Plasmid minipreparations (Methods 2.5.6) of the moderate thermophile strains BC1, TH1, LM1, ALV and TH3 were analysed by agarose gel electrophoresis (Figure 6.4A) and the DNA from this gel was transferred to nitrocellulose (Methods 2.7).

The 2.6 kb HincII fragment from pFEG7 was isolated; this represented the entire pBC1 plasmid. The fragment was radiolabelled with ^{32}P by nick-

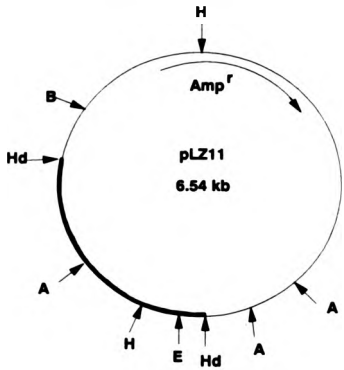
Figure 6.2 The Vectors pLZ11 and pLZ5.

Both vectors contain the entire plasmids pACYC177 (3.94 kb; Chang & Cohen, 1978; thin lines) and pBC1 (2.6 kb; thick lines). These plasmids were ligated via the unique HindIII site of both plasmids. The kanamycin resistance gene of pACYC177 was insertionally inactivated. Amp^r indicates the ampicillin resistance gene of pACYC177.

pLZ11 and pLZ5 (6.54 kb) differ in the orientation of the pBC1-derived DNA insert.

Key to restriction enzymes::

A = AvaI
B = BamHI
E = EcoRV
H = HincII
Hd = HindIII


H
Amp r
B
Hd
pLZ11
6.54 kb
A
A
H
E
Hd
A

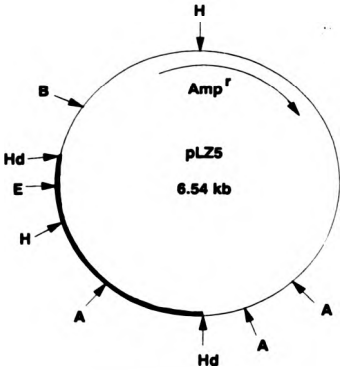
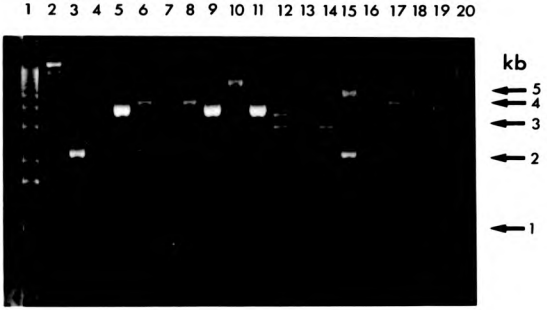

H
Amp r
B
Hd
E
H
pLZ5
6.54 kb
A
A
A
Hd

Figure 6.3 Restriction Endonuclease Digestion of pACYC177 and pFEG7.

The figure shows the analysis by agarose gel electrophoresis, of fragments of DNA produced following restriction endonuclease digestion of pACYC177 and pFEG7.

The agarose gel contained the following samples:

1) 1 kb ladder (BRL)
2) Lambda DNA cut with HindIII
3) Uncut pACYC177
4) Uncut pFEG7
5) pACYC177 cut with HindIII
6) pFEG7 cut with HindIII
7) pFEG7 cut with HindIII and HincII
8) pFEG7 cut with HindIII and BamHI
9) pACYC177 cut with BamHI
10) pFEG7 cut with BamHI
11) pACYC177 cut with SmaI
12) pFEG7 cut with SmaI
13) pFEG7 cut with HincII and SmaI
14) pFEG7 cut with BamHI and SmaI
15) pACYC177 cut with EcoRV
16) pFEG7 cut with EcoRV
17) pFEG7 cut with HindIII and EcoRV
18) pFEG7 cut with BamHI and EcoRV
19) pACYC177 cut with HincII
20) 1 kb ladder (BRL)
21) 1 kb ladder (BRL)
22) Lambda cut with HindIII
23) Uncut pACYC177
24) Uncut pFEG7
25) pACYC177 cut with HincII
26) pFEG7 cut with HincII
27) pACYC177 cut with AvaI
28) pFEG7 cut with AvaI


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
kb
5
4
3
2
1

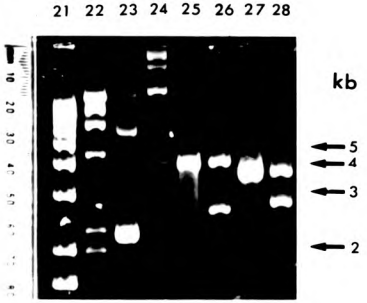

21 22 23 24 25 26 27 28
kb
5
4
3
2

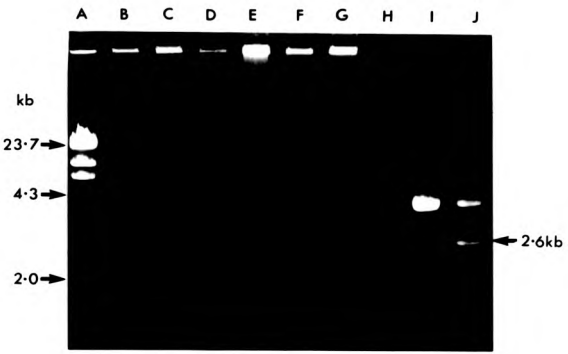
Figure 6.4 Examination of DNA from the Moderate Thermophile Strains for Homology to pBC1-Derived DNA.

DNA isolated by plasmid minipreparations from the moderate thermophile strains was analysed by agarose gel electrophoresis as in A), and Southern blotted onto nitrocellulose. The resulting blot was probed at high stringency (90%) with the 2.6 kb HincII fragment of pFEG7 (which represents the entire pBC1 plasmid). The resulting autoradiogram is shown in B).

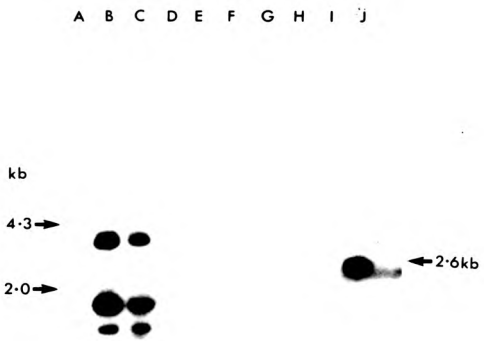
Key to tracks:

A) Lambda DNA cut with HindIII
B) DNA from strain BC1
C) DNA from strain TH1
D) DNA from strain LM1
E) DNA from strain LM2
F) DNA from strain ALV
G) DNA from strain TH3
H) -
I) pACYC177 cut with HincII
J) pFEG7 cut with HincII

(A)



(B)



translation (Methods 2.7.1) and hybridized to the DNA samples on the nitrocellulose filter. The filter was subsequently washed to a stringency value of 90% (Methods 2.7.2). The nitrocellulose filter was exposed to X-ray film and a photograph of the autoradiogram is shown in Figure 6.4B. The plasmid sample from strain TH1 hybridized to the pBC1 radiolabelled DNA and remained bound at the high stringency wash indicating that the two plasmids were probably identical.

Figure 6.4A shows plasmid bands in DNA samples from strains BC1 and TH1 and these probably represented CCC DNA (lower band near to the 2.0 kb marker), and OC DNA (near to the 4.3 kb marker). Chromosomal DNA was also visible (level with the 23.7 kb marker). Interestingly, a third plasmid band became more apparent following autoradiography, below the band of CCC plasmid.

Ethidium bromide does not intercalate in single-stranded DNA and the latter is not usually visible in agarose gels stained using this dye. However, a small amount of ethidium bromide may intercalate where internal double-stranded secondary structures occur in the single-stranded DNA (Kim et al., 1990). When agarose gels contain ethidium bromide single-stranded plasmids migrate further than CCC plasmids during electrophoresis. The agarose gel in Figure 6.4A contained ethidium bromide prior to electrophoresis and the bottom plasmid band in Figure 6.4B probably represented single-stranded plasmid. No homology was detected between pBC1 and the DNA samples isolated from the moderate thermophile strains LM1, LM2, ALV, and TH3.

The identity of the strains used for the the plasmid minipreparations in the previous experiment was confirmed by SDS-polyacrylamide gel electrophoresis (Methods 2.9.1) of lysed cell samples (Figure 6.5). Strains BC1 and TH1, which are closely related, gave identical bands on the gel and the sample from strain LM1 was similar. In a separate experiment samples of total DNA (chromosomal and plasmid) from strain BC1 were digested with HincII and hybridized to the same radioactive probe used to investigate the plasmid minipreparation samples

Figure 6.5 SDS-PAGE of Proteins From the Moderate Thermophiles.

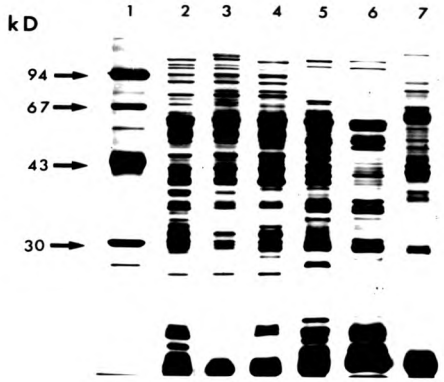
The figure shows the results of SDS-PAGE (10% [w/v] gel) of protein samples obtained from six of the moderate thermophile strains.

Cells were grown heterotrophically on 0.025% (w/v) yeast extract in Salts Medium at pH 2.0 and the gel was silver stained.

The protein standards used were: Phosphorylase b (94 kD); Bovine serum albumin (67 kD); Ovalbumin (43 kD) and Carbonic anhydrase (30 kD), supplied by Pharmacia.

Key to tracks:

1) Protein markers
2) Strain BC1
3) Strain TH1
4) Strain LM1
5) Strain ALV
6) Strain LM2
7) Strain TH3


kD
1
2
3
4
5
6
7
94
67
43
30

(i.e. the 2.6 kb HincII fragment of pFEG7). Figure 6.6 shows a single 2.6 kb band in total DNA from strain BC1 was homologous to the 2.6 kb pBC1-derived DNA of pFEG7. This confirmed the plasmid bands seen in Figure 6.4B (in the sample from strain BC1) were due to different conformations of one plasmid and these were linearized by HincII in Figure 6.7. Differences in sample buffers probably caused a slight change in mobility in sample 3 compared to samples 5 and 7 (see autoradiogram in Figure 6.6).

6.2.5 Investigation of pBC1 Host Range.

During attempts to characterize the host range of pBC1 recombinant vectors were constructed and their replication in E. coli and B. subtilis was investigated.

6.2.5.1 Cloning pBC1 into pBR325.

The origin of replication was not easily removed from recombinant vectors based on pACYC177. The pBC1 DNA was therefore cloned into an alternative E. coli vector. pLZ11 (Figure 6.2) was digested with restriction enzyme HindIII and the 2.6 kb pBC1-derived DNA purified by agarose gel electrophoresis. This fragment was ligated to pBR325 (6.0 kb; Tc^r, Cm^r, Amp^r) which had been linearized using the restriction enzyme HindIII and then dephosphorylated (Methods 2.4). Following transformation of E. coli DH1 (Methods 2.6.1), clones containing the recombinant plasmids pBRBC1 and pBRBC2 were identified by insertional inactivation of the Tc^r gene of pBR325. pBRBC1 and pBRBC2 differ in the orientation of the pBC1 insert (Figure 6.7).

6.2.5.2 Deletion of the pBR325 Origin of Replication.

The origin of replication of the pBR325 vector was deleted from pBRBC1 and pBRBC2 as outlined in Figure 6.8. pBRBC1 and pBRBC2 (8.6 kb)

Figure 6.6 Probing Total DNA from Strain BC1 With the 2.6 kb HincII Fragment From pFEG7.

(A) shows an agarose gel produced following electrophoresis of DNA samples:

Key to samples:

1) Lambda cut with HindIII
2) 1 kb ladder (BRL)
3) 2.6 kb pFEG7 HincII fragment
5) Total DNA from strain BC1 cut with HincII
7) Same as 5)
9) pACYC177 (3.94 kb) cut with HincII (contaminated with chromosomal DNA)
11) E. coli HB101 DNA cut with HincII

(B) The autoradiogram produced following transfer of the DNA samples in (A) on to nitrocellulose, and hybridization to the ^{32}P-labelled 2.6 kb HincII fragment from pFEG7. The nitrocellulose filter was subjected to a 90% stringency wash.

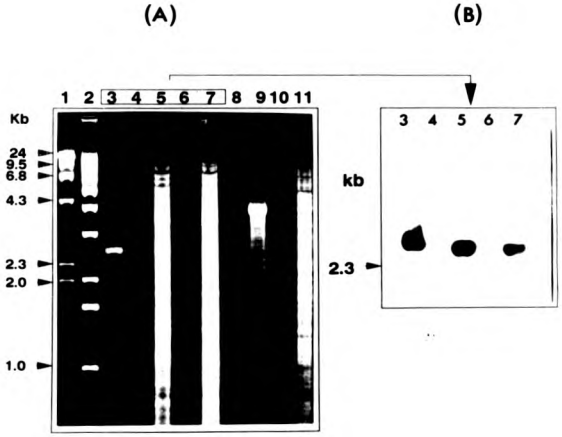

(A)
(B)
1 2 3 4 5 6 7 8 9 10 11
Kb
24
9.5
6.8
4.3
2.3
2.0
1.0
3 4 5 6 7
kb
2.3

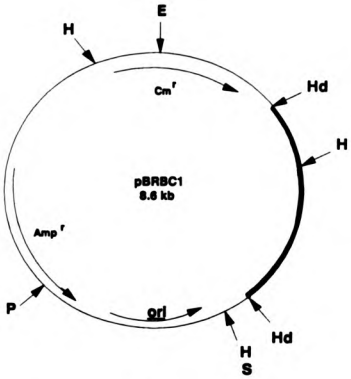
Figure 6.7 The Vectors pBRBC1 and pBRBC2.

Both vectors contain the entire plasmids pBR325 (6.0 kb; Bolivar, 1978; thin lines) and pBC1 (2.6 kb; thick lines). These plasmids were ligated via their unique HindIII sites. The tetracycline resistance gene of pBR325 was insertionally inactivated. Amp^r indicates the ampicillin resistance gene and Cm^r the chloramphenicol resistance gene of pBR325. ori indicates the origin of replication of pBR325.

pBRBC1 and pBRBC2 (8.6 kb) differ in the orientation of the pBC1-derived DNA insert.

Restriction enzymes are as follows:

E = EcoR1
H = HincII
Hd = HindIII
S = SalI
P = PstI


H
E
Cm r
Hd
H
pBRBC1
8.6 kb
Amp r
P
ori
H
S
Hd

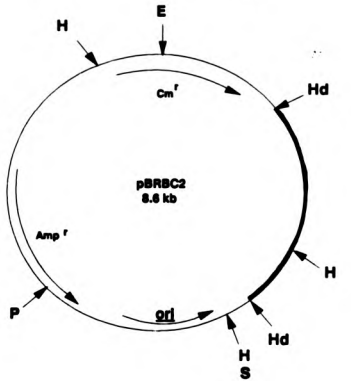
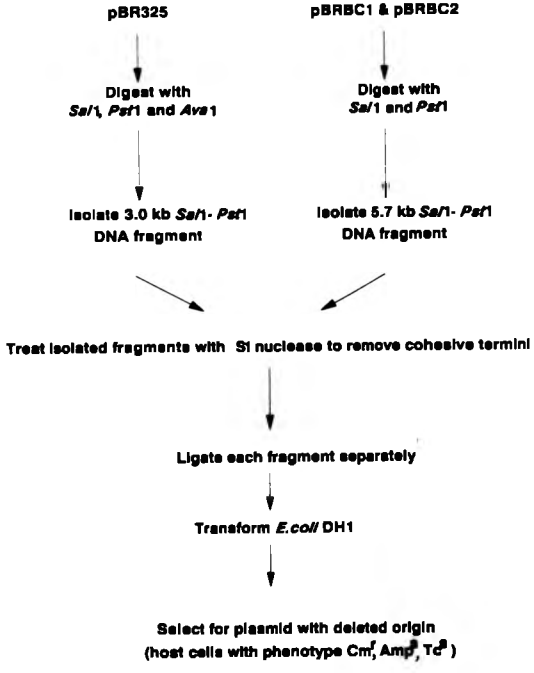

H
E
Cm r
Hd
pBRBC2
8.6 kb
Amp r
H
P
ori
Hd
H
S

Figure 6.8 Removal of the pBR325 Origin of Replication.

The figure indicates the manipulations carried out and DNA fragments isolated so that vectors derived from pBR325, pBRBC1 and pBRBC2 were created. The vectors created were deleted of the SalI-PstI fragment which contained the origin of replication of pBR325. These vectors were then transformed into E. coli DH1.

pBR325 was digested with AvaI in addition to SalI and PstI so that only one of the two PstI- SalI fragments of 3.0 kb could be identified and isolated.


pBR325
pBRBC1 & pBRBC2
Digest with
Sal1, Pst1 and Ava1
Digest with
Sal1 and Pst1
Isolate 3.0 kb Sal1- Pst1
DNA fragment
Isolate 5.7 kb Sal1- Pst1
DNA fragment
Treat isolated fragments with S1 nuclease to remove cohesive termini
Ligate each fragment separately
Transform E.coli DH1
Select for plasmid with deleted origin
(host cells with phenotype Cm^r, Amp^S, Tc^S)

were digested with the restriction enzymes SalI and PstI; the origin of replication derived from pBR325 is contained in a 3.0 kb SalI-PstI fragment. The 5.6 kb fragment was isolated by agarose gel electrophoresis and purified.

As a control, pBR325 was digested with SalI, PstI and AvaI. Digestion with SalI and PstI produces two similar sized fragments which cannot be differentiated easily by agarose gel electrophoresis. Digestion of the DNA with AvaI enabled the differentiation of the two fragments. The 3.0 kb fragment was isolated by agarose gel electrophoresis and purified.

The 5.6 kb fragment from pBRBC1 and pBRBC2, and the 3.0 kb fragment from pBR325, were each treated with S1 nuclease to remove the single stranded cohesive termini generated by the enzymes SalI and PstI. Each DNA fragment was then ligated and a sample of the ligated DNA analysed by agarose gel electrophoresis. In each sample multiple bands were visible in the gel indicating that ligation had occurred. The ligated DNA samples were transformed into E. coli DH1 by electroporation (Methods 2.6.2). The resulting cells were screened for recombinants replicating via the pBC1 replicon. Since some of the Amp^r gene was removed during deletion of the origin of replication of pBR325, recombinants replicating via the pBC1 replicon would be Amp^s, Tc^s and Cm^r. No colonies with this phenotype were detected. As expected no colonies resulted from the transformation of E. coli with the ligated 3.0 kb DNA from pBR325 because pBR325 treated in this way had no origin of replication. A control of pBR325 electrotransformed into E. coli DH1 did however give a transformation frequency of 2×10^6 cells/μg DNA indicating that successful transformation of E. coli DH1 with DNA had occurred.

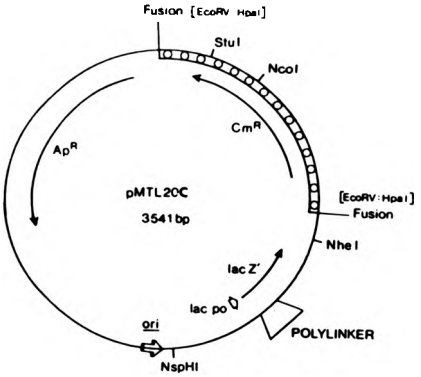
6.2.5.3 Investigation of the Replication of pBC1 in B. subtilis.

The plasmid pMTL20C (Figure 6.9; Swinfield et al., 1990) is replication-deficient in Gram positive hosts and is maintained in E. coli. pMTL20C contains pMTL20 (Chambers et al., 1988) derived from

Figure 6.9 The Gram Positive Replication-Deficient Plasmid pMTL20C.

The figure (taken from Swinfield *et al.*, 1990) shows the plasmid pMTL20C and below the restriction sites present in the polylinker sequence of the vector.

The 1.07 kb *Hpa*II fragment (boxed circles) carrying the pC194 chloramphenicol resistance gene (Cm^r; isolated from pBD64; Gryczan *et al.*, 1980) was inserted into the unique *Eco*RV site of pMTL20 (Chambers *et al.*, 1988). The position of the *lac* promoter/operator region is indicated by an open arrow and the ColE1 replication origin (*ori*) by a arrow on the circumference of the plasmid circle.



XhoI AccI SalI SmaI

HindIII SphI PstI StuI BglII NcoI MluI AatII XbaI BamHI KpnI SstI EcoRI

GCCAAGCTTGCATGCCTGCAGGCCTCGAGATCTCCATGGACGCGTTGACGTCSACTCTAGAGGATCCCCGGGTACCGAGCTCGAATTCAC

lacZ'

pBR322, the chloramphenicol acetyltransferase gene (Cm^r) derived from pC194 (isolated from S. aureus, see Horinouchi & Weisblum, 1982) and a synthetic polylinker within a lacZ' gene. The lacZ' gene produces a functional polypeptide which complements a defective lacZ gene carried by the E. coli host genome or resident episome. Colonies carrying plasmid DNA are therefore blue in the presence of the chromogenic substrate X-gal. DNA fragments cloned into the linker region cause insertional inactivation of lacZ' resulting in colourless (white) colonies in the presence of X-gal. In E. coli, pMTL20C expresses both Amp^r and inducible Cm^r.

Recombinant vectors constructed by insertion of a plasmid from a Gram positive host into the polylinker of pMTL20C can be transformed into a Gram positive host and may replicate (Swinfield et al., 1990). Detection of the recombinant plasmid in the Gram positive host is by selection for inducible Cm^r (the Amp^r gene does not function in Gram positive bacterial hosts).

6.2.5.4 Cloning pBC1 into pMTL20C.

pBC1 isolated from strain BC1 was linearized using the restriction enzymes HincII, KpnI and XbaI. Similarly, pMTL20C was linearized using the restriction enzymes SmaI, KpnI and XbaI and dephosphorylated (Methods 2.4). The linearized pBC1 was ligated to the linearized pMTL20C using the appropriate compatible termini i.e. HincII-SmaI, KpnI-KpnI and XbaI-XbaI. The ligated vectors were then transformed by electroporation into E. coli TG1 (lacZ deficient; Methods 2.6.2). Following electroporation, white colonies containing recombinants were selected by the expression of Amp^r and Cm^r. Four recombinant plasmids were identified; pMTL20CK1 and pMTL20CK2 which contain pBC1 cloned into the polylinker of pMTL20C via the KpnI site of pBC1. They differ in the orientation of the pBC1 insert (Figure 6.10A). Similarly, recombinants pMTL20CH1 and pMTL20CH2 contain pBC1 cloned into the polylinker of pMTL20C via the HincII site of pBC1.

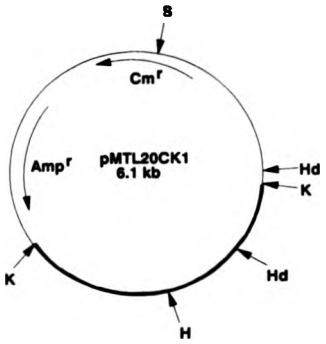
Figure 6.10A The Vectors pMTL20CK1 and pMTL20CK2.

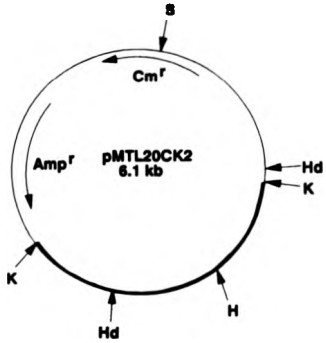
Both vectors contain the entire plasmids pMTL20C (3.54 kb; Swinfield et al., 1990; thin lines) and pBC1 (2.6 kb; thick lines). These plasmids were ligated via the unique KpnI site of both plasmids. Amp^r indicates the ampicillin resistance gene and Cm^r, the chloramphenicol resistance gene derived from pMTL20C.

pMTL20CK1 and pMTL20CK2 differ in the orientation of the pBC1-derived DNA insert.

