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BACILLUS SUBTILIS AND BACILLUS LICHENIFORMIS

AS HOSTS FOR GENETIC MANIPULATION

By

DAVID A. BARSTOW, B.Sc. (Warwick)

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SUMMARY

Because of the potential use of the Bacilli for genetic manipulation, experiments were undertaken to investigate the usefulness of Bacillus subtilis and Bacillus licheniformis strain LO2, as hosts.

Attempts at shotgun-cloning directly in B. subtilis met with repeated failure. However, subsequently the Φ 3T-thyP3 gene, pC194 chloramphenicol acetyl transferase gene, B. licheniformis 749/C penP gene and the E. coli lacZ gene were expressed in B. subtilis when cloned into the plasmid vector pAB224 or one of its derivatives. Consequently, such plasmids are useful vectors for genetic manipulation in B. subtilis.

The properties of the thyP3-containing hybrids were investigated. Of particular interest was the finding that monomeric plasmid DNA, containing the thyP3 gene, was active in the transformation of competent B. subtilis cells. Transformation resulted in integration of the thyP3-containing region of the plasmid into the host chromosome.

The secretion of fusion proteins by B. subtilis was investigated employing the B. licheniformis 749/C penicillinase protein signal-peptide. This resulted in the secretion of the E. coli β -galactosidase enzyme from the B. subtilis cell.

The thermotolerant Bacillus licheniformis strain LO2 was investigated as a possible host for genetic manipulation. A series of mutant strains were isolated but the induction of competence in two such strains could not be achieved. Additionally, transformation of protoplasts of strain LO2 could not be demonstrated. Thus contrary to previous hopes, at present this strain does not appear to be suitable as a host for genetic manipulation.

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DECLARATION

The work contained in this thesis was the result of original research conducted by myself under the supervision of Professor A. Atkinson and Dr. S.B. Primrose. All sources of information have been specifically acknowledged by means of reference.

None of the work contained in this thesis has been used in any previous application for a degree.

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ABBREVIATIONS

ccc	covalently closed circular
Md	megadaltons
s	sensitive
r	resistant
p.s.i.	pounds per square inch
S	Svedberg units
ΔG	free energy of interaction
Kcal	Kilo calories
w/v	weight to volume ratio
rpm	revolutions per minute
hrs	hours
oz	ounce
OD	optical density
UV	ultra violet
mRNA	messenger RNA
rRNA	ribosomal RNA
Tc	tetracycline
Pc	penicillin
Ap	ampicillin
Km	kanamycin
Fus	fusaric acid
Sm	streptomycin
Cm	chloramphenicol
Neo	neomycin
Cad	cadmium
PEG	polyethylene glycol

DTT	DL-dithiothreitol
ONPG	o-nitrophenyl- β -D-thiogalactopyranoside
BCIG	5-bromo-4-chloro-3-indolyl- β -D-thiogalactopyranoside
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
IPTG	isopropyl β -D-thiogalactopyranoside
EDTA	ethylene diamine tetraacetic acid
SDS	sodium dodecyl sulphate
U	uracil
A	adenine
G	guanine
T	thymine
C	cytosine
CAT	chloramphenicol acetyl transferase
SOD	superoxide dismutase

The following are used in restriction endonuclease cleavage maps:

E	<u>EcoRI</u>
B	<u>BglII</u>
Ba	<u>BamHI</u>
Bs	<u>BstEII</u>
H	<u>HindIII</u>
S	<u>SalI</u>
Sm	<u>SmaI</u>

Other symbols and units used are as detailed in the *Biochemical Journal* (1981) 193: 1-21.

CHAPTER I

GENERAL INTRODUCTION

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1.1 GENETIC MANIPULATION IN *E. coli* USING PLASMID VECTORS

The term plasmid, originally used by Lederberg (1952) to describe all extrachromosomal hereditary elements of bacteria, is now restricted to the extrachromosomal, autonomously replicating, genetic elements (Broda, 1979). Plasmids are found in a wide variety of Gram-positive and Gram-negative bacteria where they exist within the cell as covalently closed circular (ccc) DNA molecules in the form of supercoils (Clewell and Helinski, 1969; Blair *et al.*, 1972). They range in size from 1 to greater than 200 Md (Broda, 1979) and determine a wide variety of traits.