Restriction enzymes are as follows:

K = KpnI
H = HincII
Hd = HindIII
S = StuI


S
Cm^r
Amp^r
pMTL20CK1
6.1 kb
Hd
K
K
Hd
H


S
Cm^r
Amp^r
pMTL20CK2
6.1 kb
Hd
K
K
Hd
H

They also differ in the orientation of the pBC1 insert (Figure 6.10B). No recombinants cloned into pMTL20C via the XbaI site were identified.

6.2.5.5 Transformation of B. subtilis with the pMTL20C Vectors.

Transformation of naturally competent cells of B. subtilis 168 (Spizizen, 1958) was carried out according to the method of Anagnostopoulos & Spizizen (1961; Methods 2.6.5).

pC194 isolated from B. subtilis was incorporated as a control and gave $4x10^2$ transformants/μg DNA. pMTL20CK1, pMTL20CK2, pMTL20CH1, pMTL20CH2 and pMTL20C all isolated from E. coli were used to transform B. subtilis 168. No transformants were obtained using pMTL20C DNA (replication deficient in Gram positive bacteria, incorporated as a negative control), or using the recombinant vectors. On the basis of these experiments it would seem that pBC1 does not replicate in B. subtilis 168.

6.2.6 The Antibiotic and Metal Resistances of E. coli Containing pFEG7.

A small amount of work was carried out to investigate expression of antibiotic and metal resistance genes from pFEG7 in E. coli.

E. coli HB101 containing pFEG7 and E. coli HB101 were streaked onto solid media containing antibiotics and metals. The following substances were investigated: Tetracycline (10 μg/ml), chloramphenicol (10 μg/ml), erythromycin (50 μg/ml), ampicillin (50 μg/ml), spectinomycin (50 μg/ml), copper sulphate (10 mM), sodium arsenate (1 mM), mercuric chloride (5 mM), uranyl sulphate (1 mM) and silver nitrate (10 mM).

Either E. coli HB101 containing pFEG7 never grew in the presence of the test substance or growth was comparable with the control, E. coli HB101 (data not shown). No further work was done to investigate in vivo expression of pBC1 genes in heterologous hosts. Studies of the antibiotic resistance and metal resistance profiles of strain BC1 are detailed elsewhere (Chapter 4).

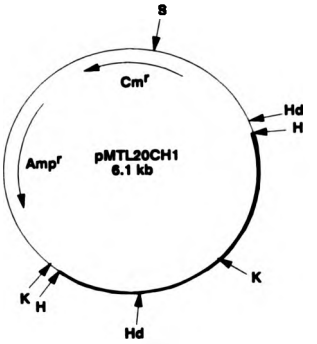
Figure 6.10B The Vectors pMTL20CH1 and pMTL20CH2.

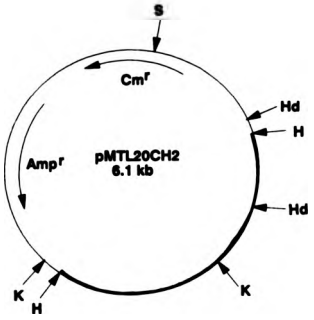
Both vectors contain the entire plasmids pMTL20C (3.54 kb; Swinfield *et al.*, 1990; thin lines) and pBC1 (2.6 kb; thick lines). These plasmids were ligated via the unique HincII site of pBC1 and the unique SmaI site of pMTL20C. Amp^R indicates the ampicillin resistance gene and Cm^R, the chloramphenicol resistance gene derived from pMTL20C.

pMTL20CH1 and pMTL20CH2 differ in the orientation of the pBC1-derived DNA.

Restriction enzymes are as follows:

K = KpnI
H = HincII
Hd = HindIII
S = StuI


S
Cm^r
Hd
H
Amp^r
pMTL20CH1
6.1 kb
K
K
H
Hd


S
Cm^r
Hd
H
Amp^r
pMTL20CH2
6.1 kb
Hd
K
K
H

6.2.7 In Vitro Expression of pBC1 Genes.

pBC1 was identified as a potential cloning vector for use in some of the moderate thermophile strains. In an attempt to further characterize the plasmid, the expression of genes from recombinant vectors containing pBC1 was investigated. The expression of polypeptides from these vectors using an E. coli in vitro transcription and translation system might provide information about the pBC1 DNA in the vectors, and might also indicate the potential of this system for other genetic studies of strain BC1.

6.2.7.1 In Vitro Transcription and Translation of CCC DNA.

Recombinant vectors containing pBC1 were incorporated into the prokaryotic DNA-directed translation kit (Amersham; Methods 2.9). The radioactive polypeptides produced were analysed by SDS-PAGE using a 12% (w/v) gel which was then fluorographed (Methods 2.9.1 & 2.9.2) and exposed to X-ray film. The results of autoradiography are shown in Figure 6.11A with a diagrammatic representation of these results in Figure 6.11B for clarity.

pACYC177 and pBR325 were used as controls since the recombinant plasmids used in this experiment contain these plasmids. The protein products of the antibiotic resistance genes of these plasmids were clearly visible i.e. ampicillin resistance protein (31.5 kD), kanamycin resistance protein (31.1 kD) and chloramphenicol resistance protein (25 kD; Murray et al., 1988). The molecular weights of ampicillin and kanamycin resistance determinants were predicted from the nucleotide sequence of pACYC177. The Tc^r gene product of pBR325 was not visible (Figure 6.11A). A sample containing no DNA was also used as a control and this sample did not produce any polypeptides (data not shown). pAT153 DNA was provided with the in vitro transcription and translation kit and the polypeptides produced from this plasmid are shown in Figure 6.11A.

The β-lactamase polypeptide (Amp^r) of both pBR325 and pAT153

Figure 6.11 In Vitro Translation Products of Plasmids Containing pBC1.

The fluorograph (A) shows the various ^{35}S-methionine labelled plasmid encoded products.

The $M_r \times 10^3$ (or kD) of polypeptides in the marker sample are indicated by arrows. Also, the position of the polypeptides translated from the ampicillin resistance genes (A), kanamycin resistance genes (K) and chloramphenicol resistance genes (C) of the plasmids are indicated. The tetracycline resistance gene product of pBR325 was not present.

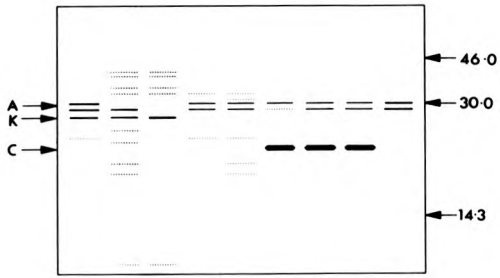
A schematic representation of the fluorograph (B) is shown.

Key to tracks:

1) pACYC177 encoded products
2) pFEG7 encoded products
3) pFEG15 encoded products
4) pLZ5 encoded products
5) pLZ11 encoded products
6) pBR325 encoded products
7) pBRBC1 encoded products
8) pBRBC2 encoded products
9) pAT153 encoded products
10) Polypeptide markers (not visible).

(A)

(B)



appeared to be present as two bands (a duplet), also, with less exposure to X-ray film than shown in Figure 6.11A, the kanamycin resistance polypeptide of pACYC177 was seen as a duplet (data not shown).

pFEG7 produced several polypeptides following *in vitro* transcription and translation other than the polypeptides encoded by the pACYC177 DNA of the recombinant vector (Figure 6.11A, tracks 1 & 2). The sizes of polypeptides were estimated by drawing a graph of distance migrated against the log of molecular weight, and the polypeptide markers were used to construct a standard curve (data not shown). The approximate molecular weights (kD) of putative polypeptides encoded by pFEG7 were; 42, 40, 36, 34 kD, two proteins representing Kan^r and four polypeptides with molecular weights 20-26 kD. These polypeptides are indicated in the the diagrammatic representation of the results (Figure 6.11B). The pFEG15 sample produced in addition, a polypeptide of about 12 kD (Figure 6.11A, track 3) which could partially be seen in the pFEG7 sample (Figure 6.11A, track 2) although somewhat obscured. During a longer exposure of the gel to X-ray film, the larger polypeptides expressed by pFEG7 (42, 40, 36, 34 kD) were visible in the sample produced from the *in vitro* transcription and translation of pFEG15 (Figure 6.12). The pLZ5 sample (Figure 6.11A, track 4) produced two bands (34 and 25 kD) besides pACYC177 specific polypeptides. These were also seen in the *in vitro* transcription and translation sample produced from pLZ11 (Figure 6.11A, track 5), and in addition, a polypeptide of about 33 kD and two polypeptides (20 and 22 kD), the latter two were also observed in the sample from pFEG7. The *in vitro* transcription and translation of pBRBC1 and pBRBC2 produced only those polypeptides specific to the pBR325 DNA (Figure 6.11A; tracks 6, 7 & 8).

It was highly unlikely that all of the putative polypeptides mentioned were produced by pBC1-derived DNA and many of the polypeptides probably represent those produced by aberrant initiation and termination of transcription and translation. The large polypeptide (42 kD) produced by pFEG7 and pFEG15 or the antibiotic resistance proteins may be the mature polypeptides of many of the smaller polypeptides observed in these

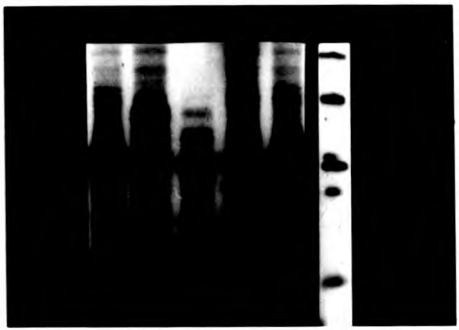
Figure 6.12 *In Vitro* Translation Products of Some Plasmids Containing pBC1 (a Long Exposure of The Fluorograph).

The fluorograph shows the various ^{35}S-methionine labelled plasmid encoded products.

The $M_r \times 10^3$ (or kD) of polypeptides in the marker sample are indicated by arrows. Also, the position of the polypeptides translated from the ampicillin resistance genes (A) and kanamycin resistance genes (K) of the plasmids are indicated.

Key to tracks:

1) pACYC177 encoded products
2) pFBG7 encoded products
3) pFBG15 encoded products
4) pLZ5 encoded products
5) pLZ11 encoded products
10) Polypeptide markers



samples. It is likely that the 42 kD polypeptide was translated from a ribosome binding site within the pBC1-derived DNA because the polypeptide was expressed from recombinant vectors when pBC1 was cloned into pACYC177 in both orientations (pFEG7 and pFEG15), although weaker expression occurred from the recombinant pFEG15. The absence of the 42 kD protein from the in vitro transcription and translation of the other recombinants (pLZ5, pLZ11, pERBC1 and pERBC2) indicated that cloning of the pBC1 DNA via the single HindIII site prevented expression of the 42 kD polypeptide. The 34 kD polypeptide produced from pFEG7, pFEG15, pLZ5 and pLZ11 but not pACYC177, indicated that this polypeptide may also be a mature polypeptide expressed from the recombinant plasmids.

6.2.7.2 In Vitro Transcription and Translation Using Linear DNA Templates.

Further studies using the in vitro transcription and translation system were carried out to investigate primarily the 42 kD polypeptide expressed by pFEG7. By digesting DNA with restriction enzymes before in vitro transcription and translation, the regions of DNA encoding specific polypeptides might be determined.

pFEG7 was digested with the restriction enzymes HincII, HindIII, EcoRV, KpnI, XbaI and AvaI. The resulting DNA fragments were phenol extracted, ethanol precipitated, resuspended in TE buffer (Methods 2.4) and used as templates for in vitro transcription and translation (Methods 2.9). Protein products were analysed by SDS-PAGE on a 12% (w/v) gel which was fluorographed (Methods 2.9.1 & 2.9.2) and exposed to X-ray film (Figure 6.13). The quantity of sample applied to the gel was calculated following TCA precipitation (Methods 2.9). 20,000 cpm of each sample were loaded onto the polyacrylamide gel. Anomalous SDS-PAGE profiles were produced; some samples requiring more exposure to X-ray film than others. This substantiates the suggestion that many small polypeptides were present in some samples producing a high scintillation count following TCA precipitation but proteins were not visible on the polyacrylamide gel

Figure 6.13 In Vitro Translation Products of Linear DNA Fragments of pFEG7.

The fluorograph shows the various ^{35}S-methionine labelled polypeptide products of restriction endonuclease digested pFEG7 templates. The size (kD) of polypeptides in the marker sample are indicated by arrows.

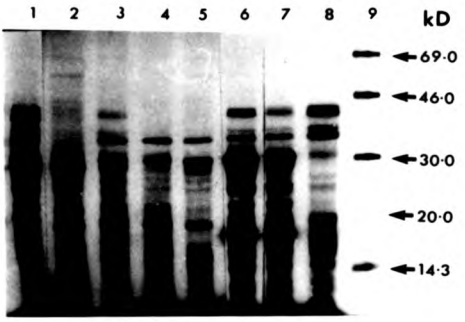
A partial restriction map of the 2.6 kb HincII fragment of pFEG7 representing pBC1 is shown below. The horizontal arrows indicate regions of DNA which might encode some of the putative polypeptides seen in the fluorograph (see text).

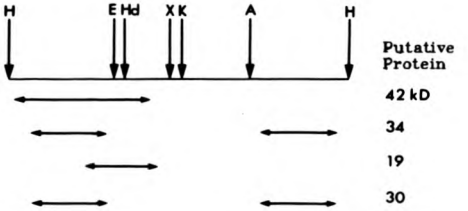
Key to tracks:

1) pFEG7 unrestricted
2) pACYC177 cut with HincII
3) pFEG7 cut with HincII
4) pFEG7 cut with HindIII
5) pFEG7 cut with EcoRV
6) pFEG7 cut with KpnI
7) pFEG7 cut with XbaI
8) pFEG7 cut with AvaI
9) Polypeptide markers

Key to restriction enzymes:

A = AvaI
E = EcoRV
K = KpnI
H = HincII
Hd = HindIII
X = XbaI


1
2
3
4
5
6
7
8
9
kD
69·0
46·0
30·0
20·0
14·3


H
E
Hd
X
K
A
H
Putative Protein
42 kD
34
19
30

and autoradiogram. The autoradiogram in Figure 6.13 thus resulted from different exposures of each sample to the X-ray film so that polypeptide profiles could be compared.

The position of the restriction sites within the pBC1 DNA in pFEG7 are indicated in Figure 6.13. The β-lactamase (31.5 kD) gene of pACYC177 contains a HincII restriction site, cleavage at this site would produce a truncated polypeptide of 9.2 kD. The kanamycin resistance (31.1 kD) gene of pACYC177 contains a HindIII site and two AvaI sites which, when cleaved, would produce truncated polypeptides of 20.9, 1.0 and 2.0 kD respectively. These polypeptides were not visible (Figure 6.13). A band representing a pACYC177 specific polypeptide of about 18 kD can be seen in Figure 6.13; tracks 2, 3, 4 and 5.

Since many polypeptides are visible in Figure 6.13, to save confusion, only those which meet the following criteria will be discussed in detail;

A) Those encoded by pFEG7 when it is cut with HincII and thus probably expressed by the pBC1 DNA.

B) Those not expressed by pACYC177.

Many truncated or nascent polypeptides are probably produced during this experiment and these may account for some of the bands on the autoradiogram (Figure 6.13).

The 42 kD polypeptide produced by the pBC1 DNA in pFEG7 (last Section) failed to appear on the polyacrylamide gel when the plasmid was digested with HindIII or EcoRV (Figure 6.13). This implied that the 42 kD polypeptide was encoded by a region of the pBC1 DNA including these restriction enzyme sites, and this is indicated in the schematic diagram in Figure 6.13. The same observation applied to the 40 and 36 kD polypeptides indicating that these polypeptides may be derivatives of the DNA encoding, or nascent forms of the 42 kD polypeptide.

A polypeptide of about 34 kD was produced by pFEG7 when digested with all the enzymes used, and a polypeptide band which migrated to the same position as the 30 kD protein marker appears in all samples except pACYC177. This latter protein had previously been described as one of two

bands representing the kanamycin resistance protein (last Section). Both AvaI and HindIII cleave within the kanamycin resistance gene of pACYC177 yet, the 30 kD polypeptide is produced by samples of pFEG7 digested with these enzymes (Figure 6.13, tracks 4 and 8). This putative polypeptide was therefore positioned on a map of the pBC1 DNA in Figure 6.13, in a similar way to the 34 kD polypeptide (when the size of the polypeptide and the DNA required to encode such a polypeptide were taken into consideration).

The level of expression of the 30 kD polypeptide can be compared to that of the kanamycin resistance polypeptide within the same sample. Comparatively stronger expression of the 30 kD polypeptide occurred from pFEG7 DNA that had been digested with KpnI and XbaI (Figure 6.13, tracks 6 & 7) than pFEG7 cut with HincII (Figure 6.13, track 3). This may indicate the presence of stronger signals for transcription and translation of the 30 kD polypeptide in the former two samples than those for the expression of the antibiotic resistance gene.

A polypeptide of about 19-20 kD was produced by all samples of pFEG7, except when the DNA was digested with HindIII and EcoRV. This polypeptide was probably a nascent form of the 42 kD polypeptide (Figure 6.13). A band on the autoradiogram represented a polypeptide of about 14 kD (Figure 6.13, tracks 6, 7 and 8). The 14 kD polypeptide produced by *in vitro* transcription and translation was not produced when pFEG7 was digested with HincII, HindIII or EcoRV.

6.3 Discussion.

Investigations of pBC1 were aimed at characterizing the plasmid, trying to identify the position of the essential replication machinery of the plasmid, and also identifying polypeptides encoded by pBC1-derived DNA.

pBC1 was first inserted into an *E. coli* vector so that large quantities of pBC1-derived DNA could be produced and also to begin the vector construction programme. The production of shuttle vectors for use

in E. coli and strain BC1 was considered beneficial and the genetic manipulation of this kind of vector could be carried out in E. coli using well established methods. The comparative difficulty of producing larger quantities of strain BC1 and growing strain BC1 particularly on solid media would also be avoided. Once DNA manipulations were completed the shuttle vector could be transferred into strain BC1.

Several unique restriction sites were identified within the pBC1-derived DNA of pFEG7 and pFEG15. This information was useful for much of the work described in this thesis.

It was important to construct several different vectors based on pBC1, these might be useful during future work and were also used in attempts to define the host range of pBC1. Unfortunately, initial efforts to transfer pBC1-derived vectors into E. coli and B. subtilis gave no additional information.

Rawlings et al. (1984a) had shown that a T. ferrooxidans plasmid inserted into pBR325 could replicate in E. coli when the origin of replication of pBR325 was deleted. In a similar experiment pC194, isolated from S. aureus (Iordanescu, 1975), was found to replicate in E. coli (Goze & Ehrlich, 1980) and pE194 also isolated from S. aureus replicated in several species of Gram negative bacteria (see Sozhamannan et al., 1990) but pC221 also isolated from S. aureus (Iordanescu et al., 1978) did not replicate in E. coli (Goze & Ehrlich, 1980).

The results presented here implied that pBC1 did not replicate in E. coli DH1. However, cloning pBC1 via the HindIII site might have disrupted the replication machinery of pBC1 and DNA sequencing results obtained at a later stage indicated the DNA region around the HindIII is essential for replication of pBC1 (Chapter 8). Therefore, to conclude that pBC1 could not replicate in E. coli DH1, further work would be necessary in which the essential replication machinery of pBC1 remains intact.

The frequency of plasmid-mediated transformation of B. subtilis is low; from $1x10^{-3}$ to $1x10^{-1}$ of the cells at saturating amounts of DNA (see Bron, 1990). One reason for the low efficiency is the requirement for

plasmid multimers (Canosi et al., 1978) or monomers containing internal repeats (Michel et al., 1982). When plasmids bind to competent cells, the plasmid DNA becomes randomly linearized and only one strand is taken up (de Vos et al., 1981). Recircularization to re-establish the plasmid requires a region of homology which can only be provided by fragments of different polarity or by internally repeated molecules (see Bron, 1990).

The recombinant vectors based on pMTL20C did form multimers in E. coli TG1 (as seen on agarose gels, data not shown) and would have been expected to transform B. subtilis 168 with lower frequencies than pC194 because the recombinant plasmids were isolated from a heterologous host. It is noteworthy that vectors based on the E. coli vector pBR322 and pC194 or pUB110 (ssDNA plasmids, see Chapter 1) form multimeric molecules in E. coli and such DNA is usually efficient in B. subtilis competent cell transformation (see Bron, 1990). B. subtilis 168 contains a weak restriction system (the primary function of these systems is to degrade incoming foreign DNA; Gryczan, 1982; Uozumi et al., 1977; Bron et al., 1988) but, Swinfield et al. (1990) used recombinant vectors based on pMTL20C (containing a replicon from a Gram positive plasmid maintained and isolated from E. coli TG1) to successfully transform B. subtilis 168.

Results in this work, indicated that pBC1 would not replicate in B. subtilis 168. However it cannot be ruled out, that the replication machinery of pBC1 was made ineffective by linearization of the plasmid by the restriction enzymes HincII and KonI during vector construction. Nucleotide sequencing of pBC1 carried out at a later stage indicated that these restriction sites do not occur within any essential genes but the spatial relationships of 'replication elements' may be altered in the pMTL20C-based recombinants, and this may be significant. Also, the restriction system of B. subtilis 168 may have digested specific sequences in the pBC1-derived DNA (Bron et al., 1988). Other plasmids from Gram positive bacteria do not replicate in B. subtilis, e.g. clostridial replicons (Young et al., 1989; Minton et al., 1990), or are unstable, e.g. pC221, pT181, pC194 (Bron, 1990).

The small plasmid in strain TH1 exhibited homology to pBC1 and these

plasmids are probably identical. This was interesting because strain BC1 was isolated from Warwickshire and strain TH1 from Iceland (see Chapter 1). This observation might also imply that pBC1 serves a useful function in the host cell. pBC1 however, remained a cryptic plasmid and attempts to cure strain BC1 of the plasmid were not made during the course of this work.

Single-stranded plasmid was probably present in DNA samples from strains BC1 and TH1 (Figure 6.4) and single-stranded plasmid has been identified in plasmid samples from Gram positive bacteria (te Riele et al., 1986a).

Antibiotic and heavy metal resistance genes are often carried by bacterial plasmids (for reviews see Couturier et al., 1988; Stanisich, 1984) but basic replicons, i.e. the genes and sites required for autonomous replication and its control, are usually 2-3 kb in length (Couturier et al., 1988), and it would therefore seem unlikely that pBC1 would possess additional characteristics. Small plasmids are however known to express resistance to antibiotics, e.g. pC194 is 2.9 kb and carries the Cm^r gene (Horinouchi & Weisblum, 1982), or bacteriocin synthesis and immunity (see Giambiagi-Marval et al., 1990). Bacteriocins generally have a narrow spectrum of activity and are lethal only for bacteria which are closely related to the strains which produce them. Bacteriocin production seems to be a characteristic of both Gram negative (Hardy, 1975) and Gram positive bacteria (Tagg et al., 1976). Heavy metal resistance has been assigned as a phenotypic property of several plasmids from Gram positive bacteria (for reviews see Silver et al., 1989; Foster, 1983; Silver & Misra, 1988), but it is unlikely that pBC1 would contain heavy metal resistance genes because they usually form an operon consisting of over 2 kb of DNA (Silver & Misra, 1988). The expression of resistance from pFEG7 to some antibiotics and metals was not detected during simple assays on solid media.

Results indicated that pBC1 DNA was expressed in an *E. coli* *in vitro* transcription and translation system, but many aberrant polypeptides made results difficult to interpret. There are many reports of "extra"

polypeptide bands on autoradiograms following this type of experiment. These bands frequently occur when DNAs from heterologous sources are expressed in E. coli in vitro transcription and translation systems, particularly DNA derived from Gram positive bacteria (Leventhal & Chambliss, 1979; Pratt et al., 1981). The DNA-dependent RNA polymerase of E. coli is able to initiate transcription at sites on the DNA not normally used in the host bacterium (Pratt et al., 1981), and prematurely terminated polypeptides have also been reported (Sancar et al., 1981; Stoker et al., 1984), along with degradation products (Thompson et al., 1984) or nascent peptides (Collins, 1979). High magnesium ion concentrations facilitate initiation of transcription and translation at unauthentic sites and reduce the fidelity of protein synthesis (Pratt, 1984). It would therefore seem likely that many of the polypeptides produced from the recombinant DNA samples used in these experiments are artefacts of the in vitro transcription and translation system.

Comparisons of the expression of the 30 kD polypeptide from pBC1-derived DNA and the kanamycin resistance determinant within the same sample might imply that element(s) which repressed expression of the 30 kD polypeptide had been removed in those samples where strong expression of this polypeptide occurred, i.e. pFEG7 digested with KpnI and XbaI (Figure 6.13). Such elements might include stem-loop structures or RNA countertranscripts. Stem-loop structures in DNA or RNA species do control the levels of expression of many bacterial genes (McClure, 1985), and RNA countertranscripts have been implicated in the control of expression of genes from plasmids of Gram positive bacteria (see Chapter 1).

To summarize, several polypeptides were identified following the in vitro transcription and translation of pFEG7 linear templates. The genes of some of these putative protein products of pFEG7 have been assigned to regions of the pBC1 DNA (Figure 6.13). The fact that the genes of many of these putative polypeptides are placed within the same region of the pBC1 DNA suggested that they may be immature forms of the 42 kD polypeptide. Some of the polypeptides produced by pFEG7 are further discussed in Chapter 9 (General Overview) in relation to data obtained

following the determination of the nucleotide sequence of pBC1 (Chapter 8).

Attempts to transfer pBC1-derived vectors into the moderately thermophilic strains ALV and BC1 (next Chapter) were carried out simultaneously with much of the work discussed in this chapter. The inability to detect transformants of strains BC1 and ALV prompted the determination of the nucleotide sequence of pBC1. It was possible that this would help define the minimal replicon of pBC1. The characterization of vectors for genetic manipulation is important for the development of genetic systems and further characterization of pBC1 is discussed in relation to the nucleotide sequence in Chapter 8.

If pBC1 was used as a cloning vector for strain BC1, the expression of genes inserted into the plasmid could be investigated *in vitro* in an *E. coli* system, although an *in vitro* expression system based on *B. subtilis* (Leventhal & Chambliss, 1979) might be more accurate due to limitations of the *E. coli* system which have been discussed.

Chapter 7.
Electroporation of Strains ALV and BC1.

7.1 Introduction.

The introduction of DNA into bacteria is essential for the production of recombinant strains and until recently the most common methods for the introduction of plasmids into bacteria were conjugation (and mobilization) and natural or chemically induced transformation. The development of these methods can be time consuming and they are usually restricted to specific strains of bacteria.

Recently, electrotransformation or electroporation has been used to successfully transfer plasmids into many species of both Gram negative and Gram positive bacteria (see Table 1.2, Chapter 1). The electroporation of bacteria is expressed as a frequency (transformants/survivors), or more commonly as an efficiency (transformants per μg of DNA) because in most experiments the total number of transformants that can be obtained is the critical parameter and DNA is usually limiting. Efficiencies of up to 10^{10} transformants/μg DNA have been reported (Dower et al., 1988; O'Callaghan & Charbit, 1990).

The Biorad Gene Pulser apparatus is the most widely used electroporation equipment. In conjunction with the Biorad Pulse Controller, the capacitor discharge device produces exponentially declining pulses of field strengths up to 12.5 kV/cm. The time needed for a given pulse to decline to 37% from its initial setting is displayed by the apparatus as the time constant in milliseconds (ms). The duration of the pulse primarily depends on the resistance setting but also the conductivity of the buffer and choice of capacitor. The pulse is delivered to a sterile disposable cuvette which contains electrodes 0.4 cm or 0.2 cm apart, and the voltage gradient between the electrodes

determines the field strength (E) which is:

$$E = \frac{\text{Voltage}}{\text{Distance}}$$

where the voltage is in kilovolts and the distance is the gap between the electrodes in cm. Dower et al. (1988) have described the electrical circuit and cuvettes.

7.2 Experimental Considerations.

The electrical parameters of central importance for bacterial electroporation are:

a) The field strength

b) The time constant

Overall, using short pulses, increases in the field strength lead to an increased transformation efficiency. Alternatively, at low field strength, increased pulse lengths are usually beneficial. Thus, electric field strength and time constant have compensatory effects (Dower et al., 1988) but the range of this compensation is limited and transformants may not be obtained with field strengths of less than 3 kV/cm, even with very long pulses.

The preparation of bacterial cells for electroporation appears to be a key factor for the success of the method and bacterial cells are usually grown in a rich complex medium to early or mid-exponential growth phase (Dower et al., 1988; Brigidi et al., 1990; Suvorov et al., 1988; Liebl et al., 1989; Dunican & Shivnan, 1989; Haynes & Britz, 1989) and concentrated to a high cell density of about 10^7-10^{14} cells/ml (Brigidi et al., 1990; Harlander, 1987; Liebl et al., 1989; Kim & Blaschek, 1989). It is also important to reduce the ionic strength of the cell suspension by washing the cells in a low ionic strength solution (Biorad Pulse Controller Manual). With the moderate thermophiles it was also important to remove ferrous and ferric ions from the growth medium. Several bacterial species including E. coli, Bacillus thuringiensis, Clostridium perfringens, Corynebacterium glutamicum,

Brevibacterium lactofermentum, Staphylococcus epidermidis, Agrobacterium tumefaciens, and Bacteroides uniformis have all been electroporated in 10-20% (w/v) glycerol alone (Dower et al., 1988; Masson et al., 1989; Kim & Blaschek, 1989; Liebl et al., 1989; Haynes & Britz, 1989; Augustin & Gotz, 1990, Wen-jun & Forde, 1989; Thompson & Flint, 1989).

Highly purified CCC plasmid DNA is usually used for electrotransformation and there does not appear to be a requirement for a pre-pulse incubation of cells and DNA (Dower et al., 1988). Following electroporation, cells can be immediately transferred to growth medium (Dower et al., 1988; Augustin & Gotz, 1990). An expression period is then allowed so that plasmid replication and the expression of reporter genes (usually antibiotic resistance) can occur prior to selection of electrotransformants on an appropriate selective solid medium.

Electroporation was used while attempting to transform the moderate thermophile strains ALV and BC1. An initial protocol was developed based on observations made by other workers who had successfully electrotransformed bacterial species (see Table 1.2 in Chapter 1 and the Biorad Pulse Controller Manual). The main steps of this protocol were (Methods 2.6.3):

a) Growth of the cell population to early/mid-exponential growth phase.
b) Harvesting of cells and concentration to a high cell density after washes to reduce the ionic strength of the cell suspension and remove iron from the cells.
c) Electroporation of the cells with plasmids (containing antibiotic resistance gene[s]).
d) Selection of antibiotic resistant electrotransformants.

Experiments indicated that the minimum inhibitory concentration (MIC) of chloramphenicol for strains ALV and BC1 was 2 μg/ml (Results Chapter 4, Section 4.2.2). Chloramphenicol is normally used at a concentration of about 5-10 μg/ml for the selection of Gram positive

transformants (Gasson & Anderson, 1985; Imanaka et al., 1981; Kok et al., 1984; Gleave et al., 1990). Some inactivation of chloramphenicol occurred during incubation in the growth medium (pH 1.7 at 45°C) but a concentration of about 0.1 µg chloramphenicol/ml appeared to remain active for about 10 hours (Results Chapter 4, Section 4.2.2) and this concentration could therefore be used where necessary as a subinhibitory concentration for the induction of chloramphenicol resistance.

The MIC of kanamycin for strains ALV and BC1 was 2 mg/ml during a 30 hour incubation (Results Chapter 4, Section 4.2.3); kanamycin is normally used at a concentration of 10-30 µg/ml for the selection of Gram positive transformants (Gleave et al., 1990; Imanaka et al., 1981; Bingham et al., 1979; Gasson & Anderson, 1985) but 1 mg/ml has been used for Enterococcus faecalis (Krah & Macrina, 1989) and Acidiphilium sp. (F. F. Roberto, pers. comm.). Kanamycin was highly unstable and was probably a poor choice for the selection of transformants of strains ALV and BC1. Due to a restricted range of characterized antibiotic resistance genes which express in Gram positive bacteria, kanamycin was investigated as a selection agent with the assumption that as long as the time at which wild type bacteria began to grow was known, then the time limit for the selection of transformants was also known.

No transformants of the moderate thermophile strains ALV or BC1 were produced so a method was developed which indirectly allowed the detection of plasmid transfer into the moderate thermophile strains by electroporation, but which did not require plasmid replication or the expression of antibiotic resistance genes. This method was based on the principle that if bacteria were successfully electroporated, plasmid molecules that entered the bacterial cells would be protected from digestion by exogenous DNase; these intracellular plasmids could then be recovered by plasmid minipreparation of the cells. The quantity of plasmid recovered might have been insufficient to detect by agarose gel electrophoresis so a more sensitive method of detection was used by electrotransformation of E. coli with the plasmid minipreparation DNA. Thus, plasmids transferred during the initial (or primary)

electroporation would be detected by a secondary electroporation of E. coli. Unfortunately, the amount of plasmid transferred during the initial electroporation could not be accurately quantified because detection was limited by the efficiency of the secondary electroporation. However, because the electrotransformation efficiency of E. coli was high, relative efficiencies of the primary electroporation could be estimated. In addition, because replication of plasmids was not required in the initial bacterial host, plasmids which electrotransform E. coli with high efficiency, such as pBR- or pUC-based vectors (Dower et al., 1988), were used.

7.2.1 Plasmids.

For the successful production of electrotransformants of the moderate thermophile strains ALV and BC1, vectors which replicated and expressed a selectable antibiotic resistance were required. Before the initiation of a lengthy and time consuming programme for the production of suitable recombinant vectors, some commonly used vectors for Gram positive bacteria were investigated particularly those with a broad host range and a chloramphenicol or kanamycin resistance gene.

pC194 (2.9 kb) and pC221 (4.5 kb) were isolated from S. aureus (Iordanescu, 1975; Iordanescu et al., 1978) and replicate in B. subtilis (Ehrlich, 1977). In addition pC194 replicates in B. thuringiensis (Bone & Ellar, 1989; Mahillon et al., 1989), Lactobacillus acidophilus (Luchansky et al.,1988), Lactobacillus leichmannii (Cardy, 1989b) and Streptococcus pneumoniae (Ballester et al., 1986). Both plasmids carry an inducible gene for chloramphenicol acetyltransferase and 386 bp of pC221 were deleted to form the higher copy number vector pC221cop903 (Projan et al., 1985).

pCK1 (5.5 kb; Gasson & Anderson, 1985) is a recombinant plasmid which contains the replication region of a cryptic streptococcal plasmid pSH71 (Gasson, 1983) and the antibiotic resistance genes (Kan^r and Cm^r) from the Bacillus vector pBD64 (Gryczan et al., 1980). pCK1 replicates in

B. subtilis, Streptococcus lactis, E. coli (Gasson & Anderson, 1985) and L. leichmannii (Cardy, 1989b).

pMTL20CK1, pMTL20CK2, pMTL20CH1 and pMTL20CH2 (6.1 kb; this thesis Figures 6.10A & 6.10B) contain the pC194 Cm^r gene and the 2.6 kb pBC1-derived DNA. The plasmids differ in the arrangement of the pBC1 DNA.

pAT187 (10.5 kb) contains the origins of replication of the E. coli vector pBR322 and the broad host range streptococcal plasmid pAMβ1, and a kanamycin resistance gene derived from a plasmid of Campylobacter coli which is known to be expressed in both Gram positive and Gram negative bacteria (Trieu-Cuot et al., 1987). pAT187 has been transferred into E. coli, Enterococcus faecalis, S. lactis, Streptococcus agalactiae, Bacillus sphaericus, B. thuringiensis, Listeria monocytogenes and S. aureus (Trieu-Cuot et al., 1987).

7.3 Results.

7.3.1 The Survival Of Cell Suspensions On Ice.

Cells of strain ALV and BC1 were prepared for electroporation (Methods 2.6.3) and a dense cell suspension of about 2 x 10^7 cells/ml produced. Aliquots (50 µl) of this cell suspension were kept on ice in Eppendorf tubes for 30, 60, 90 and 120 minutes.

Samples were transferred to flasks containing Salts Medium at pH 1.7 and supplemented with ferrous sulphate and yeast extract (Methods 2.2.2 and 2.2.3) and growth was monitored by assaying the ferrous iron oxidized. A control consisted of cells not incubated on ice but immediately used as an inoculum.

All samples exhibited identical growth rates and no lag phase was evident (data not shown). Therefore, incubation of the cells on ice probably had no significant effect on cell numbers, cell viability and subsequent growth in liquid media. However, heavy inocula were used and some damage to the cells may have been masked by the growth of large quantities of unaffected bacteria.

7.3.2 The Survival Of Strains ALV and BC1 Following Electroporation.

Cells of strains ALV and BC1 were prepared for electroporation (Methods 2.6.3) and 40 µl aliquots electroporated using a Biorad Gene Pulser, Pulse Controller Unit and 0.2 cm disposable cuvettes. Plasmid DNA was omitted from samples.

Following electroporation, samples were immediately added to 5 ml of Salts Medium at pH 1.7 and supplemented with ferrous sulphate and yeast extract (Methods 2.2.2 & 2.2.3). The samples were then incubated for 6 hours (about 2 doubling-times) at 45°C, serially diluted in Salts Medium at pH 1.7 (to 10^{-8}) and 100 µl of each dilution were used as inocula for pour plates (Methods 2.2.2).

The number of colony forming units (cfu) in each of the electroporated samples was determined following an eight day incubation period at 45°C and expressed as a percentage of the number of cfu in a control which was not electroporated. As expected strain BC1 produced only a few single colonies due to poor growth on this type of medium (Chapter 3, Section 3.2.6), but the results for strain ALV are shown in Figure 7.1. Overall, survival rates of strain ALV decreased with higher field strengths.

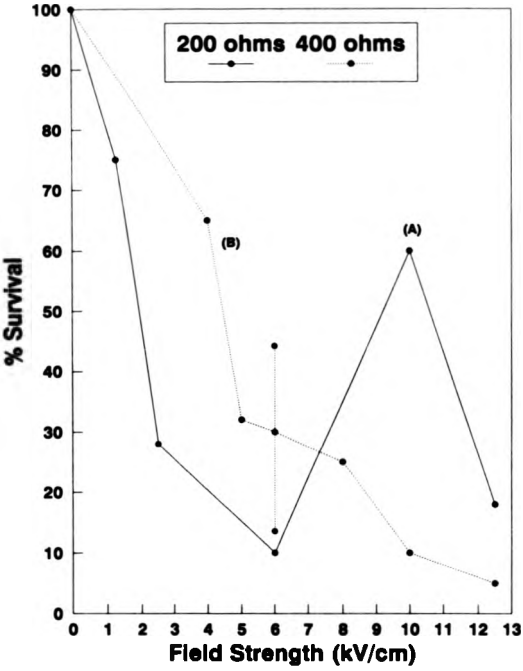
7.3.3 The Selection Of Electrotransformants Using Chloramphenicol.

Initial electroporation experiments did not result in electrotransformants of strain ALV, i.e. no resistant colonies were obtained on pour plates containing chloramphenicol. Chemolithoheterotrophic growth of strain BC1 on solid media was poor (Results Chapter 3). Selection of electrotransformants in liquid media was therefore attempted.

It was desirable (for convenience) to introduce an overnight break

Figure 7.1 The Effect Of Electroporation On Strain ALV.

The figure illustrates the percentage of cfu of strain ALV which survive electroporation at different field strengths. The field strength (E) was calculated by dividing the voltage applied to the sample by 0.2 cm (the electrode gap). The effective resistance placed in parallel with the electrodes determined the time constant of the pulse and in (A) the resistance was 200 ohms which gave a time constant of about 4.5 ms. In (B) the resistance setting was 400 ohms, which gave a time constant of about 9 ms. For all samples the capacitor was 25 μF and the buffer was 10% (v/v) glycerol.


200 ohms 400 ohms
% Survival
Field Strength (kV/cm)
(A)
(B)
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13

into the electrotransformation protocol and after electroporation was considered to be the most appropriate time for this. Typically, following growth of the cells overnight, monitoring the cells to the appropriate growth phase through to electroporation took 8 hours. In addition, an expression period of about 6 hours was thought to be required, a stage which could possibly be carried out overnight. However, if the electroporated cells were incubated with an overnight expression period at 45°C in a ferrous iron medium, this would probably result in much growth of the cells and the formation of a large amount of ferric iron which would then inhibit the bacteria. In addition, the loss of some plasmids (in transformants) could occur because there would be no selection pressure to promote their retention. Therefore, an expression period at 30°C was proposed to reduce the overnight growth, followed by selection of transformants using chloramphenicol in liquid media at 45°C.

It has been demonstrated that cells are killed during the electroporation procedure and for a given quantity of cells surviving electroporation, it was necessary to determine when wild-type bacteria would grow in liquid media containing heat- and acid-inactivated chloramphenicol.

Cells of strain ALV were prepared for electroporation (Methods 2.6.3) and 40 µl aliquots electroporated (without plasmid) and subsequently treated as indicated in Table 7.1A. The stages of the protocol can best be described as time periods: expression, induction and selection as a simulation of the stages of an electrotransformation experiment when plasmid was used. The expression period and induction period occurred simultaneously in Samples 6-10 and a sub-inhibitory concentration of antibiotic was used for induction. In some instances (Samples 1-5) these two periods were carried out separately (see Table 7.1A). In a second experiment, strain BC1 was treated in a similar fashion although in this case all samples except two (controls) were subjected to a simultaneous expression and induction period as for Samples 7-10 in Table 7.1A. The controls were treated as for Samples 1 and 6 in Table 7.1A.

Table 7.1 The Treatment and Growth of Electroporated Cells of Strain ALV in Medium Containing Chloramphenicol

The experiment was designed to simulate electrotransformation but with no plasmid used during the electroporation. The main objective was to determine whether the conditions could be used for selection of recombinants following electroporation and to investigate whether a sufficient period would be allowed to select for transformants prior to the growth of wild-type cells. As such, although no plasmid was present the treatment of samples is referred to as expression, induction and selection.

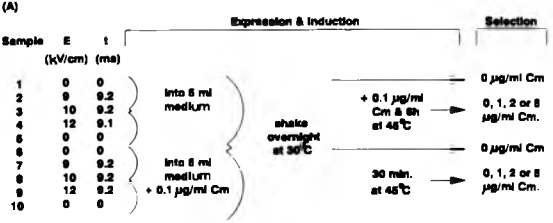
(A) Outline of the treatment of samples following electroporation.

Following expression and induction, 1 ml of samples 2-5 and 7-10 were used to inoculate four flasks containing growth medium and chloramphenicol which was added to a final concentration of 0, 1, 2 or 5 µg/ml for selection. These flasks were labelled with the sample number and a, b, c and d respectively. 1 ml of samples 1 and 6 were used to inoculate similar flasks but no chloramphenicol was added. These samples had not been electroporated and were controls.

(B) Growth of strain ALV in flasks during the selection period.

The growth of cultures was monitored using the amount of ferric iron in the flasks i.e. the amount of red/brown colouration in the flasks. The field strength (E) used during electroporation of the samples and the concentration of chloramphenicol in the flasks is indicated. The time of observation refers to the time when growth was first observed in the flasks after inoculation (t = 0). The Table includes only those flasks in which growth was observed. Typically, + indicates early exponential growth phase, ++ indicates mid-exponential growth phase and +++ indicates late exponential to stationary growth phase.

(A)



(B)

I) Samples which had separate expression and induction periods (samples 1-5)

Flask No.	E (kV/cm)	Cm (µg/ml)	Time of observation (h). 16	18	42	66	72	86	95
1	0	0	++	++	+++	+++	+++	+++	+++
2a	9	0			++	+++	+++	+++	+++
2b	9	1						+	++
3a	10	0				+++	+++	+++	+++
4a	12	0				+++	+++	+++	+++
5a	0	0	++	++	+++	+++	+++	+++	+++
5b	0	1			+	++	+++	+++	+++

II) Samples which had simultaneous expression and induction periods (samples 6-10)

Flask No.	E (kV/cm)	Cm (µg/ml)	Time of observation (h). 22	24	31	48	114
6a	0	0	++	+++	+++	+++	+++
7a	9	0		+	++	+++	+++
8a	10	0			+	++	+++
9a	12	0			+	++	+++
10a	0	0	++	+++	+++	+++	+++
10b	0	1				++	+++
10c	0	2					+

Following the expression and induction stages of the protocol, 1 ml of each sample was used to inoculate flasks containing 0, 1, 2 or 5 μg Cm/ml for 'selection' and growth was monitored visually as red/brown ferric iron production. The time at which growth was seen in samples of strain ALV is shown in Table 7.1B and similar results were obtained using strain BC1.

7.3.4 Electroporation Of Strains ALV and BC1 With Plasmids Encoding Chloramphenicol Resistance.

Plasmids were isolated from their host bacteria (Materials and Methods, Table 2.2) and purified by caesium chloride density gradient centrifugation (Methods 2.5.1, 2.5.2 and 2.5.5).

Cells of strains ALV and BC1 were prepared for electroporation (Methods 2.6.3) and stored on ice. 40 μl of cell suspension were mixed with 5 μl (2.5 μg) of plasmid DNA and immediately transferred to a chilled 0.2 cm electroporation cuvette and electroporated as in Table 7.2.

Following electroporation, samples were removed from the electroporation cuvette into 5ml prewarmed (30°C) Salts Medium at pH 1.7 and containing ferrous iron and yeast extract (Methods 2.2.2 & 2.2.3). Chloramphenicol (Cm) was added to a final concentration of 0.1 μg/ml and the samples incubated overnight at 30°C and then 30 minutes at 45°C. Selection of electrotransformants was carried out as described (Methods 2.6.3) using 0, 1, 2 or 5 μg Cm/ml and growth was monitored by visually estimating the amount of iron-oxidized in the flasks after, 21, 24, 27, 43, 60, 84, 100 and 124 hours. The time when growth was observed in cultures containing 0 and 1 μg Cm/ml is indicated in Table 7.2. Flasks containing 2 or 5 μg Cm/ml showed no growth over the 124 hour period of observation. Some samples of strains ALV and BC1 electroporated with plasmid and inoculated into flasks containing 1 μg Cm/ml grew before the corresponding controls (cells electroporated without plasmid).

A half of all samples which grew in the 124 h period of observation

Table 7.2 The Electroporation of Strains ALV and BC1 with Plasmids Specifying Chloramphenicol Resistance.

Strains ALV and BC1 were electroporated using the electrical parameters indicated and 25 μF capacitance setting. The time constant given is the average for the two samples electroporated, i.e. strain ALV and strain BC1. Those samples which contained no plasmid were controls as were samples which contained plasmid but which were not electroporated. The former of these controls was used to indicate when growth of wild-type bacteria occurred. The time when growth was first seen in the samples containing 0 and 1 μg Cm/ml is indicated. - indicates that no growth was observed during the 124 hour period of observation. The plasmids have been described previously (Section 7.2.1).