The use of plasmids for genetic manipulation stems from two major properties which are common to most plasmids. Firstly, they can often be purified easily from a bacterial strain and subsequently introduced into either the same strain or into a different strain, species or genus and secondly, many but not all plasmids have easily selectable genetic markers, the most important being antibiotic resistance. Such markers greatly facilitate the selection of transformed clones.

With the discovery of site-specific restriction endonucleases (see Roberts, 1981) particularly type II enzymes (Smith and Wilcox, 1970), it became possible to controllably and specifically manipulate DNA molecules *in vitro*. Type II enzymes cleave duplex DNA to produce either fully double-stranded (flush or blunt-ended) termini or fragments with single-stranded, self-complementary (cohesive or sticky-ended) termini. The restriction endonuclease EcoRI was shown to produce DNA molecules with cohesive termini after cleavage (Mertz and Davis, 1972) which could be reannealed by the use of *E. coli* polynucleotide ligase (Dugaiczky *et al.*, 1975) and introduced into bacterial cells. Blunt-ended DNA fragments also can be joined by the use of T4 polynucleotide ligase (Heyneker *et al.*, 1976). Other methods for linking together DNA molecules have subsequently been

developed. The enzyme terminal-deoxynucleotidyl transferase (Chang and Bollum, 1971) can be used to add homopolynucleotide tails to DNA fragments to be joined. After mixing and annealing such DNA molecules then can be used directly to transform E. coli where repair of single-stranded gaps occurs in vivo (e.g. Hutchison and Halvorson, 1980). Synthetic DNA fragments (linkers) also can be used for linking together DNA molecules (e.g. Maniatis et al., 1978).

The choice of a vector in which to clone foreign DNA is principally determined by four factors. Firstly, the vector DNA must be able to infect a suitable host organism and replicate within that host. Secondly, the vector must possess at least one but preferably two selectable markers such as those conferring resistance to antibiotics. These markers enable identification of host cells which have taken up the required DNA molecule. Thirdly, the vector must possess suitable restriction endonuclease sites for the insertion of foreign DNA; ideally a unique site which lies within an antibiotic-resistance gene. By cloning foreign DNA into a site which lies within a second marker, it is possible to screen transformants for those which harbour recombinants. The undamaged marker is used to select transformants which then can be screened for loss of the second marker. Fourthly, insertion of foreign DNA must not impair essential functions such as control of plasmid replication.

To date most work involving the manipulation of DNA molecules has made use of E. coli K12 and its associated plasmids and bacteriophages. The major reason for this is that our genetic and biochemical knowledge of this organism is far greater than that of any other. This bacterium provided the basis for the classical genetic studies of Jacob and Monod and much of our understanding of gene structure and gene regulation stems from work carried out using E. coli. Furthermore, simple methods for introducing DNA into E. coli cells have been developed (e.g. Mandel and Higa, 1970; Cohen et al., 1972). A large variety of plasmid and bacteriophage vectors are available for molecular cloning in this host but probably the most widely used plasmid vector is pBR322 (Bolivar et al., 1977)

and its derivatives such as pAT153 (Twigg and Sherratt, 1980) whereas bacteriophage λ (for review see Brammar, 1979) is the most widely used bacteriophage vector.

In summary, the discovery of restriction endonucleases and other DNA modifying enzymes has made the rearrangement of DNA molecules in vitro a relatively simple procedure. Fragments of DNA from any source can be covalently joined to a suitable vector molecule and introduced into a bacterial cell. Cells containing a particular DNA fragment then can be isolated and further investigated as required. To date most of the developments in genetic manipulation have been made in E. coli. However, more recently other organisms have been investigated with the aim of developing genetic manipulation systems similar to those developed for use in E. coli.

1.2 ALTERNATIVE HOST-VECTOR SYSTEMS

The host-vector systems of E. coli have a wide range of useful features but are unlikely to be ideally suited for every purpose. Furthermore, E. coli may not prove to be the most suitable organism for the production of cloned gene products on an industrial scale since it has several unwanted attributes.