Plasmid	resistance (ohms)	voltage (kV)	Field strength (kV/cm)	time (ms)	Time taken for sample to grow: Strain ALV 0 (µg Cm/ml)	Strain ALV 1 (µg Cm/ml)	Strain BC1 0 (µg Cm/ml)	Strain BC1 1 (µg Cm/ml)
None	0	0 0	0 0	0 0	21	43	21	43
None	200	2 5	12 5	4 8	27	124	24	100
None	400	1 0	5 0	8 5	27	124	24	124
None	400	2 0	10 0	8 3	27	124	24	124
None	400	2 5	12 5	7 8	43	124	43	=
pC194	0	0 0	0 0	0 0	21	43	21	43
pC194	200	2 5	12 5	4 5	27	60	=	=
pC194	400	1 0	5 0	8 6	24	60	24	84
pC194	400	2 0	10 0	8 5	27	60	27	100
pC194	400	2 5	12 5	8 4	43	100	43	100
pC221*	0	0 0	0 0	0 0	21	43	21	43
pC221*	200	2 5	12 5	4 3	27	60	24	60
pC221*	400	1 0	5 0	8 5	24	60	24	60
pC221*	400	2 0	10 0	8 2	24	60	24	84
pC221*	400	2 5	12 5	8 0	43	84	43	84
pCK1	0	0 0	0 0	0 0	21	43	21	43
pCK1	200	2 5	12 5	4 2	24	60	24	84
pCK1	400	1 0	5 0	8 7	24	43	24	100
pCK1	400	2 0	10 0	8 5	27	60	24	124
pCK1	400	2 5	12 5	7 8	43	100	43	124

Plasmid	resistance (ohms)	voltage (kV)	Field strength (kV/cm)	time (ms)	Time taken for sample to grow: Strain ALV 0 (µg Cm/ml)	Strain ALV 1 (µg Cm/ml)	Strain BC1 0 (µg Cm/ml)	Strain BC1 1 (µg Cm/ml)
pMTL20CK1	0	0 0	0 0	0 0	21	43	21	43
pMTL20CK1	200	2 5	12 5	4 5	27	100	27	124
pMTL20CK1	400	1 0	5 0	8 5	27	100	27	100
pMTL20CK1	400	2 0	10 0	8 4	27	100	27	100
pMTL20CK1	400	2 5	12 5	8 0	43	84	43	100
pMTL20CK2	0	0 0	0 0	0 0	21	43	21	43
pMTL20CK2	200	2 5	12 5	4 5	27	60	43	=
pMTL20CK2	400	1 0	5 0	8 6	24	60	27	84
pMTL20CK2	400	2 0	10 0	8 5	27	60	43	100
pMTL20CK2	400	2 5	12 5	8 4	43	100	43	124
pMTL20CH1	0	0 0	0 0	0 0	21	43	21	43
pMTL20CH1	200	2 5	12 5	4 3	27	84	27	100
pMTL20CH1	400	1 0	5 0	8 6	24	60	27	124
pMTL20CH1	400	2 0	10 0	8 5	27	84	27	124
pMTL20CH1	400	2 5	12 5	8 0	43	84	43	124
pMTL20CH2	0	0 0	0 0	0 0	21	43	21	43
pMTL20CH2	200	2 5	12 5	4 2	43	124	24	100
pMTL20CH2	400	1 0	5 0	8 6	27	100	24	100
pMTL20CH2	400	2 0	10 0	8 5	27	100	27	100
pMTL20CH2	400	2 5	12 5	8 5	43	100	43	124

pC221* = pC221cap903

= = no growth observed

was subjected to a plasmid minipreparation and the DNA isolated was analysed by agarose gel electrophoresis (Methods 2.5.6). Only chromosomal DNA was apparent in the agarose gels and no plasmid DNA was observed except pBC1 from strain BC1 which was linearized with HincII and gave a band in agarose gels at about 2.6 kb (data not shown). Aliquots (2 ml) of the same samples were used to inoculate flasks containing 1 μg Cm/ml but growth was not observed before that of a control which contained wild-type bacteria and 1 μg Cm/ml.

7.3.5 Electroporation Of Strains ALV and BC1 With Plasmids Encoding Kanamycin Resistance.

Plasmids pAT187 and pCK1 were isolated from their E. coli hosts and purified by caesium chloride density gradient centrifugation (Methods 2.5.1 and 2.5.2). 5 μl (2.5 μg) of each plasmid was used per electroporation of strains ALV and BC1.

Electroporation was carried out as in the previous section except no antibiotic resistance induction was necessary and cells were incubated overnight at 30°C without antibiotic for the expression period. Following this incubation, 1 ml of each sample was used to inoculate four flasks containing 0, 1, 2, and 3 mg kanamycin/ml.

All samples had grown following a three day period of observation and the cultures which might have contained putative electrotransformants did not grow before the appropriate controls, i.e. cells electroporated without plasmid DNA. However, 50 ml of all samples were subjected to a plasmid minipreparation (Methods 2.5.6) and the resulting DNA analysed by agarose gel electrophoresis. No plasmid DNA bands were apparent except pBC1 from strain BC1 which was linearized using HincII and gave a band in gels at about 2.6 kb (data not shown).

7.3.6 The Development of a Method to Detect Electro-Transfer of Plasmids.

The scheme proposed to detect transfer of plasmids into bacteria by electroporation (Section 7.2) was first tested using E. coli.

7.3.6.1 DNase Treatment of Samples Following Primary Electroporation of E. coli.

E. coli DH1 cells were prepared and 40 µl electroporated with 5 µl (1 µg) of pBR325 (6.0 kb; Cm^r, Amp^r, Tc^r; Methods 2.6.2). Following electroporation, the cells were resuspended in 1 ml of 10% (v/v) glycerol, treated with DNase (as in Methods 2.6.4) and subsequently plasmid DNA was isolated (Methods 2.5.6). The isolated DNA was resuspended in 20 ul of TE buffer and 5 µl were used for a secondary electroporation of E. coli DH1 (Methods 2.6.2). Electrotransformants were selected on solidified L-broth (Methods 2.2.2) containing chloramphenicol, ampicillin and tetracycline (Methods 2.2.3). The results and the various controls used in this experiment are indicated in Figure 7.2.

The remaining 15 µl of isolated DNA (from cells following primary electroporation) was digested with EcoR1 and analysed by agarose gel electrophoresis. A single plasmid band of about 6.0 kb represented pBR325 in sample (A) (as in Figure 7.2) and no plasmid was observed in the other samples (data not shown). This indicated that the primary electrotransformation of E. coli was very efficient and the quantity of DNA transferred could be detected on agarose gels.

7.3.6.2 Electro-Transfer of pBR325 into Strains ALV and BC1.

The method described in the previous section was repeated except strains ALV and BC1 were the target cells in the primary electroporation. Cells of these bacteria were prepared for electroporation as described in Methods 2.6.3. These cells were electroporated with pBR325 as in

Figure 7.2 Investigation of a Method for the Indirect Detection of Electrotransformation.

The figure outlines the steps of the protocol used with E. coli DH1. As the method was being tested in this experiment E. coli DH1 was used for both the initial (primary) and secondary electrotransformations (see text). The number of transformants detected from each sample is shown at the bottom of the figure.

EtOH ppt. = Ethanol precipitated.
r/s = resuspended.
r.t. = room temperature.
miniprep. = mimipreparation.

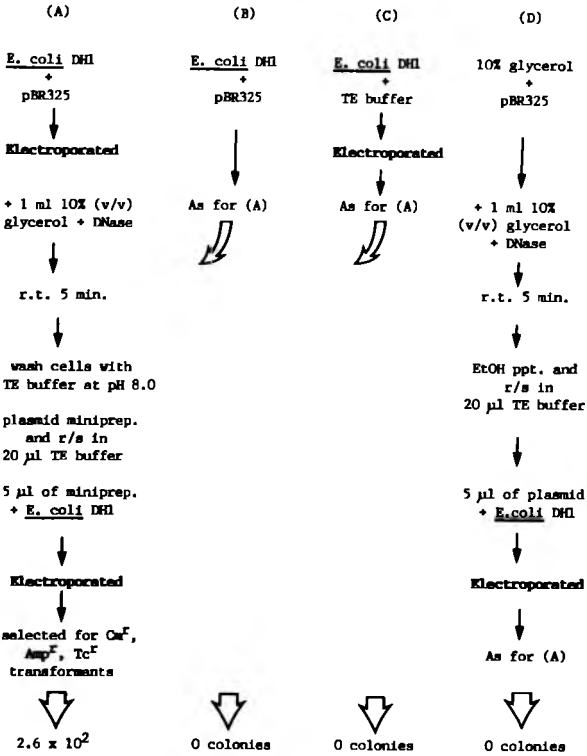

(A)
E. coli DH1
+
pBR325
Electroporated
+ 1 ml 10% (v/v) glycerol + DNase
r.t. 5 min.
wash cells with TE buffer at pH 8.0
plasmid miniprep. and r/s in 20 µl TE buffer
5 µl of miniprep. + E. coli DH1
Electroporated
selected for Cm^r, Amp^r, Tc^r transformants
2.6 x 10^2
(B)
E. coli DH1
+
pBR325
As for (A)
0 colonies
(C)
E. coli DH1
+
TE buffer
Electroporated
As for (A)
0 colonies
(D)
10% glycerol
+
pBR325
+ 1 ml 10% (v/v) glycerol + DNase
r.t. 5 min.
EtOH ppt. and r/s in 20 µl TE buffer
5 µl of plasmid + E.coli DH1
Electroporated
As for (A)
0 colonies

Table 7.3, DNase treated (Methods 2.6.4) and plasmid DNA isolated by plasmid minipreparation (Methods 2.5.6). Plasmids isolated were resuspended in 20 µl of TE buffer and 5 µl of this DNA was used for a secondary electroporation of E. coli DH1. Following the secondary electrotransformation of E. coli DH1, transformants were obtained as in Table 7.3.

Controls of each strain were included and steps of the protocol were omitted or adjusted as follows:

a) A sample was not subjected to the primary electroporation.

b) No pBR325 was added prior to the primary electroporation.

c) No cells. In this case plasmid alone was treated with DNase, subjected to ethanol precipitation (Methods 2.4.2) and resuspended in 20 µl TE buffer and 5 µl were used for the secondary electroporation.

For all controls, no E. coli electrotransformants were detected following the secondary electroporation.

Following the primary electroporation and isolation of DNA from strains ALV and BC1, 15 µl of minipreparation DNA was analysed by agarose gel electrophoresis. The results are shown in Figure 7.3. The DNA isolated from strain BC1 clearly showed the presence of a plasmid band which can be attributed to pBC1. pBR325 (6 kb) was not apparent in any of the DNA samples.

7.4 Discussion.

Initial experiments were aimed at investigating aspects of the electrotransformation protocol which had been devised for the electroporation of the moderate thermophile strains ALV and BC1. Brief incubations of dense cell suspensions on ice for about two hours did not appear to pose a problem. Most published electrotransformation experiments involve electroporation at 0-4°C (see references Table 1.2, Chapter 1).

Bacterial species are probably best harvested by chilling and centrifugation but the effect of temperature on electrotransformation

Table 7.3 Electro-Transfer of pBR325 into Strains ALV and BC1

Sample No.	Strain	E (kV/cm)	R (ohms)	t (ms)	No. of *E. coli* transformants.
1	ALV	12.5	200	4.5	316
2	ALV	6.25	400	8.5	116
3	ALV	8.00	400	8.4	128
4	ALV	12.5	400	8.5	160
5	BC1	12.5	200	4.5	132
6	BC1	6.25	400	8.6	44
7	BC1	8.00	400	8.5	64
8	BC1	12.5	400	8.4	80

The Table indicates the electrical parameters used in the primary electroporation of strains ALV and BC1 with pBR325 (1 μg). In all cases the capacitance setting was 25 μF. The Table also shows the number of Cm^r, Amp^r, Tc^r transformants produced by the secondary electroporation of *E. coli* DH1. The actual number of antibiotic resistant colonies counted on plates was multiplied by four because only 25% of the DNA isolated from strains ALV and BC1 was used for the secondary electroporation. A direct calculation of the efficiency of transfer into strains ALV and BC1 cannot be made because the quantity of plasmid used, and thus the efficiency of the secondary electroporation was not known.

The field strength (E) was calculated by dividing the voltage applied to the samples by 0.2 (the electrode gap in cm). R indicates the resistance setting of the apparatus, this is the primary determinant of the time constant (t).

Figure 7.3. An Agarose Gel of the DNA Isolated From Strains ALV and BC1 Following Electroporation with pBR325.

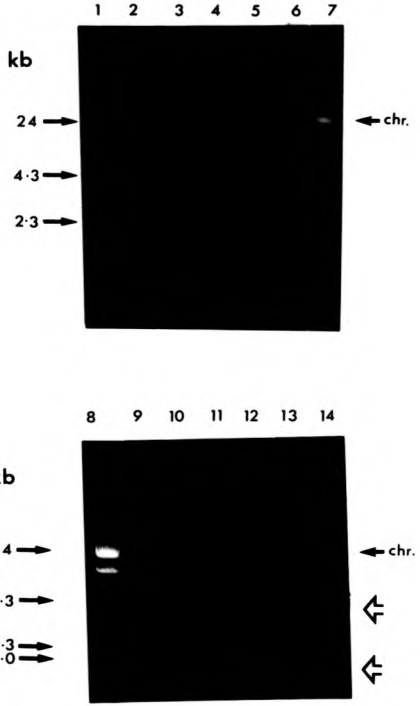
Some of the samples in this figure correspond to the electroporation parameters described in Table 7.3.

(A) The tracks are:

1) Lambda cut with HindIII.
2) Strain ALV Sample 1 in Table 7.3
3) Strain ALV Sample 2 in Table 7.3
4) Strain ALV Sample 3 in Table 7.3
5) Strain ALV Sample 4 in Table 7.3
6) Strain ALV control (electroporated as for Sample in track 3 but no pBR325 added).
7) Strain ALV control (not electroporated)

8) Lambda cut with HindII marker.
9) Strain BC1 Sample 5 in Table 7.3
10) Strain BC1 Sample 6 in Table 7.3
11) Strain BC1 Sample 7 in Table 7.3
12) Strain BC1 Sample 8 in Table 7.3
13) Strain BC1 control (electroporated as for Sample in track 10 but no pBR325 added).
14) Strain BC1 control (not electroporated)

The black arrows indicate chromosomal DNA (chr.) and the open arrows indicate the position of CCC and OC pBC1.


1 2 3 4 5 6 7
kb
2·4
4·3
2·3
chr.
8 9 10 11 12 13 14
kb
2·4
4·3
2·3
2·0
chr.

efficiency has rarely been studied and many workers use frozen stocks of cells, stored and electroporated in glycerol which acts as a cryoprotectant and a suitable buffer for the electroporation of many species (see Section 7.2). It was of primary importance that the ionic strength of the cell suspension was reduced by washing cells in a low ionic strength solution or distilled water because components of the electroporation medium have a pronounced effect on the efficiency of transformation. Ionic strength, osmolarity, pH and other parameters may require adjustment for optimal transformation efficiency (Biorad Pulse Controller Manual).

Buffers used for electroporation are usually about pH 7.5 and contain some of the following chemicals: glycerol, sucrose, sodium phosphate, Tris-HCl, HEPES, magnesium chloride and other ions (Miller et al., 1988; Dower et al., 1988; Powell et al., 1988; Biorad Pulse Controller Manual). It was noted that electrotransformation of Campylobacter sp. was strongly inhibited by Ca^{2+}, Mg^{2+}, or Mn^{2+} at less than 1 mM (Miller et al., 1988), while E. coli transformation was unaffected by up to 10 mM of these cations (Biorad Pulse Controller Manual). 10% (v/v) glycerol alone was used as a buffer for all electroporations described in this work.

Cells of strain ALV were clearly not refractory to the effects of electroporation and exposure to various voltages at resistance settings of 200 ohms and 400 ohms clearly had an effect on the cells; the survival of bacteria decreased in relation to increased field strengths (Figure 7.1). Cell death does occur during electroporation and when using conditions which produce electrotransformants survival rates of 20 to 80% can be expected (Biorad Pulse Controller Manual) and if no cell death occurs, the pulse may be too weak to cause 'porations' in the cell membrane. Maximal electrotransformation efficiencies of E. coli, B. thuringiensis, C. glutamicum, Bacteroides ruminicola and S. epidermidis occur when 40, 2.5, 99-55, 85 and 50% (respectively) of the cells survive pulses of between 10 and 12.5 kV/cm (Dower et al., 1988; Masson et al., 1989; Liebl et al., 1989; Thompson & Flint, 1989;

Augustin & Gotz, 1990 respectively). Bonamy *et al*. (1990) however, suggested that lethality was not necessarily linked to efficient electrotransformation of corynebacteria.

Previous experiments had indicated that for the moderate thermophiles the number of cfu produced on solid media did not accurately reflect the number of viable cells present in cell suspensions (Results Chapter 3). Strains ALV and BC1 usually existed in pairs or short chains when grown chemolithoheterotrophically and Holo & Nes (1989) noticed that chain shortening of lactococci occurred as a result of electroporation. These factors thus reduce the accuracy of the results shown in Figure 7.1. It was noted that a wide deviation from the mean occurred when strain ALV was electroporated at 25 µF, 400 ohms and 6 kV/cm, when cell survival in the range of 14 to 44% was recorded. Overall however, strain ALV was susceptible to a range of field strengths and time constants of about 4.5 and 9 ms when cells were harvested at mid-exponential growth phase (Figure 7.1) and electroporated in 10% (v/v) glycerol.

Following electroporation of the moderate thermophile strains ALV and BC1, the choice of method for the selection of transformants was restricted by their inability to grow adequately on solid media. In addition, the prevailing conditions of low pH and high temperature which inactivate many antibiotics produced a narrow set of options available for the selection procedure. The use of chloramphenicol and chloramphenicol acetyltransferase (*cat*) reporter genes appeared the only reasonable avenue available when the relatively small range of characterized antibiotic resistance genes from Gram positive sources were considered. Unfortunately some of the *cat* genes used in the experiments required induction and this made the selection process slightly more complex. In retrospect, similar plasmids which do not require induction should have been used. Nevertheless, a selection procedure in liquid media was developed where antibiotic resistant electrotransformants would be identified by their ability to grow before wild-type bacteria in a medium containing antibiotic.

Preliminary experiments indicated that following electroporation

(E = 9, 10 or 12 kV/cm), surviving wild-type cells exposed to sub-inhibitory concentrations of chloramphenicol (0.1 μg/ml) began to grow 24 hours (Sample 7a, Table 7.1B) to 66 hours (Samples 3a and 4a, Table 7.1B) after initiation of the 'selection' stage of the procedure (the term 'selection' is ambiguous because no additional chloramphenicol was added to the samples used here as examples). As expected, cell suspensions exposed to higher field strengths generally took longer to produce visible growth because a smaller percentage of bacteria survived the pulse. When 1 μg Cm/ml was used during the selection stage, growth of an electroporated wild-type population occurred in only one sample (Sample 2b, Table 7.1B) where growth was seen after 86 hours. These results indicated that induction with 0.1 μg Cm/ml could be carried out overnight at 30°C or for 6 hours at 45°C and that a long period was available for the growth of electrotransformants prior to the growth of wild-type bacteria.

Cells suspensions of strain ALV and BC1 were subsequently electroporated with plasmids using field strengths of 5-12.5 kV/cm (Table 7.2) and some cultures which contained potential transformants produced growth before appropriate control samples (cells electroporated without plasmid) indicating that electrotransformants might be present. However, analysis of these cultures failed to detect any novel DNA in the bacteria and transformants were not detected during subsequent growth in the presence of antibiotic. Several factors must be taken into consideration here. Firstly, the plasmids may have been unstable or incompatible with endogenous plasmids (e.g. pBC1) and also, the quantity of DNA in the putative transformants may have been insufficient for detection by agarose gel electrophoresis. In retrospect, transformed plasmid DNA might have been identified by making a Southern blot of this gel and then by the use of a suitable radioactive probe.

Chloramphenicol acetyltransferase is an intracellular enzyme which modifies chloramphenicol (Leslie et al., 1988; Lovett, 1990). Presumably the intracellularly modified drug is released from the cells (which contain the Cat protein) so that the antibiotic in the media is slowly

inactivated. It is possible that transformed cells were initially selected by 1 μg Cm/ml and the antibiotic was later inactivated by the high temperature and low pH (and by putative transformants), resulting in non-selective conditions and the loss of the plasmids from the transformed cell population.

If transformants of strain ALV and BC1 were not produced following electroporation with the plasmids specifying chloramphenicol resistance, it is difficult to explain why, in many cases, non-transformed cells grew in a medium containing chloramphenicol significantly earlier than control samples which had been treated in a very similar fashion (the only difference was that no plasmid was used during electroporation, Table 7.2). However, the degree of lethality caused by the electroporation pulse may have been different in the samples and this may account for the ambiguous results.

Although most of the plasmids used had a broad host range they might not have been able to replicate in strains ALV and BC1. The pMTL20C-derived vectors contain pBC1 but the probability of these plasmids replicating in strain ALV was unknown even though 16S rRNA analysis indicated that strains ALV and BC1 were more closely related to each other than to any other bacterial strain tested (see Chapter 1, Figure 1.1). Assuming that one of the pMTL20C-derived vectors contained the functional replicon of pBC1 (see Chapter 6), one might expect them to be good candidates for electrotransformation of strain BC1. However as strain BC1 contains pBC1, plasmid incompatibility and/or homologous recombination might be likely events (see Sections 1.6.1.3 and 1.6.1.5, Chapter 1).

A further problem might be the ability of strains ALV and BC1 to express cat genes. Different levels of expression of the pC194 cat gene have been observed in different bacterial species; Ballester et al. (1990) suggested that the expression of cat in streptococci was severely impaired. The thermostability of the cat gene or its product does not appear to be a problem as Wu & Welker (1989) working with B. stearothermophilus reported that chloramphenicol itself was active up

to 70°C and that some cat genes and their products were active in vivo at this temperature. Additionally, Shaw & Brodsky (1968) reported that the cat gene of S aureus was resistant to thermal inactivation at 75°C. In contrast, a tetracycline resistance determinant was not thermostable but tetracycline itself was not inactivated by high temperatures (Wu & Walker, 1989). The thermostability of plasmids was another factor for consideration, as high temperature treatment is a method used to cure bacterial hosts of their plasmids.

Selection using kanamycin was an attractive alternative to chloramphenicol because:

a) the genes do not require induction
b) broad host range plasmids are available which express kanamycin resistance in both Gram negative and Gram positive hosts
c) kanamycin was used to select transformed Acidiphilium sp. at pH 3.5
d) the mode of resistance means that aminoglycoside antibiotics are not normally inactivated or detoxified in culture media.

Following the electroporation of strains ALV and BC1 with pCK1 and pAT187 no electrotransformants were detected. The inability to produce electrotransformants could have been caused by plasmid instability or incompatibility or by the inability to express kanamycin resistance at 45°C. More importantly, kanamycin is less stable than chloramphenicol at high temperature and inactivation of kanamycin at elevated temperatures has been reported (Wu & Walker, 1989). Kanamycin resistance proteins from different bacterial sources also display various levels of thermostability and the enzyme produced by a plasmid isolated from a thermophilic bacillus was more stable than that encoded by pUB110 from a mesophile, yet the proteins had only a single amino acid difference (Matsumura et al., 1984).

It was important to understand why no electrotransformants were detected using the proposed method for electrotransformation of strains ALV and BC1. Most importantly, it was critical to determine whether DNA could be transferred into strains ALV and BC1 by electroporation. A method was therefore developed to determine if transfer of plasmid DNA

into the moderate thermophiles occurred. The 'DNase Method' was investigated using E. coli and results suggested that plasmids entering cells during an initial electroporation could subsequently be selectively identified by a second electroporation. The 'DNase Method' was repeated using strains ALV and BC1 and a limited number of initial electroporation conditions. The results of this experiment indicated that plasmids were transferred into the moderate thermophile strains by electroporation (Table 7.3). Relative comparisons of the results were made, based on the assumptions that:

a) plasmids were efficiently isolated from all samples.

b) DNase digestion of plasmids by residual enzyme did not occur during the plasmid minipreparation.

c) E. coli DH1 was efficiently electrotransformed during the secondary electroporation.

A short pulse (4.5 ms) at the maximum field strength of the apparatus (12.5 kV/cm) was probably more efficient than pulses of a longer duration (8.5 ms) and cells of strain ALV probably acted as better recipients for pBR325 than did strain BC1. Unfortunately this method could not be used to directly quantify the amounts of plasmid transferred during the primary electroporations because detection of these plasmids was probably limited by all or some of the points mentioned above. A good alternative would have been to use radioactive DNA and following the primary electroporation, plasmids transferred into cells could have been directly quantified by the amount of radioactive DNA in recipient cells. The attraction of this kind of experiment was that plasmid replication or the expression of marker genes was not required in the primary host and the plasmids transferred could be detected even when they were not seen on agarose gel electroporesis.

The failure to detect transformants in earlier experiments may have been because of the inefficient transfer of plasmid during electroporation of strains ALV and BC1 and the results of the 'DNase Method' supported this hypothesis because, relative to the efficiencies reported by other workers, the efficiencies of electro-transfer into

strains ALV and BC1 were probably low. In future it may be possible to alter the conditions and parameters used during electroporation of strains ALV and BC1 to increase the efficiency of the method.

Apart from the electrical parameters, the choice and source of plasmid DNA was important. Restriction and modification of DNA can reduce efficiencies of transformation. In the experiments described here for strains ALV and BC1 all DNA samples were isolated from heterologous hosts. Restriction of DNA along with other aspects of electroporation which may increase the efficiency of electrotransformation are discussed in the General Overview (Chapter 9).

Strain ALV did not appear to contain small plasmids and grew better than strain BC1 on solid media (Chapters 3 and 5). Initial results suggested that strain ALV was slightly more amenable to electrotransformation than strain BC1 (Table 7.3), therefore efforts to produce transformants might be better directed at this organism. In addition, the post-electroporation treatment of strains ALV and BC1 requires investigation as does the method for the selection of electrotransformants. The transfer of cells to a low pH medium following the pulse is probably not a problem to at least some of the cell population (depending on the size and length of the pulse) and Roberto *et al*. (1989) have transferred *Acidiphilium* sp. to medium at pH 3.5 immediately after electroporation.

Chapter 8.
Determination and Analysis of the Nucleotide Sequence of pBC1.

8.1 Introduction.

Attempts at electroporation of the moderate thermophile strains ALV and BC1 had so far proved unsuccessful (Chapter 7). The lack of a cloning vector which would replicate in the bacteria and/or express a reporter gene may have been reasons for the inability to detect transfer of plasmids into the bacteria. For this reason it was decided further characterization of pBC1 by nucleotide sequencing was necessary. By determining the DNA sequence of pBC1 further information about this plasmid might be obtained:

a) The region of pBC1 that comprised the minimal replicon might be identified.

b) Information gained from DNA sequencing might lead to an understanding of the mechanisms involved in the replication of the plasmid, this in turn would be beneficial to the vector construction programme.

c) The plasmid could be examined for open reading frames (ORFs) to which functions might be assigned, e.g. bacteriocin production or antibiotic resistance.

d) Knowledge of restriction sites present in the plasmid would help vector construction.

e) Information about the transcription and translation signals used by strain BC1 might be obtained.

8.2 Results and Discussion.

8.2.1 pBC1 Nucleotide Sequence Determination.

The plasmid isolation techniques used for the moderate thermophile strain BC1 had proved relatively inefficient (Chapter 5). The 2.6 kb

plasmid had however been cloned into the E. coli vector pACYC177 via a single HincII restriction site to form vector pFEG7 (Figure 6.1). This recombinant plasmid was chosen as a suitable source of the 2.6 kb of DNA derived from pBC1. Propagation of pFEG7 in E. coli would enable isolation of sufficient quantities of the 2.6 kb HincII fragment for nucleotide sequence determination. Using the restriction map of pFEG7 (Figure 6.1), restriction enzyme sites (particularly those sites within the 2.6 kb HincII fragment) were used as a basis for the nucleotide sequencing strategy outlined in Figure 8.1A.

The restriction fragments required for sequencing were all derived from the plasmid pFEG7. Each specific restriction fragment (as outlined in Figure 8.1A) was prepared by digestion of pFEG7 with the appropriate restriction enzyme. When isolation of a fragment of DNA would have been difficult due to the presence of many sites for the enzyme in the vector, or digestion would result in fragments of similar sizes, pFEG7 was first digested with HincII and the 2.6 kb fragment isolated using agarose gel electrophoresis (Methods 2.4.7). This 2.6 kb fragment was then digested with restriction enzymes so that the required fragment for DNA sequencing was isolated. Fragments of DNA were purified (Methods 2.4.9) and subcloned into the polylinker regions of bacteriophage vectors M13mp10, mp11, mp18 and mp19 and served as templates for 'chain termination' sequencing reactions (Methods 2.8). The nucleotide sequence data obtained was compiled into one complete sequence using the Microgenie Sequence Analysis Program of Queen and Korn (1984). Analysis of the sequence revealed the presence of several more restriction sites subsequently exploited for subcloning into M13 vectors and sequence determination. The DNA sequence determination of the pBC1 derived DNA was also assisted by using oligonucleotide primers:

		pBC1 sequence ref.
P1	5' GTGGCGCCGATCCTTACG 3'	311-328
P2	5' TGACCGCCTTCGGGGGC 3'	913-929
P3	5' CCTTCCAGTGGCCGCTCAGGAGC 3'	2190-2212

Figure 8.1 Sequencing Strategy for the Determination of The Nucleotide Sequence of pBC1.

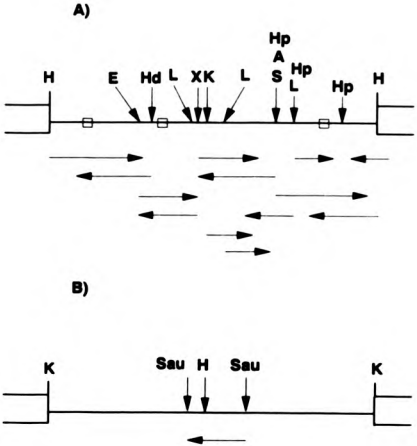
A restriction map of the 2.6 kb HincII DNA fragment of pFEG7 is shown in A). The nucleotide sequence of the regions indicated by arrows was determined by DNA sequencing.

B) indicates the 456 bp Sau3A fragment of pMTL20CK2 which was sequenced.

Restriction sites are as follows:

A = AvaI
E = EcoRV
H = HincII
Hd = HindIII
K = KonI
A = LspI (AsuII)
Hp = HpaII
S = SmaI
Sau = Sau3A
X = XbaI

The regions of sequence corresponding to oligonucleotide primers used during sequencing are indicated by open boxes in A).


A)
H
E
Hd
L
XK
L
Hp
A
S
Hp
L
Hp
H
B)
K
Sau
H
Sau
K

These primers were synthesized (Methods 2.8.7) and annealed to template (instead of M13 Universal primer) during the nucleotide sequencing protocol (Methods 2.8.3). The use of the synthesized primers enabled the sequence determination of larger subclones (i.e. over 400 bases). Furthermore, some regions of the nucleotide sequence produced abnormal banding in sequencing gels and the DNA sequence in these regions was confirmed using dITP instead of dGTP in the sequencing reaction (Methods 2.8.6).

8.2.2 Nucleotide Sequence Determination of the HincII Region of pBC1.

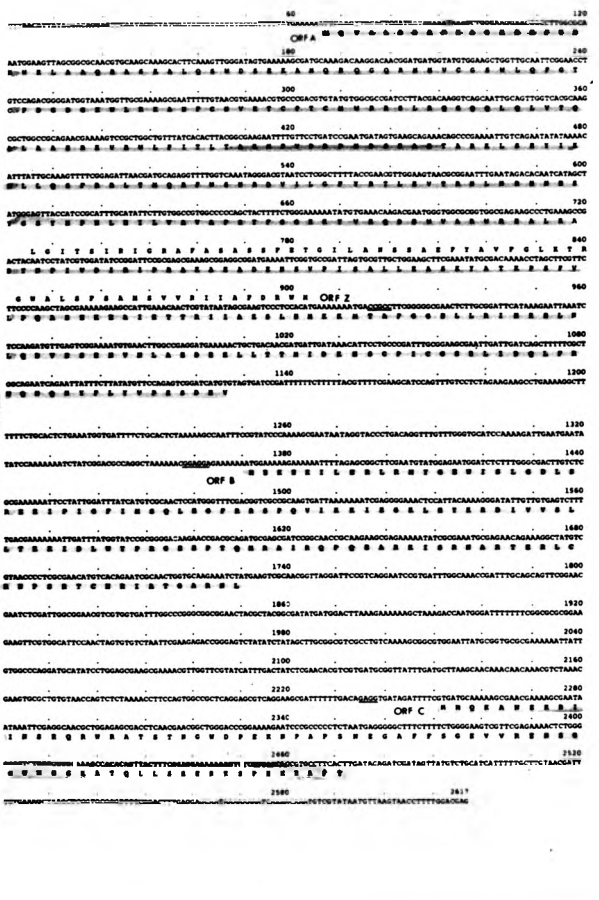
pBC1 from strain BC1 was initially cloned into E. coli vector pACYC177 using the restriction enzyme HincII. It was assumed that the enzyme HincII recognized a single site in pBC1 and would therefore linearize the entire plasmid. There was a small chance that two or more HincII sites might exist close together within the plasmid and be detected as one HincII restriction site by agarose gel electrophoresis. It was therefore necessary to confirm the presence of a single HincII site within pBC1.

pBC1 had been cloned into the vector pMTL20C via the single KpnI site of pBC1 to produce the recombinant plasmid pMTL20CK2 (Figure 6.10A). A large scale plasmid preparation of pMTL20CK2 from E. coli was used to produce large quantities of vector and the 2.6 kb KpnI fragment was isolated and digested with Sau3A to produce 9 fragments including a 456 bp fragment containing the HincII restriction site (Figure 8.1B). The 456 bp fragment was subsequently isolated and the nucleotide sequence determined, as described in Section 8.2.1. The results confirmed the presence of a single HincII site within pBC1.

The entire nucleotide sequence of pBC1 was determined for both strands (Figure 8.2) and included a previously undesignated ClaI restriction site (ATCGAT) at nucleotide position 2481. This site has an overlapping GATC nucleotide sequence and was therefore sensitive to methylation by dam methylase (see Maniatis et al., 1982).

Figure 8.2 The Nucleotide Sequence of pBC1.

The complete nucleotide sequence of the + strand (5'-3') of pBC1 is shown, together with the four putative open reading frames; ORF A, ORF B, ORF C and ORF Z. ORF Z was positioned on the minus strand of the plasmid (3'-5'). Regions of sequence underlined indicate the putative ribosome binding sites (RBS) for ORFs B, C, and Z. Each nucleotide was counted from the first base of the HincII restriction site (GTTAAC).


ORF A
ORF Z
ORF B
ORF C

8.2.3 Features of the pBC1 Nucleotide Sequence.

The complete nucleotide sequence of pBC1 was determined to be 2,617 bp which agreed with the size of linearized pBC1 estimated by agarose gel electrophoresis (2,600 bp). A circular restriction endonuclease map is given in Figure 8.3 and this diagram also shows the position of the putative open reading frames identified from the pBC1 sequence.

The overall nucleotide content of pBC1 was determined and numbered from the first base of the HincII restriction site.

	Mol %					
	dA	dC	dG	dT	dA+dT	dC+dG
pBC1	30.5	19.5	25.7	24.3	54.8	45.2
BC1 chromosome	24.0	24.9	25.1	25.3	50*	50*

* indicates + or - 1%

The mole percentages of deoxynucleotides in chromosomal DNA derived from strain BC1 were determined on enzymatically digested DNA by means of high-pressure liquid chromatography (Harrison, 1986b). The values for nucleotides present in pBC1 are most markedly different from the BC1 chromosome in the increased content of dA and decreased content of dC. Bouia et al. (1989) noted that the mol% G+C content of small plasmids found in Gram positive bacteria are usually lower than that of the host bacteria, and this was true for pBC1. The values for the mol% G+C content of some Gram positive bacteria and plasmids found in Gram positive bacteria are given in Table 8.1. The mol% G+C content of pBC1 was significantly higher than that of the other plasmids in Table 8.1.

Figure 8.3 Physical Map and Genetic Organization of pBC1.

A circular restriction map of pBC1 is shown, together with the positions of the four putative open reading frames of pBC1. The restriction enzymes are as follows:

Restriction Enzyme Site		Nucleotide Position
A	= AvaI	1835
Bc	= BclI	1064
Bg	= BglI	1834
C	= ClaI	2481
E	= EcoRV	738
H	= HincII	1
Hd	= HindIII	805
K	= KpnI	1273
S	= SmaI	1835
X	= XbaI	1177
Xh	= XhoII	1417

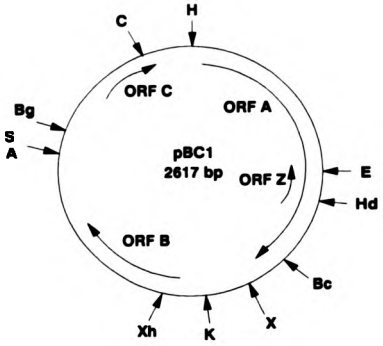

C
H
ORF C
ORF A
Bg
S
A
pBC1
2617 bp
ORF Z
E
Hd
ORF B
Bc
Xh
K
X

Table 8.1 The Mol% G+C Content of DNA From Gram Positive Bacteria.

Plasmid	Host	Size (kb)	Mol% G+C	Reference
pBC1	Strain BC1	2.6	45.2	This work
pT181	*S. aureus*	4.4	30.2	Khan & Novick, 1983
pLP1	*Lactobacillus plantarum*	2.1	38.3	Bouia *et al.*, 1989
pLJ1	*Lactobacillus helvelicus*	3.3	35.3	Takiguchi *et al.*, 1989
pC194	*S. aureus*	2.9	29.4	Horinouchi & Weisblum, 1982
pUB110	*S. aureus*	4.5	32.0	McKenzie *et al.*, 1986

Gram positive bacteria	Mol% G+C	Reference
S. aureus	30	from Projan *et al.*, 1987
B. subtilis	43	"
L. plantarum	44-46	from Kandler & Weiss, 1986
B. stearothermophilus	49.5	Friedman, 1968

8.2.4 Genetic Organization of pBC1.

Open reading frames (ORFs) were identified on the basis of size (>30 codons) and appropriate translational initiation signals (McLaughlin et al., 1981a; Moran et al., 1982). In addition, the sequence was screened for appropriately placed transcription initiation signals, i.e. up to 200 bases upstream of the putative ribosomal binding site (Graves & Rabinowitz, 1986; Moran et al., 1982). Overall codon usage of the ORFs was not used because no DNA from strain BC1 had previously been sequenced and therefore data indicating any codon biases were not available.

The principal feature of the nucleotide sequence was a putative large open reading frame (ORF) accounting for about 40% of the plasmid (ORF A). Three other putative ORFs were identified and labelled ORF B, ORF C and ORF Z. ORFs A, B and C were identified on the same strand of pBC1 (5' - 3') sometimes called the coding strand and in this work referred to as the plus (+) strand. ORF Z was located on the opposite strand (3' - 5') sometimes called the non-coding strand and in this work referred to as the minus (-) strand. The region encoding ORF Z was within the area of sequence (on the opposite strand of DNA) which encoded ORF A (Figure 8.3). The predicted molecular weights of the ORF products are shown in Table 8.2.

Table 8.2 The Predicted Molecular Weights and the Number of Amino Acids in the ORF Products of pBC1.

ORF	Molecular Weight (Daltons)	Amino acids
A	41,112	354
B	14,227	120
C	8,288	78
Z	6,538	60

The average mol% G+C content of the four ORFs was 46.6% and this was comparable to that of the plasmid as a whole (45.2%). The average G+C content of the intergenic regions was 42.7%. This value was slightly lower than that of the plasmid as a whole. More specifically, the intergenic regions had a G+C content of less than 40%, except the region 1731-2250 which had a value of 48.5 mol% G+C.

8.2.5 Translation Signals and Codon Usage.

The 5' ends of the four ORFs were screened for sequences complementary to the 3' end of the 16S rRNA of B. subtilis and in most cases a suitably positioned ribosome binding site (RBS) was found (Table 8.3). The predicted free-energy of base-pairing (ΔG) of ribosome binding sites in B. subtilis with B. subtilis 16S rRNA calculated according to the rules of Tinoco et al. (1973) range from -14 to -23 kcal/mol (Moran et al., 1982). As for the translation initiation signals of genes from Gram positive bacteria (McLaughlin et al., 1981a; Moran et al., 1982), significant complementarity was evident between the putative RBS of each ORF and the 16S rRNA of B. subtilis with ΔG values of about -17.0 kcal/mol (see Tinoco et al., 1973). The initiation of translation of ORF A will be discussed in more detail in Section 8.2.6.

The codon usage of the four ORFs was analysed using the program of Microgenie and data are presented in Table 8.4. Codon usage appeared to reflect the nucleotide content of the plasmid and there was a preference for codons containing the nucleotide dA. When the third ('wobble') position within a codon was filled by any of the four nucleotides there was a preference for G (36.7% G, 22.4% A, 20.8% C, 20.1% T). However, when the choice in the 'wobble' position was between nucleotides A or G about 70% of codons had A, whereas about 30% had G. Similarly, when the choice in the 'wobble' position was between nucleotides T or C, T occurred more frequently (65% T, 35% C). The frequency of codon usage in pBC1 was compared with that of E. coli (Ikemura, 1981; Grosjean & Fiers, 1982) and major differences were seen for most amino acids with the

Table 8.3 The Ribosome Binding Sites of ORFs B, C and Z.

ORF	ΔG value (kcal/mol)
B (1353-1373)	-17.8
AAAACGGAGGAGAAAAAAATG	
C (2229-2253)	-17.8
TTGACAGAGGTGATAGATTTTCGTG ATG	
Z (927-902)	-17.4
CCCCGAAGGCGGTCATTTTTTTCATG	

3' end of B.subtilis 16S rRNA.
UCUUUCCUCCACUAG (3'-5')

The location of the regions of DNA within the plus strand of pBC1 is indicated in brackets. Boldface type indicates sequences complementary to the 3' end of B. subtilis 16S rRNA (Moran et al., 1982). Initiation signals for the start of translation are underlined. The putative ribosome binding site of ORF A is discussed in the text (Section 8.2.6). ΔG values were calculated according to Tinoco et al. (1973).

Table 8.4 Codon Usage in the ORFs of pBC1

Amino Acid	Codon	Occurrence in ORF A	B	C	Z	% use of one codon relative to all others within one amino acid group pBC1	B.s	E.c
Arg	CGU	2	1	1	0	7.3	35.2	58.0
	C	4	1	1	1	12.7	17.5	35.0
	A	8	5	2	1	29.1	9.1	2.3
	G	7	4	0	1	21.8	11.1	3.2
	AGA	4	4	1	1	18.2	27.7	1.1
	G	2	2	1	1	10.9	9.4	0.3
Leu	CUU	5	2	1	0	15.4	26.1	8.6
	C	5	2	0	0	13.5	9.8	6.6
	A	0	2	0	3	9.6	6.3	1.8
	G	6	0	0	0	11.5	21.8	69.0
	UUA	6	2	1	0	17.3	22.4	5.8
	G	13	3	0	1	32.7	13.6	8.1
Ser	UCU	1	2	4	1	17.4	24.5	26.6
	C	3	0	0	4	15.2	12.0	25.6
	A	2	0	1	2	10.9	18.7	8.3
	G	4	4	2	1	23.9	10.0	11.4
	AGU	6	1	0	0	15.2	10.7	6.5
	C	7	0	0	1	17.4	24.1	21.6
Thr	ACU	1	1	1	0	10.7	14.9	23.8
	C	2	0	1	1	14.3	14.1	50.6
	A	5	2	1	0	28.6	43.3	5.9
	G	8	2	1	2	46.4	27.9	19.7
Pro	CCU	4	1	1	0	25.0	33.6	9.0
	C	2	1	2	0	20.8	9.8	6.0
	A	2	0	0	0	8.3	19.1	19.9
	G	6	4	1	0	45.8	37.5	65.1
Ala	GCU	3	0	1	6	20.0	27.5	27.9
	C	9	0	3	1	26.0	20.0	18.7
	A	5	2	0	2	18.0	27.1	22.9
	G	13	3	2	0	36.0	25.4	30.5
Gly	GGU	2	1	0	1	12.5	25.4	47.8
	C	2	1	2	1	18.8	29.6	40.8
	A	4	3	0	3	31.3	31.5	4.6
	G	5	2	5	0	37.5	13.5	6.8

Codon usage in the putative ORFs of pBC1 (A, B, C and Z) is compared to codon usage of B. subtilis (B.s) and E. coli (E.c) (taken from Garnier & Cole, 1988a). See also overleaf.

Table 8.4 continued.

Amino Acid	Codon	Occurrence in ORF A	B	C	Z	% use of one codon relative to all others within one amino acid group pBC1	B.s	E.c
Val	GUU	5	1	1	2	31.0	31.4	37.5
	C	2	0	2	1	17.2	24.7	12.8
	A	3	0	0	0	10.3	24.5	22.9
	G	9	2	1	0	41.4	19.4	26.8
Ile	AUU	9	6	0	1	44.4	50.0	37.3
	C	4	5	0	2	30.6	39.4	62.2
	A	3	1	2	3	25.0	10.6	0.5
Lys	AAA	23	10	3	0	81.8	75.4	76.7
	G	4	0	3	1	18.2	24.6	23.3
Asn	AAU	14	2	3	0	73.1	53.1	24.2
	C	2	1	3	1	26.9	46.9	75.8
Gln	CAA	14	4	2	0	71.4	54.2	26.6
	G	6	1	1	0	28.6	45.8	73.4
His	CAU	8	2	0	0	71.4	68.6	38.9
	C	2	1	1	0	28.6	31.4	61.6
Glu	GAA	19	8	5	2	79.1	69.5	73.4
	G	4	2	3	0	20.9	30.5	26.6
Asp	GAU	13	2	0	0	68.2	63.8	51.0
	C	4	1	1	1	31.8	36.2	49.0
Tyr	UAU	6	2	0	1	64.3	61.8	40.6
	C	4	1	0	0	35.7	36.2	59.4
Cys	UGU	4	2	0	0	66.7	45.7	42.0
	C	3	0	0	0	33.3	54.3	58.0
Phe	UUU	8	2	1	3	60.9	64.0	43.5
	C	4	0	2	3	39.1	36.0	56.5
Met	AUG	8	4	1	2	100	100	100
Trp	UGG	11	2	3	2	100	100	100

exception of those encoding lysine and glutamate (Table 8.4). A similar comparison with B. subtilis codon usage (Ogasawara, 1985) revealed major differences for most amino acids except lysine, histidine, aspartate, tyrosine and phenylalanine (Table 8.4). Overall, pBC1 exhibited a more evenly distributed codon usage than E. coli and the same was observed during a comparison of B subtilis and E. coli (Ogasawara, 1985).

The frequency of amino acid usage in the predicted polypeptides encoded by pBC1 was examined (Table 8.5). The protein products of the plasmid contained more hydrophobic amino acids than any other type probably due to the fact that the protein product of ORF A was highly hydrophobic and contained about 35% hydrophobic amino acids. Also 50% of the amino acids in ORF Z product, which was encoded by the same region of sequence as ORF A albeit on the opposite strand, were hydrophobic amino acids.

8.2.6 Initiation of Translation of the ORFs of pBC1.

ORFs B and Z of pBC1 possessed typical methionine start codons (AUG, Table 8.3), ORF C however, appeared to start with the atypical methionine codon GUG (Table 8.3, Shuttleworth et al., 1987; McLaughlin et al., 1981a). The putative GUG start codon of ORF C preceded an AUG triplet which may also have been the start codon of ORF C. The spacing between the RBS and the start codon of translation was an important factor for consideration (Kozak, 1983; McLaughlin et al., 1981a). Moran et al. (1982) analysed several genes from B. subtilis and found the distance between the ribosome binding sites and initiation codons similar to that observed for E. coli: as measured from the first base to the right (3') of AGGA or its equivalent (in accordance with the convention for E. coli mRNAs) through the adjacent base to the initiation codon, the distance between the ribosome binding sites and initiation codons ranged from 7-14 bases. For ORF C, measured according to convention, the distance from the ribosome binding site to the GTG codon was 14 bases (Table 8.3) and the

Table 8.5 The Frequency of Amino Acid Usage in the Putative ORF products of pBC1.

		No. of amino acids in ORF product. A	B	C	Z	%*
A. Strongly Basic Amino Acids	Lysine (K)	27	10	6	1	
	Arginine (R)	27	17	6	5	16.3
B. Strongly Acidic Amino Acids	Aspartate (D)	17	3	1	1	
	Glutamate (E)	23	10	8	2	10.7
C. Hydrophobic Amino Acids	Alanine (A)	0	5	6	9	
	Isoleucine (I)	16	12	2	6	
	Leucine (L)	35	11	2	4	34.2
	Phenylalanine (F)	12	2	3	6	
	Tryptophan (W)	11	2	3	2	
	Valine (V)	19	3	4	3	
D. Polar Amino Acids	Asparagine (N)	16	3	6	1	
	Cysteine (C)	7	2	0	0	
	Glutamine (Q)	20	5	3	0	24.8
	Serine (S)	23	7	7	9	
	Threonine (T)	16	5	4	3	
	Tyrosine (Y)	10	3	0	1	

* This value represents the total amount of amino acids of each group present in the ORF products, and was calculated as a percentage of the total number of amino acids in all the ORF products.

distance from the ribosome binding site to the following triplet (ATG) was thus 17 bases. This observation gave support to GTG as the start of ORF C and for the purposes of this work, initiation of translation of ORF C will be discussed as occurring at the atypical GTG codon.

The initiation of translation of ORF A remained somewhat of an enigma. A suitably positioned RBS (i.e. AGGAG) was identified 12 bases upstream of an ATG translation start codon at position 23-35. This RBS had a ΔG value of -13.8 kcal/mol (Tinoco et al., 1973) and appeared a likely candidate for the initiation of translation of ORF A. If the translation of ORF A was initiated at this ATG, a protein product of 42,532 Daltons would be produced. This is in agreement with a polypeptide of about 42,000 Daltons which was observed during in vitro transcription and translation experiments of pFEG7 (Chapter 6). During these experiments it was also observed that the 42,000 Dalton polypeptide was produced by linear templates generated when pFEG7 was cut with restriction enzyme HincII. Examination of the pBC1 sequence (Figure 8.4) revealed that the distance between the HincII site (ie GTT/AAC at location 1-6) and the RBS (AGGAG) at location 17-21 would provide insufficient nucleotide sequence for the position of a promoter between the HincII site and the RBS; promoters span a region about 45 bases upstream of the RBS (for the consensus sequences of promoters in Gram positive bacteria see later, Table 8.6).

The protein product of ORF A exhibited homology with several replication proteins produced by other Gram positive plasmids (discussed later in Section 8.2.9.3). More specifically the amino acids GKERPWRK encoded by the nucleotide sequence 99-122 (Figure 8.4) were similar to the amino acid sequences of other plasmid replication proteins (see later; Figure 8.6). Furthermore the fifth amino acid of the ORF A product, Leucine (L), and the seventh amino acid aspartate (D), were probably conserved (Figure 8.4).

The nucleotide sequence of pBC1 was examined for an alternative translation start codon and appropriately positioned RBS upstream of the

A)

```
         10          20          30          40
GTTAACATTA TACGACAGGA GTTCAATACG CTATGGATAC
         50          60          70          80
TATAATATAC GATTTTTATG AAAAAATTCA AGTTGCACTC
                                 I  Q    V  A  L
         90         100         110         120
GATGATAGAA ATAAAGCTGG GAAGGAACGG CCTTGGCGCA
 D  D  R    N  K  A  G    K  E  R    P  W  R
        130
AATGGAAGTT
K  W  K
```

B)

```
TAATATACGATTTTTATGAAAAAATT 43-68

   ATTTCTNNNNNTATANNNNNNNNNATG a.r.b.s.
```

Figure 8.4 The N-Terminus and Nucleotide Sequence Upstream of ORF A.

A) The N terminus of the ORF A protein and the nucleotide sequence of pBC1 (1-130) are illustrated. Amino acids conserved within plasmid replication proteins are shown in boldface text (see later, Figure 8.6). The putative translation start codons (ATG and ATT) which are in the correct reading frame are underlined. The HincII restriction site is also underlined (GTT/AAC).

B) Region 43-68 of the nucleotide sequence of pBC1 is aligned with the atypical ribosome binding site (a.r.b.s, de la Campa et al., 1990). Boldface letters indicate similarity. N indicates any of the four nucleotides.

regions exhibiting homology with other proteins. An atypical ATT translation start codon (Kozak, 1983) was identified at nucleotide position 66-68 (Figure 8.4). B. subtilis and other Gram positive organisms use non ATG initiation codons more frequently than E. coli (29% to 9%; Hager & Rabinowitz, 1985a, 1985b). A RBS complementary to the 3' end of B. subtilis 16S rRNA was not identified upstream of the ATT codon.

Gram positive bacteria usually have highly conserved ribosome binding sites (Moran et al., 1982). More recently, de la Campa and co-workers (1990) identified an atypical ribosome binding site (a.r.b.s.) in the streptococcal plasmid pLS1. The a.r.b.s. was positioned upstream of the plasmid replication protein and had the sequence 5'-ATTTCT-$N_{4/5}$-TATA-$N_{9/10}$-ATG, where N represented any nucleotide. The atypical ribosome binding site was found to be functional in other S. pneumoniae genes (de la Campa et al., 1987) and functioned in E. coli, and probably B. subtilis besides S. pneumoniae (de la Campa et al., 1990). Among plasmids of Gram positive bacteria the replication genes (rep) of the pT181 family showed a RBS sequence (Projan & Novick, 1988), whereas the rep genes of pC194 and pE194 showed neither RBS or a.r.b.s sequences (Horinouchi & Weisblum, 1982; Villafane et al., 1987; de la Campa et al., 1990). The nucleotide sequence of pBC1 upstream of the ATT putative translation start codon was examined for the presence of the atypical ribosome binding site. The nucleotides 43-68 (Figure 8.2) showed some homology, probably not significant, with the atypical ribosome binding site of S. pneumoniae (Figure 8.4B). For the purposes of this work the initiation of translation of ORF A has been assumed to occur at position 66-68 (codon ATT) of the nucleotide sequence and to date, persuasive evidence for the position of a ribosome binding site has not been obtained.

8.2.7 Putative Transcriptional Regulatory Signals.

The sequences upstream of the ORFs were screened for possible promoters by comparison with the consensus sequence for 'extended'

promoter elements found in Gram positive genes (Graves & Rabinowitz, 1986) and likely candidates are shown in Table 8.6. Sequence conservation outside of the canonical -10 and -35 regions appears to be evident particularly around the -10 region. Two different promoters are given for ORF A in Table 8.6. The first at position 2613-1-46 includes the HincII site of pBC1 and removal of the 5' end of this promoter by HincII digestion might prevent DNA-dependent RNA polymerase recognizing this promoter, particularly because the 'A' cluster at positions -41 to -45 are conserved in the promoters of Gram positive genes (Graves & Rabinowitz, 1986). Expression from this promoter might not occur in an *in vitro* transcription and translation system (as was found in Chapter 6). The second promoter for ORF A at position 10-53 therefore seemed more suitable and matched the Gram positive consensus sequences to a greater extent than the promoter at position 2613-1-46. However, transcription from the second putative promoter would probably give a messenger with inadequate nucleotide sequence for recognition by rRNA before the ATT start codon for translation.

Compared to the promoters of other plasmids from Gram positive bacteria (Garnier & Cole, 1988a; Khan & Novick, 1983; Horinouchi & Weisblum 1982), the promoters of pBC1 showed less conservation to the Gram positive -10 and -35 consensus sequences (Graves & Rabinowitz, 1986). This however may be a consequence of the higher G+C content of pBC1 (see Table 8.1). Imanaka *et al.* (1986) had difficulty detecting the promoter sequence for the replication protein (RepA) of pRAT11. pRAT11 RepA was derived from pTB19, isolated from a thermophilic bacillus, and plasmids containing RepA were maintained at a low copy number (about 8 per chromosome) in *B. subtilis*. RepA had the atypical GTG translation start codon (see Imanaka *et al.*, 1986).

The nucleotide sequence of pBC1 was examined for dyad symmetries which could correspond to rho-independent transcriptional terminators (Platt, 1986; Rosenberg & Court, 1979). The sequence of one candidate which included the terminal sequence of ORF A had a free energy of

Table 8.6 Putative Promoter Sequences of pBC1.

```
     -45          -35                        -15   -10   -5
      |            |                          |     |     |
1) ACGAGGTTAACA TTATAC GACAGGAG TTCAATACGC TATGGATACTATAAT
2) ATACGACAGGAG TTCAAT ACGCTAT       GGATAC TATAAT  ATACGAT
3) TGACAGGTTTGT TTGGGT GCATCCA   AAAGATTGA ATGAAT  ATATCCA
4) GTTATTTGATGC TTAAGC AACAAAC    AACAAACG TCTAAA CGAAGTGC
5) CAAGTTCACATT TTCCGA CTCAACA   TCTTGGAGA TTTAAT TCTTTATG

6) Ta AAAAA      TTGAcA    a  A     a T TG  TATAAT AAtAt

7)    a       t  TTGACa t  t          t tg  TAtAAT
```

These putative promoters are located upstream of the following ORFs:

1) Sequence 5'-3' (2613-1-46) upstream of ORF A.
2) Sequence 5'-3' (10-53) upstream of ORF A.
3) Sequence 5'-3' (1280-1326) upstream of ORF B.
4) Sequence 5'-3' (2121-2167) upstream of ORF C.
5) Sequence 5'-3' (992-945*) upstream of ORF Z.
6) The Gram positive 'extended' consensus promoter sequence.
7) The E.coli consensus promoter sequence

The boldface letters in 1) to 5) indicate bases conserved with the consensus sequence for Gram positive promoters. * The coordinates for the promoter of ORF Z refer to + strand. The form of letters in the consensus sequences in 6) and 7) indicates the relative degree of conservation in 29 promoters analysed by Graves and Rabinowitz (1986) i.e. bases typed in small letters were in more than 41% of promoters, those in capital letters were in over 50% of promoters and those typed in boldface were present in over 75% of Gram positive promoters.

interaction (ΔG) of -12.8 kcal/mol (Tinoco et al., 1973) and is shown below:

Nucleotide position 1112-1157 (see Figure 8.2)
5' AGAGTCGGATCATGTGTAGTGATCCGATTTTTTCTCACGTA 3'

The consensus sequence TCTG occurred before and after the termination points of some genes (see d'Aubenton et al., 1990). The sequences TGTG and TCCG occurred before and after the termination of ORF A (see Figure 8.2).

8.2.8 Additional Sequence Features.

Inverted repeats which form 'stem-loop' or 'hairpin loop' structures play a role in the control of gene expression, for example, in attenuation, and they also occur in the operator sequences of some genes (for reviews see McClure, 1985; Platt, 1986). There are numerous examples in E. coli where proteins and/or secondary structures regulate translational initiation (see Hager & Rabinowitz, 1985a; Gold et al., 1981). Inverted repeat regions have also been implicated as plasmid incompatibility determinants (Imanaka et al., 1986).

The minus origins of replication (MO) of plasmids which replicate via rolling-circle replication (ssDNA plasmids, see Chapter 1) are characterized by a region of about 200 bases containing many imperfect palindromic sequences (see Section 1.6.1.1). In addition, several plasmids encode putative RNA transcripts which contain 'stem-loop' structures (Novick et al., 1985; Bron, 1990; Lacks et al., 1986). These RNA transcripts are thought to be involved in copy number control of the plasmids by controlling the synthesis of the Rep proteins (see Section 1.6.1.2).

Using the computer program Microgenie the remainder of the pBC1 sequence was examined for sequences that might contribute to the formation of secondary structures either at the DNA or RNA level and

perhaps have some regulatory role, i.e. direct repeats and palindromic elements (Table 8.7). The analysis revealed many inverted repeat sequences and most of these were probably of no significance. Subsequent analysis was therefore limited to invert repeats with a minimum match of 10 nucleotides in the 'stem' and a 'loop' or 'hairpin loop' of 0-10 nucleotides. However, a structure with a 'stem' of only 8 nucleotides length is given in Table 8.7M. Microgenie calculates the ΔG value of invert repeats according to Tinoco et al. (1973) but does not add kilocalories to account for the 'hairpin loop'. Tinoco et al. (1973) suggested the addition of about 4-8 kcal. depending on the 'loop size'. Additional factors contribute to the ΔG value, for example, 'loops' containing the sequence AUG are 1 or 2 kilocalories more stable than loops containing C (see Tinoco et al., 1973). Adjustments for 'hairpin loops' also depend on whether the 'loop' is closed by A-T or G-C and the ΔG values calculated by Microgenie have not been adjusted to take account of the unpaired bases in the 'loops' of these structures (Table 8.7).

About 50 nucleotides upstream of ORF A was a possible 'stem-loop' structure with a 'stem' of ΔG value -21.2 kcal/mol. (Table 8.7A). A putative promoter of ORF A (Table 8.6) occurred within this structure (Table 8.6) and it may therefore play some role in the regulation of expression of ORF A. This structure was not however essential for expression of ORF A because it included the HincII restriction site and DNA 5' to the HincII site was probably not essential for in vitro expression of ORF A (see Section 8.2.6). The same structure was also located about 100 nucleotides downstream of ORF C (Figure 8.2).

Within the nucleotide sequence of ORF A several possible invert repeats were identified (Table 8.7B, C, D, E , F and G) and most notable was a possible secondary structure in the region 333-368 with a ΔG value of -16.2 kcal/mol (Table 8.7B). At the C terminus of ORF B a putative invert repeat was identified with a ΔG value of -7.8 kcal/mol (Table 8.7H). About 100 bases upstream of ORF C and in the same region as the -35 transcription signal of ORF C was a putative invert repeat with a ΔG value of -6.2 kcal/mol (Table 8.7J). The most significant invert

Table 8.7 Invert repeats within the DNA sequence of pBC1.

	Sequence Position	ΔG (kcal/mol)
A) 5'-TGTCGTATAATGTTAAGTAA------CC-TT |||||||||||| ||| || : 3'-ACAGCATATTACA----ATTGGAGCAGG-TT	2585-17	-21.2
B) 5'-GGTCAGCAATTGCAGT-TG || |||| |||| || G 3'-CCGGTCGCGAACG-CA-CT	333-368	-16.2
C) 5'-GCAGAGGTTTTGGTC-AAA || |||| || ||| T 3'-CGGCTCCTAATGCAG-GGA	512-548	-10.0
D) 5'-TCGTGGATATCC ||| ||| |||| 3'-AGCGCCT-TAGG	733-755	-5.6
E) 5'-ATCCG-----GATTCCGC-G ||||| | || ||| : 3'-TAGGCGGAGGCGAAAGCG-A	741-773	-7.6
F) 5'-GCTTCGTTCTTC-CCCAA ||||| || ||| G 3'-CGAAG-AAAAAG-CGATC	832-865	-5.6
G) 5'-AAC-TCTTGCGGATTCATAAAG ||| ||||| |||| ||| : 3'-TTGTAGAACCTCTAA--ATTAA	931-971	-5.4

It is unlikely that all the invert repeats form 'stem loop' structures but to save confusion all invert repeats have been depicted in a similar fashion. Each invert repeat structure reads 5' to 3'. | indicates the palindromic regions which may form 'stem' structures. The position of the putative structures in the sequence of pBC1 is shown. The ΔG values were calculated for the 'stem' of structures by the Microgenie program according to Tinoco et al. (1973).

Table 8.7 continued.

	Sequence Position	ΔG (kcal/mol)
H) 5'-ACAGAATCGCAACTGGTGCAAGAAATC-T || |||| ||| | ||| || || A 3'-TGCCTTAGGATTGGCAACG--CTGAAG-T	1702-1756	-7.6
I) 5'-ACGTTGGTTCG---TA-TCAT |||| ||||| || T 3'-TGCA--CAAGCTCTAT-CAGT	2076-2111	-7.6
J) 5'-GTTATTTGATGCTT ||| |||| ||||| 3'-CAACAAACAACGAA	2121-2148	-6.2
K) 5'-AAAGAATCCCGCCCCCTC-TA ||| ||||||| : 3'-TTT-------CGGGGGAG-TA	2334-2366	-24.0
L) 5'-CCCGGAAAAGAA-------TC-CCGCCCC ||| ||||||| || : 3'-GGGTCTTTTCTTTCGGGGGAG-TAATCTC	2328-2376	-14.4
M) 5'-GCCCCCTC-TA ||||||| : 3'-CGGGGGAG-TA	2344-2363	-27.6

repeat sequences occurred at position 2328-2376 within ORF C and a secondary structure in this region was likely because three putative 'stem loops' with low ΔG values were predicted. These are depicted in Table 8.7K, L and M, with ΔG values of -24.0, -14.4 and -27.6 kcal/mol respectively.

The DNA sequence (nucleotides 2400-2420, see Figure 8.2) caused extensive four tracking and 'pile ups' during the determination of the sequence of the minus (-) strand. This region contained the sequence shown below (nucleotide sequence and position refers to + strand as in Figure 8.2) and a direct repeat sequence:

2395 5'TCTGGGGGGG TCTGGGGGGG 3' 2414

Interestingly, sequences based on a consensus sequence 5' AAAAGCGAA 3' occurred frequently (10 times) within pBC1 (Table 8.8). A similar analysis of the nucleotide sequence of pUB110, a ssDNA plasmid, revealed a 7-mer sequence which was repeated frequently in the plasmid sequence and McKenzie et al. (1986) suggested the sequence played a role in DNA-membrane binding. Other repeated sequences identified in the nucleotide sequence of pBC1 were:

1201 5' T T T T C T G C A C T C T G A A A 3' 1217
1224 5' T T T T C T G C A C T C T A A A A 3' 1240

1766 5' G T G A T T T G G C 3' 1775
1808 5' G A T T G G C 3' 1814
1826 5' G T G A T T T G G C 3' 1835

Table 8.8. A 9-Mer Repeated Sequence in pBC1 DNA.

	5'		3'	
258	AAATGGTTGCG	**AAAAGCGAA**	TTTTTGTA	285
1039	GCCCGATTTGC	GG**AAGCGAA**	TTGATTGA	1066
1227	TCTGCACTCTA	**AAAAGCCAA**	TTTCCGTA	1254
1248	TTCCGTATCCC	**AAAAGCGAA**	TAATAGGT	1275
1623	CGGCAACCGCA	AG**AAGCGAG**	AAAAATAT	1650
2055	ATATCCTGGAG	CG**AAGCGAA**	AACGTTGG	2082
2206	CAGGAGCGTCA	GG**AAGCGAT**	TTTTTGAC	2233
2248	TTCGTGATGCA	**AAAAGCGAA**	CGAAAAGC	2275
2259	AAAAGCGAACG	**AAAAGCGAA**	TAATAAAT	2286
165	TGGGATAGTGA	**AAAAGCGAT**	GCAAAGAC	192

Consensus:

AAAAGCGAA A/T rich

Regions of the nucleotide sequence of pBC1 which contained the 9-mer sequence AAAAGCGAA or close variants of this sequence are indicated. The 9-mer sequence was usually followed by an A/T rich nucleotide sequence.

2436 5' G G A G G A A A A A A A 3' 2447
2559 5' G G A G G A A T A A A A 3' 2570

The function of these repetitive DNA sequences in pBC1 was unknown. Similar repeated sequences in plasmids from Gram negative bacteria play a role in plasmid replication and/or incompatibility (e.g. R6K, Shaffermen et al., 1987) and plasmid transfer, e.g. oriT of R46 (Coupland et al., 1987). Direct repeats also occur in the replication origins of plasmids pRAT11 (Imanaka et al., 1986), pLS1 (Lacks et al., 1986) and pIP404 (Garnier & Cole, 1988a) which are from Gram positive bacteria.

8.2.9 Analysis of the pBC1 ORF Products.

8.2.9.1 Amino Acid Sequence Comparisons.

The NEWSWEEP program was used to search the OWL 10 composite sequence database (SERC facility, Daresbury, Warrington; 7th March 1991), which includes protein databases, e.g. NBRF, SWISSPROT, and BROOKHAVEN. NEWSWEEP searches the OWL 10 database for entries which display homology with a probe sequence. The NEWSWEEP program is also a SERC facility.

The protein product of ORF A was similar to several replication proteins (Rep) of plasmids from Gram positive bacteria (Table 8.9) and it seems likely that ORF A encoded the replication protein of pBC1.

The putative protein product of ORF B was similar to several ribosomal proteins of Xenopus laevis (African clawed frog) and of mammalian origin. Probably of more significance was a 40% similarity (20% identity) between ORF B protein and the first 100 amino acids of the threonine-tRNA synthetase (thrS) protein of B. subtilis (Ogasawara et al., 1986). The thrs protein of B. subtilis has significant homology with the N-terminal end of E. coli threonine-tRNA synthetase.

The putative protein product of ORF C was not considered significantly similar to any proteins in the database but the putative protein produced by ORF Z had 51.7% similarity (30% identity) to amino

Table 8.9 Proteins Similar to ORF A Protein.

Protein	O'Lap	% Identity
Rep of pHY300PLK	309	27.8
Rep of pFTB14	318	24.5
Rep of pLP1	270	28.1
Rep of pC30i1	270	27.8
Rep of pBAA1	290	23.4
Rep of pUB110	182	32.4
Rep of pLAB1000	167	28.7
Rep of pC194	161	25.5

The NEWSWEEP program identified proteins which shared identity with the ORF A protein product. The amino acid sequence of each protein was compared to the amino acid sequence of ORF A. The identity was calculated by aligning the two amino acid sequences so that a region called the overlap (O'Lap) was formed where the two sequences were most similar. The % identity refers to the percentage of identical amino acids in the overlap.

pC194 (Horinouchi & Weisblum, 1982) and pUB110 (McKenzie et al., 1986) were isolated from S. aureus. pFTB14 (Murai et al., 1987) was isolated from Bacillus amyloliquefaciens. pLAB1000 (Josson et al., 1990) was isolated from Lactobacillus hilgardii and pBAA1 (Devine et al., 1989) was isolated from B. subtilis. pLP1 (2,093 bp; Bouia et al., 1989) and pC30i1 (2,140 bp; Skaugen, 1989) replication proteins were 95% identical and probably the same proteins. Both were from plasmids of Lactobacillus plantarum. pHY300PLK (Ishiwa & Shibahara-Sone, 1986) is a recombinant shuttle vector and contains pAMβ1 (Clewell et al., 1975) from Streptococcus faecalis.

acids 301-450 in the protein product of fecA of E. coli (Pressler et al., 1988). FecA gene encodes an outer membrane receptor protein and this is one of three structural genes of the citrate-dependent iron (III) transport system of E. coli. The product of ORF Z also had 45% similarity (28.3% identity) to the amino acids 351-444 of the dicitrate transport protein of Klebsiella pneumoniae (van der Rest et al., 1990). This protein spans the cell membrane several times and allows the uptake of citrate with the concomitant export of a proton (symport system) and enables K. pneumoniae to utilize citrate as a sole carbon and energy source. The dicitrate transporter protein is in turn similar to other transport proteins of E. coli. The region of the dicitrate transporter protein (380-400) which displayed the greatest similarity to the putative protein product of ORF Z was predicted to be a membrane spanning alpha-helix (van der Rest et al., 1990). The ORF Z putative protein was highly hydrophobic (Table 8.5) and it could form a transmembrane protein.

8.2.9.2 Hydrophobicity.

Using a computer program in Microgenie devised by Kyte and Doolittle (1982) the hydrophobicity and hydrophilicity of all the putative pBC1 ORF products were evaluated. The results are displayed graphically in Figure 8.5. This kind of information is used to determine if proteins bind to or span membranes.

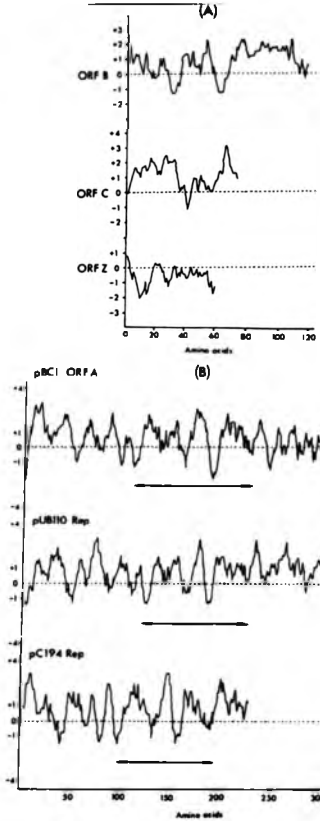
The hydrophobicity plot of ORF A was also compared to similar plots constructed for the replication proteins of pUB110 (McKenzie et al., 1986) and pC194 (Horinouchi & Weisblum, 1982), extensive similarity was evident (Figure 8.5B).

Figure 8.5 The Hydropathic Character of the Putative ORF Products of pBC1.

The hydropathy value at each point (amino acid) corresponds to the sum of hydropathy values for the amino acids over a span of 9 residues centered at that point (Kyte & Doolittle, 1982). Positive values represent hydrophilicity and negative values represent hydrophobicity.

A) Shows the hydropathic character of the putative products of ORF B, ORF C and ORF Z.

B) Shows the hydropathic character of the putative protein product of ORF A and the replication proteins of pUB110 (McKenzie et al., 1986) and pC194 (Horinouchi & Weisblum, 1982). Regions of similarity are indicated by solid arrows.


(A)
ORF B
ORF C
ORF Z
0
20
40
60
80
100
120
Amino acids
pBC1 ORF A
(B)
pUB110 Rep
pC194 Rep
50
100
150
200
250
300
350
Amino acids

8.2.9.3 The Replication Protein of pBC1.

A search of the OWL 10 database had indicated that the protein product of ORF A was similar to the replication proteins of other plasmids from Gram positive bacteria. The similarity of the putative replication protein (Rep) of pBC1 was investigated further using the IBM compatible CLUSTAL program (Higgins & Sharp, 1988). Figure 8.6 shows several Rep proteins aligned with the ORF A protein product of pBC1 and highly conserved regions are indicated by the frequency of black boxes in the Figure. The Rep protein of pBC1 thus belonged to the family of plasmids containing pC194 and pUB110 (see Gruss & Ehrlich, 1989). Interestingly, the Rep of pBC1 was larger than the other Rep proteins in Figure 8.6 and this was also indicated by the protein product of pBC1 identified by *in vitro* transcription and translation (about 42 kD; Chapter 6).

The ROOT 66 programme (J. Parkhill & D. A. Rouch, unpublished, Birmingham University Computer Centre) was used to compare Rep proteins, and to produce a dendrogram which indicated the phylogenic relationship of the Rep protein of pBC1 to other Rep proteins. The results are shown in Figure 8.7. A similarity score near to 1.0 indicated a close relationship and the Rep of pBC1 was the most distantly related of those included in the study, with a similarity score of about 0.28.

8.2.9.4 Analysis of pBC1 ORFs for DNA-Binding Regions.

McKenzie *et al.* (1986) identified a region of the replication protein of pUB110 (shown below) which was highly basic and included a sequence of 20 amino acids which satisfied the consensus DNA binding sequence (reviewed by Pabo & Sauer, 1984). A similar sequence was identified within the ORF A translation product (amino acids 82-101) and

Figure 8.6 Alignment of Plasmid Rep Proteins.

Plasmid replication proteins were aligned with the protein product of pBC1 ORF A to maximize similarity using the CLUSTAL program (Higgins & Sharp, 1988).

Larger black boxes indicate identical amino acids in, and small black boxes similarity between, at least 7 of the 9 proteins.

pC194 (Horinouchi & Weisblum, 1982) and pUB110 (pUB, McKenzie et al., 1986) were isolated from from S. aureus, pFTB14 (pFTB, Murai et al., 1987) was isolated from Bacillus amyloliquefaciens, pLAB1000 (pLAB, Josson et al., 1990) was isolated from Lactobacillus hilgardii, pRAA1 (Devine et al., 1989) and pBS2 (Darabi et al., 1989) were isolated from B. subtilis, pLP1 (Bouia et al., 1989) was isolated from Lactobacillus plantarum and pAMβ1 (Clewell et al., 1975; Ishiwa & Shibahara-Sone, 1986) was isolated from Streptococcus faecalis.

Only the replication protein region around the tyrosine residue (Y; *) in the phage ØX174 Rep protein enzymatic site is shown for pCB101 and pIJ101 (these short sequence similarities have already been described by Gruss & Ehrlich, 1989).

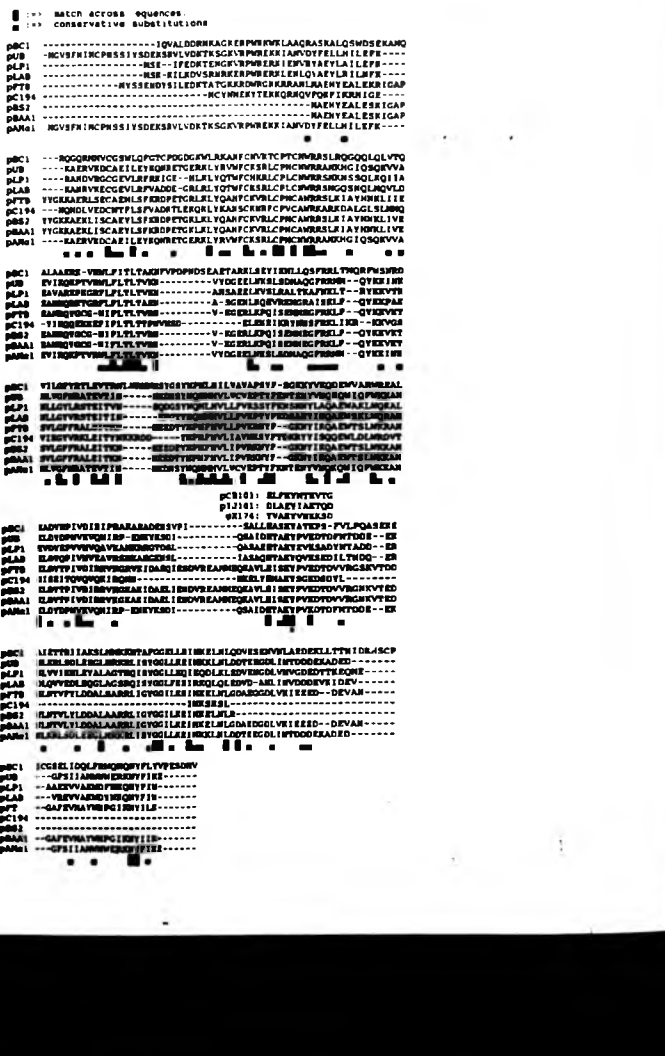

: :* match across sequences.
. :* conservative substitutions
pSC1
pUB
pLP1
pLAB
pFT8
pC194
pBS2
pBAA1
pAMa1
pSC1
pUB
pLP1
pLAB
pFT8
pC194
pBS2
pBAA1
pAMa1
pSC1
pUB
pLP1
pLAB
pFT8
pC194
pBS2
pBAA1
pAMa1
pSC1
pUB
pLP1
pLAB
pFT8
pC194
pBS2
pBAA1
pAMa1

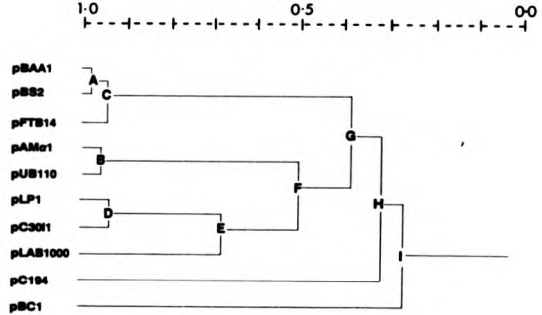
Figure 8.7 A Dendrogram Constructed from the Amino Acid Sequences of Plasmid Rep Proteins.

The dendrogram was constructed using the ROOT 66 program (J. Parkhill & D. A. Rouch, unpublished, Birmingham University Computer Centre). The relationships between proteins was suggested based on the similarity scores of a matrix comparison.

The letters (A-I) signify hypothetical amino acid sequences derived from the plasmid Rep proteins. For example, the similarity of the Rep proteins from pBAA1 and pBS2 resulted in a hypothetical protein A, this in turn was similar to the Rep protein from pFTB14, indicated by hypothetical protein C.

The horizontal distance or position of the hypothetical amino acid sequence linking Rep proteins reflected their similarity score and a score close to 1.0 indicated that proteins were very similar or closely related.

pLP1 (2,093 bp; Bouia et al., 1989) and pC3011 (2,140 bp; Skaugen, 1989) replication proteins were 95% identical and probably the same proteins. Both proteins were encoded by plasmids of L. plantarum. Similarly, pBAA1 and pBS2 were 99% identical, pBAA1 and pFTB14 were 96% identical and pUB110 and pAMα1 were 96% identical. For other references see Figure 8.6.


1·0
0·5
0·0
pBAA1
pBS2
pFTB14
pAMα1
pUB110
pLP1
pC301
pLAB1000
pC194
pBC1
A
C
B
D
E
F
G
H
I

this is compared to some other known DNA-binding proteins below (Lambda cro, trpR, lexR from Pabo & Sauer, 1984; spoIID, Lopez-Diaz et al., 1986)

	Helix 1									Turn		Helix 2									
	1				5					10				15						20	
Lambda cro	Q	T	K	T	A	K	D	L	G	V	Y	Q	S	A	I	N	K	A	I	H	
trpR	Q	R	E	L	K	N	E	L	G	P	G	I	A	T	I	T	R	G	S	N	
lexR	R	A	E	I	A	Q	R	L	G	F	R	S	P	N	A	A	E	E	H	L	
spoIID	T	R	G	F	G	H	G	V	G	M	S	Q	Y	G	A	N	F	M	A	K	
pUB110 Rep	N	W	R	R	A	M	L	H	G	I	Q	S	Q	L	V	V	A	E	V	I	Amino acids 95-144
pBC1 ORF A	M	W	R	R	S	L	R	Q	G	Q	Q	L	Q	L	V	T	Q	A	L	A	Amino acids 82-101

As indicated above, DNA-binding domains contain substantial regions of alpha-helix and a bihelical unit occurs (Pabo & Sauer, 1984). The region of the ORF A protein (82-101, as above) was analysed using the program of Microgenie for the Helix-Turn-Helix Motif. The Turn and Helix 2 were identified but the Helix 1 was not apparent. The glycine at position 9 of the DNA-binding sequence was conserved in 18 of 21 sequences analysed by Pabo & Sauer (1984) and is important in maintaining the structure of the bihelical unit. Other regions of ORF A protein product (amino acids 4-30 and 98-142) displayed a Helix-Turn-Helix character but were further removed from the consensus sequence (Pabo & Sauer, 1984) and only one region (amino acids 4-30) contained a glycine residue (at amino acid 12) within the predicted Turn. Strikingly, the ORF A protein was predicted as an alpha helix within the entire region of amino acids 260-320, which contains 60 amino acids.

The putative binding sequence of pUB110 was flanked by two hydrophobic regions (McKenzie et al., 1986) and the same observation was made for ORF A protein of pBC1 (either side of region 82-101; Figure 8.5).

8.2.10 Rolling Circle Replication and Features of pBC1.

Recent reports had indicated that several of the small (<10 kb) plasmids from Gram positive bacteria replicated by rolling-circle replication, involving a single-stranded intermediate in an analogous fashion to the bacteriophages of E.coli (see Chapter 1).

The single-stranded plasmids were assigned to families and members of a given family interacted with their cognate ori^+ sequences (origin of plus strand synthesis) which were conserved. The ori^+ sequence contained the 'nick site' where DNA was cleaved by the plasmid replication protein during rolling-circle replication (RCR) and the ori^+ was usually located upstream of, or within, the sequence encoding the plasmid replication protein (see Gruss & Ehrlich, 1989; Novick, 1989).

The minus origin of replication (MO) functioned as an initiation site for lagging strand synthesis during RCR, but MOs were usually non-essential affecting the efficiency of lagging strand synthesis. MOs were non-coding, highly palindromic, cis-acting sequences of about 200-300 bp which functioned in only one orientation. Their position relative to the primary replication functions, i.e. replication protein and ori^+, did not seem to be critical (see Bron, 1990). A number of MOs have been identified (see Gruss & Ehrlich, 1989; Bron 1990) and they appeared to function in a limited number of hosts. This is important because in the absence of a functional MO single-stranded DNA accumulated and plasmid instability occurred (see Chapter 1).

8.2.10.1 Plus Origin or 'Nick Site'.

Using the computer program of Microgenie, pBC1 was screened for the presence of a suitably located sequence that might bear some resemblance to either the ØX174 type of 'nick site' (Langeveld et al., 1978) also shared by pC194 and pUB110 (Gros et al., 1987) or the M13 type 'nick site' shared by pT181 (Koepsel & Khan, 1987), pC221 and pS194 (Gruss & Ehrlich, 1989).

Homology was found between the pBC1 nucleotide sequence at position 2463-2481 and the 'nick site' sequence common to ØX174 (Langeveld et al., 1978) and other ssDNA plasmids (Figure 8.8A). The 'nick site' of pBC1 was located about 200 bases upstream of ORF A which encoded the putative plasmid replication protein. The 'nick site' was also situated at the terminus of ORF C. This is unusual because ori$^+$ sequences are usually found in non-coding regions of plasmid DNA (see Novick, 1989).

Secondary structures in DNA sequences have been noted in the region of ori$^+$ (see Noirot et al., 1990). The putative ori$^+$ of pBC1 might also form a secondary structure (nucleotide positions 2473-2502) with a stem of ΔG value of -13.0 kcal/mol:

```
2473 5' TGATACAGAT CGATA        The underline indicates
         |||| ||||               the putative 'nick-site'.
2502 3' ACTACGTCTG TATTG
```

A putative secondary structure of ΔG value -21.2 kcal/mol discussed earlier (Table 8.8A) also occurred between the ori$^+$ of pBC1 and ORF A, the putative replication protein.

The similarity between the replication protein of pBC1 and the presence of the ori$^+$ indicated that pBC1 was a member of the family of ssDNA plasmids including pUB110 and pC194 (see Gruss and Ehrlich, 1989). However, the sequence CTCTAAT which is characteristic of the 'nick site' of plasmids within the pT181 family (see Gruss & Ehrlich, 1989) was also identified at nucleotide position 2349-2355 and occurred in the the 'loop' of a secondary structure with a stem of ΔG value -14.4 kcal/mol (Table 8.7L). The plasmids containing the CTCTAAT 'nick site' had a conserved sequence either side of this sequence (see Gruss & Ehrlich, 1989) but this was not seen in the nucleotide sequence of pBC1.

The replication proteins of Gram positive plasmids have amino acid conservation in the region thought to be responsible for recognition of the ori$^+$ and this region contains a tyrosine (Y) residue (see Gruss &

Figure 8.8 Plus Origin Sequence Homologies and Identification of The Active Site of The Replication Protein of pBC1

A) Nucleotide sequence homology in the vicinity of the nick-site of: pBC1 (nucleotide sequence 2463-2481; this study), ØX174, pCB101, pUB110, pC194, pLP1, pAA1 (taken from Gruss & Ehrlich, 1989) and pFTB14 (taken from the nucleotide sequence; Murai et al., 1987). The 'nick site' is indicated by an arrow.

B) The region containing the enzymatic active site of ØX174 Rep protein was compared to regions in the protein product of ORF A of pBC1 (amino acids 247-257) and regions from the Rep proteins of other plasmids from Gram positive bacteria (references as above). The tyrosine residue (Y) of the ØX174 Rep protein, which is covalently linked to DNA during replication, is indicated by an arrow.

A)

ØX174	5'	TGCTCC CCCAACTTG ATA TTA 3'
pCB101	5'	TTCTTT CTTATCTTG ATA ATA 3'
pUB110	5'	TTCTTT CTTATCTTG ATACATA 3'
pC194	5'	TTCTTT CTTATCTTG ATA ATA 3'
pFTB14	5'	GTCTTTTCTTATCTTG ATAC TA 3'
pLP1	5'	TTC TTCTTATCTTG ATAC TA 3'
pBAA1	5'	GTCTTTTCTTATCTTG ATAC TA 3'
pBC1	5'	TGC CTTCACTTG ATACAGA 3'

B)

ØX174	E	L	A	K	Y	V	N	K	K	S	D
pCB101	E	L	F	K	Y	M	T	K	V	T	G
pUB110	K	T	A	K	Y	P	V	K	D	T	D
pC194	E	M	A	K	Y	S	G	K	D	S	D
pFTB14	E	I	S	K	Y	P	V	K	D	T	D
pLP1	K	T	A	K	Y	E	V	K	S	A	D
pBAA1	E	I	S	K	Y	P	V	K	D	T	D
pBC1	K	A	S	K	Y	A	T	L	P	S	F

Ehrlich, 1989). The conserved amino acid sequence was present in the ORF A protein (amino acids 247-257; Figure 8.8B). The cognate tyrosine residue of ØX174 replication protein is covalently attached to the 5' end of DNA upon nicking of the replication origin during RCR (van Mansfield et al., 1986). This also occurs during RCR of plasmids in Gram positive bacteria (Thomas et al., 1990; Alonso, 1989).

8.2.10.2 Minus Origin and Copy Number Control of pBC1.

The pBC1 sequence was examined for the palA-type (159 bp), palU-type (or BA3-type; 277 bp) and palT-type (or'stab'; 169 bp) minus origins (as published by Gruss & Ehrlich, 1989 and renamed by Bron, 1990). Regions of homology between pal sequences and the nucleotide sequence of pBC1 are listed below:

Minus origin	Region of pBC1	% Homology
palA (pC194)	1093-1120	62
palU (pUB110)	660-689	62
"	1125-1168	57
"	1165-1203	62
"	1902-1939	58
palT (pBAA1)	No Homology	-

The sequence relationships of MOs are more distant than those of the ori^{+} of ssDNA plasmids and, for example, analysis of seven palA sequences revealed similarities ranging from 35-85% (Projan & Novick, 1988). In the nucleotide sequence of pBC1 (Figure 8.2) four regions of homology with palU of pUB110 (277 bp) were identified (see above). The regions of pBC1 homologous with the pal sequences were candidates for a minus origin in pBC1 and three of the regions were contained within a 110 nucleotide stretch of pBC1 (regions: 1093-1120; 1125-1168, and 1165-1203) and probably contributed to the sequence of the minus origin of pBC1 (Figure 8.9). This region spans DNA encoding 12 amino acids at the C-terminus of

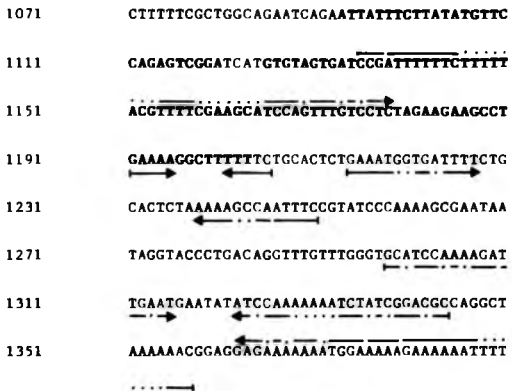


Figure 8.9 The Putative Minus Origin of pBC1.

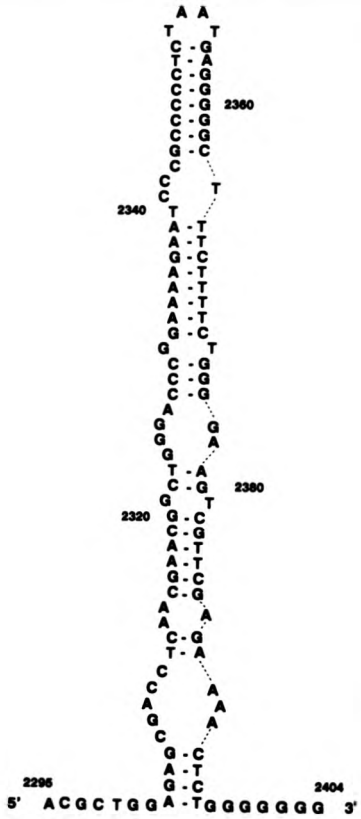
The nucleotide sequence of pBC1 is shown 1071-1420.
Bold letters indicate regions of homology with palA (1093-1120) and palU (1125-1203). Imperfect palindromic sequences are indicated by arrows.

ORF A (rep). Interestingly, of the three homologous regions within the putative minus origin of pBC1 (Figure 8.9), one was homologous to palA and two were homologous to palU. MOs can be depicted as a large secondary structure (see del Solar et al., 1987) and as noted earlier the region about 2310-2380 contained three putative secondary structures (Table 8.7K, L, & M). This region was inspected and a large secondary structure deduced (Figure 8.10). This DNA sequence may also be the MO of pBC1 or an alternative and second MO. Most plasmids contain only one MO but two are present in pMV158; the palU-type and the palA-type (del Solar et al., 1987; van der Lelie et al., 1989).

The DNA sequence depicted in Figure 8.10 was about 270 bases upstream of ORF A (rep) and could also be a copy control element or incompatibility determinant of pBC1. In some ssDNA plasmids the region directly upstream of the Rep encoding region was the template for countertranscript RNA molecules (as discussed in Chapter 1) and complex secondary structures between the rep mRNA leader (untranslated mRNA upstream of the mRNA encoding the Rep protein) and countertranscripts have been predicted by computer analysis, these structures probably controlled the rate of Rep synthesis (Novick et al., 1985; see del Solar et al., 1989). The DNA sequence upstream of ORF A was likely to form one or more secondary structures and it was interesting to postulate that a similar copy number control system might function in pBC1. However, no promoters for the synthesis of countertranscript mRNA were detected in the pBC1 DNA sequence. Studies have also indicated that some of the rep mRNA leaders might in fact produce repressor proteins instead of, or in addition to, countertranscript mRNA (del Solar et al., 1989; de la Campa et al., 1990; Byeon & Weisblum, 1990). These proteins were thought to repress Rep synthesis and were about 50 amino acids long and it was noticed that the DNA sequence depicted in Figure 8.10 was within ORF C which encodes a putative 78 amino acid product.

Figure 8.10 A Large Putative Secondary Structure Upstream of ORF A.

A large secondary structure deduced from the nucleotide sequence of pEC1 is shown and sequence coordinates are indicated. The ΔG value calculated for the whole structure using the rules of Tinoco *et al*. (1973) was -50.6 kcal/mol.


2360
2340
2380
2320
2296
2404
5' ACGCTGGA-TGGGGGGG 3'

8.2.10.3 Analysis of pBC1 for Single-Stranded Replication Intermediates.

Prompted by the discovery of the replication protein of pBC1 and a putative ori$^+$, exponential growth phase cultures of strain BC1 were examined for single-stranded plasmid, an intermediate during RCR (Methods 2.10). During the characterization of pBC1 results had indicated that a single-stranded form of plasmid might exist in plasmid preparations and this was seen as DNA which migrated further than CCC plasmid during agarose gel electrophoresis (Figure 6.4). S1 nuclease digests single-stranded DNA whilst leaving double-stranded DNA intact and this forms the basis of methods for the detection of single-stranded plasmid intermediates of RCR (te Riele et al., 1986a, 1986b), see Methods 2.10.

The results in Figure 8.11 indicated that single-stranded plasmid species were present in DNA samples from strain BC1 and they were digested by S1 nuclease. Chromosomal DNA also appeared to hybridize with the radioactive probe and this probably indicated the presence of high molecular weight DNA in the plasmid samples (see Section 1.6.1.5). Unfortunately attempts to repeat this experiment were unsuccessful. It was likely that pBC1 replicated by RCR with the production of a single-stranded intermediate in an analogous fashion to other small plasmids from Gram positive bacteria.

8.3 Summary.

The nucleotide sequence of pBC1 (Figure 8.2) was 2,617 bp with a mol% G+C value of 45.2. Four putative ORFs were identified from the pBC1 sequence and the codon usage was in most cases evenly distributed.

The signals for the control of expression of the ORFs appeared to be similar to the transcription and translation elements found in B. subtilis. However, the position of the promoters and ribosome binding site of ORF A were not deduced but the identity of this ORF was confirmed by homology to the replication proteins of pUB110, pLP1, pLAB1000, pFTB14, PC194, pRS2, pBAA1 and pAMα1 (see Figure 8.6). Some of these

Figure 8.11 Single-Stranded Plasmid from Strain BC1.

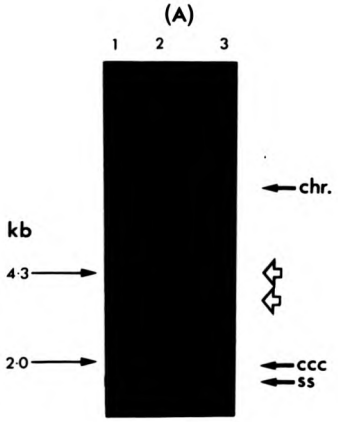
(A) An agarose gel of plasmid samples from strain BC1. The gel contained ethidium bromide prior to electrophoresis.

Key to tracks:

1 Lambda DNA cut with HindIII, the sizes of two of the fragments produced are indicated by arrows to the left of the photograph.
2 DNA from Strain BC1
3 DNA from strain BC1 (S1 nuclease treated).

The DNA in the agarose gel of (A) was transferred to nitrocellulose and hybridized to the 2.6 kb HincII fragment from pFBG7 (pBC1-derived DNA) which had been radiolabelled with ^{32}P. The nitrocellulose filter was washed to a stringency value of 80% and the resulting autoradiogram is shown in (B).

Single-stranded (ss), covalently closed circular (CCC) plasmid species and chromosomal DNA (chr.) are indicated. The open arrows indicate other forms of plasmid e.g. open circular, linear or multimer.


(A)
1 2 3
chr.
kb
4·3
2·0
ccc
ss


(B)
2 3
chr.
ccc
ss

plasmids have been extensively characterized, e.g. pC194 and pUB110 and are ssDNA plasmids which replicate by rolling-circle replication (see Chapter 1). pBC1 was probably a member of the family of ssDNA plasmids typified by pC194 and pUB110 (see Gruss & Ehrlich, 1989) and replicated via a single-stranded intermediate (Figure 8.11). The replication protein of pBC1 was least related when the replication proteins of the pC194/pUB110 family were compared (Figure 8.7). Further analysis of the pBC1 replication protein revealed a possible DNA-binding domain (Section 8.2.9.4) and a tyrosine residue in the active site of the protein (Figure 8.8) which may be covalently attached to the plasmid during replication. A large secondary structure (Figure 8.10) was implicated in the control of synthesis of the replication protein of pBC1 and sequence elements associated with rolling-circle replication of ssDNA plasmids were identified in the pBC1 nucleotide sequence, e.g. an origin of leading strand replication (*ori*$^{+}$, Figure 8.8) and a putative origin of lagging strand synthesis (MO, Figure 8.9). The minimum requirement for replication or the primary replication functions defining the minimal replicon in plasmids pC194 and pUB110 was the presence of a plus origin and a functional replication protein (see Bron, 1990). Thus, the minimal replicon of pBC1 has probably been identified.

In addition to *rep* and antibiotic resistance genes, ssDNA plasmids contain an ORF encoding a site-specific recombinase enzyme denoted Pre in pT181 (see Novick, 1989). This enzyme was believed to be involved in plasmid cointegrate formation and proteins related to Pre are encoded by several ssDNA plasmids (see Section 1.6.1.4). The Pre protein of pMV158 was recently shown to be required for conjugative mobilization in streptococci and lactococci (van der Lelie *et al.*, 1990) and may also be called Mob (Bron, 1990). Mob proteins, like Rep proteins, share sequence homology but no homology was detected between the ORF B, ORF C or ORF Z products of pBC1 and ORFs of other single-stranded plasmids present in the OWL 10 database (see Section 8.2.9.1). However, the putative protein encoded by ORF B was 40.0% similar to the N-terminus of the threonine-tRNA synthetase of *B. subtilis*, and the putative hydrophobic product of

ORF Z (Figure 8.5) was 51.7% similar to a region of FecA from E. coli. Additionally, the predicted ORF Z product was 45.0% similar to a membrane spanning region of the dicitrate transport protein of K. pneumoniae (Section 8.2.9.1).

The observation that pBC1 replicated via rolling-circle replication had important implications for the use of pBC1 as a cloning vector as most small plasmids which replicate via a rolling-circle type mechanism are unstable (see Bron, 1990; Gruss & Ehrlich, 1989 and Section 1.6.1.5). Recombination events occur as a direct consequence of rolling-circle replication (see Gruss & Ehrlich, 1989) and deletions in many plasmids, particularly recombinant vectors, have been reported (Michel & Ehrlich, 1986; Ballester et al., 1989; Peeters et al., 1988). Usually only short DNA segments are efficiently cloned in these vectors and longer segments often undergo rearrangements (see Bron, 1990). Ballester et al. (1986) noted that deletions occurred presumably by recombination between short direct repeats of 6 to 9 bp and a 9-mer direct repeat was identified in the pBC1 sequence (Table 8.8). Deletion(s) or rearrangements of DNA in pFEG7 cannot be ruled out and this vector was used as a source of DNA for the nucleotide sequencing of pBC1.

Chapter 9.
General Overview.

At the outset of the work detailed in this thesis host:vector methodology for the Gram positive iron-oxidizing moderate thermophiles did not exist, and because of the biotechnological importance of these bacteria, it seemed prudent to initiate studies towards the development of a genetic system.

Initial studies were directed towards improving growth of the moderately thermophilic strains ALV, BC1 and TH3 on solid media (Chapter 3). Results indicated that the microaerophilic nature of the bacteria, together with the requirements for low pH (1.7-2.0) and high temperature (45-50^{o}C), were contributory factors to poor growth and affected both the quality and quantity of the growth obtained. For the growth of single colonies, particularly of strain BC1, there appeared to be a requirement for a more defined set of conditions and more work would be necessary to improve the efficiency of plating of strains ALV, BC1 and TH3. However, pour plates of the bacteria were used to develop a disc assay method for the identification of potential markers which could be utilized during chemolithoheterotrophic growth in a ferrous sulphate medium at pH 1.7 (Chapter 4).

Metal ion tolerance was a particularly attractive marker for genetic studies of the moderate thermophiles because it was a useful laboratory marker for which plasmid-mediated resistance was known to occur, and also had the potential of conferring an industrially significant characteristic on the organisms. The tolerances of strains ALV, BC1 and TH3 to copper, silver, arsenate, uranium and mercury were determined (Chapter 4), and overall, strain BC1 was least tolerant of all but mercury (Figure 4.5). Metal resistances are usually encoded by an operon of over 2 kb of DNA (Silver & Misra, 1988; Silver *et al*, 1989) which may be difficult to clone. Due to the availability and more extensive use and characterization of antibiotic resistance genes as reporters, these were

a more attractive alternative. Whilst antibiotic genes were probably the best choice as reporter genes for development of a novel host:vector system, other types of reporter genes are available for use in Gram positive bacteria. XylE, which can be assayed colorimetrically has been used as a marker for the identification of B. subtilis transformants (Zukowski et al., 1983; Gleave et al., 1990) and bacterial luciferase genes which emit light are available for use in B. subtilis (Karp, 1989).

Following a comprehensive investigation of the effects of antibiotics on strains ALV, BC1 and TH3 using the disc assay technique, chloramphenicol acetyltransferase genes were identified as potential markers for cloning vectors (Chapter 4). Strains ALV and BC1 had minimum inhibitory concentrations of 2 μg chloramphenicol/ml in a medium at pH 1.7 when growth was chemolithoheterotrophic at 45°C, and Rawlings et al. (1983) had reported that chloramphenicol remained active at pH 1.8 in ferrous sulphate medium during studies using T. ferrooxidans.

Methods for the isolation of plasmids were investigated and the moderate thermophile strains ALV, BC1, TH3, NAL, TH1, LM1 and LM2 were screened for potential cloning vectors (Chapter 5). A large plasmid was isolated from strain LM2 and smaller plasmids were found in strains LM2, LM1, TH1, and BC1 (Chapter 5). The small plasmid in strain TH1 was probably identical to the small plasmid (pBC1) in strain BC1 (shown by DNA homology; Figure 6.4).

A large scale procedure was developed for the isolation of pBC1 (2.6 kb) from heterotrophically grown cells of strain BC1 (Chapter 5), and the whole of pBC1 was subsequently linearized and cloned into E. coli vectors pACYC177, pBR325 and pMTL20C, to form the pFEG- pBRBC- and pMTL20C-type recombinant vectors, respectively (Chapter 6). These vectors were used for the production of large quantities of the pBC1-derived DNA from E. coli and were to form the basis of E. coli/moderate thermophile shuttle vector(s).

The recombinant vectors containing pBC1 were used during attempts to characterize pBC1 by restriction mapping, in vitro transcription and translation using an E. coli system (Chapter 6), and DNA sequencing

(Chapter 8). Attempts to drive the replication of vectors using the replication machinery of pBC1 in E. coli DH1 and B. subtilis 168 were unsuccessful, and the host range of pBC1 may not include these bacteria (Chapter 6). However, DNA sequencing results indicated that the putative minimal replicon of pBC1 would have been inoperative in all the vectors used for the transformation of E. coli DH1 because the HindIII site of pBC1 (used to clone the 2.6 kb pBC1 DNA) was within the rep gene (ORF A) of pBC1 (see later). The results described using E. coli as a host were therefore not conclusive. The reason for the inability to detect transformants of B. subtilis 168 was not deduced and this bacterium may, or may not, serve as a host for pBC1-derived recombinant vectors.

Further investigations of pBC1 by nucleotide sequencing indicated the presence of ORF A within the pBC1 sequence and this encoded the pBC1 replication protein (Rep) of about 41 kD (Chapter 8). The in vitro transcription and translation results using pFEG7 and pFEG15 (Figures 6.11 & 6.12) confirmed the expression of a protein of about 41 kD by pBC1 (estimated as 42 kD from polyacrylamide gels). During the use of DNA templates digested with HindII or EcoRV the 41 kD protein was not produced (Figure 6.13) and the position of ORF A in the nucleotide sequence of pBC1 agreed with this result (Chapter 8). ORF A encoded the plasmid replication protein, and the replication proteins of other Gram positive plasmids have been expressed in E. coli (de la Campa et al., 1990; Bringel et al., 1989). Also, pLJ1 (3,292 bp) from Lactobacillus helveticus subsp. jugurti produced a protein of about 41 kD in an E. coli maxicell system (Takiguchi et al., 1989) and the minimal replicon of pCI305 from Lactococcus lactis subsp. lactis produced a 48 kD replication protein (RepB) following in vitro transcription and translation using an E. coli system (Hayes et al., 1990b).

The nucleotide sequence data together with the possible coding capacity of DNA indicated that many of the polypeptides produced by in vitro transcription and translation, including a 34 kD polypeptide (Figure 6.13), were probably immature forms of the pBC1 Rep protein. A polypeptide of about 14 kD was produced by some samples of pFEG7

following *in vitro* transcription and translation (Figure 6.13). Nucleotide sequence data predicted that ORF B encoded a putative product of 14 kD and ORF B was positioned on the pBC1 sequence between the (5') KpnI-AvaI (3') restriction sites (Figure 8.3). However, the 14 kD translation product appeared to be absent from samples of pFEG7 which had been digested with restriction enzymes whose sites mapped outside this region (Figure 6.13).

The nucleotide sequence of pBC1 was 2,617 bp with a G+C content of 45.2% and contained several unique restriction sites useful for vector construction (Chapter 8). The G+C content of pBC1 was high in comparison to that found in other plasmids from Gram positive bacteria (Table 8.1). Alonso (1989) suggested that ssDNA plasmids had a low G+C content because this reduced the free energy required for strand separation during plasmid replication. Indeed, increasing *in vitro* the G+C content of ssDNA plasmids appeared to result in the occurrence of aberrant replication forms (Viret & Alonso, 1987). The higher G+C content of pBC1 may have been an evolutionary consequence of the moderately thermophilic nature of strain BC1.

As expected, the codon usage within the four putative ORFs of pBC1 and the signals for transcription and translation showed a greater similarity to those found in Gram positive bacteria, for example *B. subtilis*, than those in *E. coli* and other Gram negative bacteria (Chapter 8). Evidence for the identification of sites for the initiation of transcription and translation of Rep from pBC1 (ORF A) remained inconclusive (Chapter 8, particularly Section 8.2.6) and the information used to identify these signals in the genes of other Gram positive bacteria did not seem to be applicable for ORF A. For the stable maintenance of plasmids, replication needs to be stringently controlled and excessive quantities of plasmid DNA can be stressful to host cells. This might explain why 'common' signals for expression are not apparent in the genes of some Rep proteins (see Section 8.2.6). Additionally, it is likely that some promoter consensus sequences and sigma factors are yet to be discovered or identified (see Section 1.7.1).

AUU was suggested as the translation start codon of the Rep of pBC1 but reports concerning AUU as a start codon in Gram positive bacteria were not found. However, Kozak (1983) suggested AUU could be an alternative initiator of translation and noted that it was the start codon of E. coli initiation factor IF-3. Also, the genes of Gram positive bacteria possess more rare start codons than the genes of Gram negative bacteria (see Section 1.7.3.1).

pBC1 was similar to other small plasmids (<10 kb) from Gram positive bacteria and replicated via a single-stranded intermediate and probably a rolling-circle mechanism (see Gruss & Ehrlich, 1989). Evidence for this hypothesis was the observed amino acid homology between the protein encoded by ORF A of pBC1 and the replication proteins of other Gram positive plasmids (Figure 8.6). This implied the ORF A translation product was functionally equivalent to a replication protein with topoisomerase (nick-closing) activities (see Gruss & Ehrlich, 1989). Further support came from the identification of a putative DNA-binding motif (Section 8.2.9.4). In addition, the pBC1 replication protein contained a region of homology with other Rep proteins which included a tyrosine residue (Figure 8.8); this amino acid was covalently attached to the 5' end of the leading strand during the replication of ssDNA plasmids (see Section 1.6.1.1). There was also an apparent similarity in the hydropathy profiles of some amino acids comprising the central regions of the replication proteins of pC194, pUB110, and the pBC1 ORF A product (Figure 8.5).

DNA sequences similar to the ori$^+$ 'nick-site' of some ssDNA plasmids were identified within the nucleotide sequence of pBC1, upstream of ORF A (Figure 8.8). A 'nick-site' sequence between nucleotides 2463-2481 suggested that pBC1 could be included in the pUB110/pC194 family of ssDNA plasmids (see Gruss & Ehrlich, 1989; Bron, 1990). In addition, the primary replication functions or minimal replicon of pBC1, composed of the ori$^+$ and rep (see Section 8.3), could be predicted to span from about 2450-1-1140 (Chapter 8) and this represented 50% of the plasmid DNA.

The proposed rolling-circle mechanism of replication of pBC1 was

further substantiated by the demonstration of single-stranded DNA in strain BC1 in a similar way to that demonstrated for other ssDNA plasmids from Gram positive bacteria (Figure 8.11, te Riele et al., 1986a, 1986b). Consistent with the theory of rolling-circle replication the nucleotide sequence of pBC1 was analysed for a minus origin of replication; the initiation signal for lagging strand replication. A region of the pBC1 sequence was identified which exhibited homology with the palA and palU MO-type sequences and this indicated that the putative minus origin was within the nucleotide region 1071 to 1420 (Figure 8.9), which includes the C-terminus of ORF A and the N-terminus of ORF B. Minus origins are usually in non-coding regions of DNA (see Novick, 1989) and the MO of pBC1 can probably be more accurately positioned to the region between ORF A and ORF B (see Figure 8.2).

Although experiments to detect and identify regulatory RNA countertranscripts were beyond the scope of this study it would seem feasible that replication control and incompatibility of pBC1 might be effected by short countertranscripts as for pT181 and other ssDNA plasmids (see Section 1.6.1.2). It is perhaps worth speculating that a large putative secondary structure upstream of ORF A occurred in pBC1 DNA or RNA (Figure 8.10) and could be involved in the regulation of expression of ORF A.

Comparisons of the replication protein of pBC1 with replication proteins of other ssDNA plasmids indicated that although pBC1 could be included in the pC194/pUB110 family of replication proteins, it was only distantly related (Figures 8.6 & 8.7).

The ability to transfer DNA into a target cell is critically important for the development of a host:vector system. Unfortunately, transformants of strains ALV and BC1 were not selected following electrotransformation but the transfer of pBR325 into strains ALV and BC1 was demonstrated using a novel method developed in this work, following the advice of Prof. D. E. Rawlings (University of Cape Town, see Chapter 7). Initial experiments using a limited number of electroporation conditions for the 'DNase Method' suggested that the efficiency of

electro-transfer into strains ALV and BC1 was probably low, but higher for strain ALV than strain BC1 (Table 7.3).

A limited number of broad host range plasmids and vectors containing pBC1 were used during attempts to electrotransform strains ALV and BC1 and it would probably be beneficial to investigate the use of more of these vectors, particularly those derived from thermophilic Gram positive bacteria. The latter type of vector might be useful because in general, plasmids, genes and proteins isolated from thermophiles are more stable than their mesophilic counterparts (see Brock, 1986; Imanaka & Aiba, 1986; Wu & Welker, 1989). But additionally, it has been established that vectors derived from large plasmids of Gram positive bacteria, e.g. pAMβ1 (Clewell et al., 1974) and pTB19 (Imanaka et al., 1984), are more stable than vectors derived from small ssDNA plasmids, e.g. pC194, pC221 (Janniere et al., 1990; Bron, 1990) and probably pBC1. Reports suggested that smaller plasmids electrotransformed bacterial cells at higher efficiencies than larger plasmids (Brigidi et al., 1990) but, in contrast, McIntyre & Harlander (1989a) noticed no difference in the efficiency of electrotransformation of L. lactis subsp. lactis with plasmids of 9.8 kb and 30 kb. An important consideration may not actually be the size of the plasmid but properties of the plasmid, for example, the source of the origin of replication and compatibility of the plasmid with the host cell.

The vectors used during attempts to electrotransform strains ALV and BC1 were all isolated from heterologous hosts. Where DNA from different sources has been investigated by electrotranformation considerably lower efficiencies occurred whilst using heterologous DNA (Lereclus et al., 1989; Bone & Ellar, 1989; Liebl et al., 1989; Hayes & Britz, 1990; Bonamy et al., 1990). Quarinot et al. (1990) electrotransformed Bradyrhizobium japonicum to highest efficiencies with plasmid DNA isolated from E. coli GM2163 (dcm$^-$, dam$^-$). This strain gave efficiencies 1-3 orders of magnitude greater than that observed with DNA prepared from other E. coli strains (dam$^+$, dcm$^+$).

The electroporation experiments described in Chapter 7 used mid-

exponential growth phase cells of strains ALV and BC1 because most bacteria can be electrotransformed more efficiently during early to mid-exponential growth phase (Muller et al., 1989, Fiedler & Wirth, 1988; Powell et al., 1988). But exceptions to this rule occur, and the growth phase at which cells are harvested has been related to their competence during electrotransformation. Phillips-Jones (1990) noted that C. perfringens cells harvested during late stationary growth phase gave higher electrotransformation efficiencies and, Kim & Blaschek (1989) suggested that for C. perfringens the success of electroporation of cells harvested at this growth phase was attributable to autolysis which made some of the cells more susceptible to high voltage electroporation. L. lactis subsp. lactis also gave higher transfomation efficiencies when harvested during stationary growth phase (McIntyre & Harlander, 1989a, 1989b).

Several electrotransformation methods involve the addition of cell wall 'weakening' agents to bacterial growth media or cell suspensions e.g. lysozyme (Wolf et al., 1989), glycine (Haynes & Britz, 1989, 1990; Holo & Nes, 1989; McIntyre & Harlander, 1989a, 1989b), Tween 80 (Haynes & Britz, 1989), isonicotinic acid hydrazide (Haynes & Britz, 1990) and DL-threonine (McIntyre & Harlander, 1989a, 1989b). Most bacteria do not reqire such treatments for successful electrotransformation and cell wall 'weakening' agents often resulted in osmotically sensitive cells prior to and after electroporation. These cells required osmotically protective media and/or buffers and in some cases the regeneration of spheroplasts or protoplasts was necessary.

The treatment of cells with PEG, or electroporation in the presence of PEG, has resulted in the successful electrotransformation of Bacillus sp. (Lereclus et al., 1989; Mahillon et al., 1989). But other workers have obtained higher electrotransformation efficiencies without resort to PEG treatment of Bacillus sp. (Bone & Ellar, 1989; Masson et al., 1989). It is noteworthy that Wolf and colleagues (1989) suggested the addition of PEG to cells during electroporation increased the time constant of the pulse because of a decreased conductance (by increased viscosity).

General Overview

In most reports of bacterial electrotransformation different buffers and conditions are used and exact details of experiments are omitted making a comparison of different methods difficult. It is however likely that most bacteria are suceptible to electrotransformation at a particular stage of growth, and if bacteria are refractory to electro-transformation, then cells may be made more susceptible by a treatment(s) which weakens the cell wall. Where agents have been added, most probably to weaken the cell wall of the bacteria, workers have not investigated the effects of the agent on the electrical parameters of the system and some may affect the buffering capacity (e.g. glycine) or electrical parameters of the sample (e.g. conductance or time constant of the pulse applied).

Some of the factors discussed above could be used during attempts to improve the efficiency of electro-transfer into strains ALV and BC1. These parameters could be evaluated using the 'DNase Method', or alternatively, by the electro-transfer of radioactive DNA (see Section 7.4). The production of electrotransformants of strains ALV and BC1 probably does require amendments to the electroporation methodology developed during this work (Chapter 7), and since electro-transfer into the bacteria was demonstrated, the choice of vectors, selection agent and selection conditions, are prime candidates for alteration. The pMTL20C-type recombinant plasmids used during attempts to transform pBC1 had appeared to be suitable vectors. They contained the intact domains of the putative minimum replicon of pBC1 i.e. *ori*$^{+}$ and *rep*, and a chloramphenicol acetyltransferase gene derived from pC194 which was isolated from *S. aureus* (see Figures 6.10A and 6.10B; although in pMTL20CH1 and pMTL20CH2 the spatial relationship of the two domains was altered by linearization of pBC1 using HincII). The inability to produce transformants of strains ALV and BC1 using these vectors could have been caused by restriction of the heterologous DNA by the bacteria, by the inability of the organisms to express the *cat* gene, or by the instability of the plasmids or chloramphenicol resistance determinant (Section 7.4). In addition, strain BC1 contained pBC1 and plasmid incompatibility may

have occurred and the production of a cured host strain would be beneficial for future experiments.

Kanamycin was also used as a selective agent for the electrotransformation of strains ALV and BC1 with broad host range vectors pCK1 and pAT187 (see Chapter 7). The experiments were unsuccessful and the minimum inhibitory concentration (MIC) of kanamycin for the bacteria was difficult to define (Section 4.2.3). High concentrations of kanamycin (e.g. 1.3 and 1.4 mg/ml) resulted in cultures with an extended lag growth phase and then exponential growth at rates comparable to a control (Figure 4.3). The determination of the MIC was thus confined to a 30 hour incubation period and during this length of time, the MIC of kanamycin for both strain ALV and strain BC1 was determined to be 2 mg/ml (Chapter 4). Kanamycin was an attractive selection agent for transformation of the moderate thermophiles (see Section 7.4), but the results obtained implied that kanamycin was rapidly inactivated at 45°C in a ferrous iron medium adjusted to pH 1.7. Rawlings *et al.* (1983) had also reported the instability of some aminoglycoside antibiotics at pH 1.8, including kanamycin. Thus, the choice of selection agent and the selection conditions used for the production of strain ALV and strain BC1 electrotransformants both need more extensive consideration (see Section 7.4).

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