There has always been much controversy about the use of E. coli as a host for genetic manipulation (e.g. Grobstein, 1977) since it is a normal inhabitant of the alimentary tract of man and domestic animals. Most strains of E. coli produce a lipopolysaccharide endotoxin which makes its use as a host to produce pharmaceutical products, such as interferon, possibly undesirable. The development of crippled strains such as X 1776 (Curtiss et al., cited in Brammar, 1979) reduces the risk of infection and proliferation in the gut but does not avoid the problem associated with endotoxin production. The development of genetic manipulation techniques which are applicable to other organisms greatly facilitates the study of both the genetics and biochemistry of that organism and in addition, problems with E. coli may not be present in other systems.

Plasmids, bacteriophages and viruses have been isolated from, and genetic exchange systems have been developed for a large number of both prokaryotic and eukaryotic species; these are the two basic attributes that are required for the development of a useful genetic manipulation system. Among the prokaryotes, in addition to the Bacilli, systems have been developed for organisms such as Streptococcus (Stassi *et al.*, 1981), Staphylococcus (Wilson and Baldwin, 1978), Streptomyces (Bibb *et al.*, 1980), Methylophilus (Hennam *et al.*, 1982), Haemophilus (Setlow *et al.*, 1981), Salmonella (Lederberg and Cohen, 1974) and Pseudomonas (Sakaguchi, 1981). Among the eukaryotes systems have been developed for the yeast Saccharomyces (e.g. Gerbaud *et al.*, 1979), the fungus Neurospora (Vapnek and Case, 1981), animal cells (e.g. Mulligan and Berg, 1980) and plant cells (for review see Maheshwari *et al.*, 1980). With further advances, cloning systems in organisms other than E. coli will undoubtedly assume a much more important role in the future.

1.3 WHY BACILLI?

B. subtilis is a Gram-positive, aerobic, soil bacterium and other than E. coli is the most widely studied prokaryotic organism (for reviews see Priest, 1977; Henner and Hoch, 1980; Young, 1980). Studies have focussed on many aspects of this organism including its biochemistry, physiology and genetics and a large number of mutants have been isolated and characterised. Also, transformation and transduction studies have led to the construction of a linked genetic map (Lepesant-Kejzlarova *et al.*, 1975; Henner and Hoch, 1980). Several of these advances have laid the groundwork for the development of B. subtilis as a suitable host for the cloning and expression of foreign genes.

Recently many studies on the regulation of gene expression during sporulation have made use of genetic manipulation techniques (Jayaraman *et al.*, 1981; Kerjan *et al.*, 1982; Dubnau *et al.*, 1981) and it is hoped that the cloning of

specific sporulation genes will give an understanding of the nature and function of their products.

The Bacilli already are widely used for the industrial production of a wide range of products such as antibiotics, insecticides, and enzymes and the large scale fermentation requirements of Bacilli are well known. Genetic manipulation now offers a new approach for the improvement of commercially important strains in addition to the construction of strains producing novel products. The ability of Bacilli to secrete proteins into the culture medium is of industrial importance also. Currently greater than 40 extracellular enzymes are produced commercially from Bacilli (Priest, 1977) and it is hoped to tailor strains to secrete cloned gene-products such as interferon.

The Bacilli have several advantages over E. coli in terms of safety. Unlike Gram-negative bacteria the Bacilli have a simple cell surface composed of teichoic acid and peptidoglycan neither of which are pyrogens. Although B. subtilis can exist on human skin, it is a non-pathogenic organism and there are no reports to date of it causing disease in hosts that are not compromised by pre-existing disease processes (Ehrlich, 1978b). Also, unlike E. coli which can readily transfer genetic material to many other species of Enterobacteriaceae, B. subtilis does not readily transfer genetic material to other species of Bacillus. Some thermophilic species of Bacillus have an added safety feature in that they are incapable of growth at temperatures below 40°C and hence cannot grow at human body temperature (A. Atkinson, personal communication). Human infections with such species have never been reported.

The wide use of B. subtilis on an industrial scale results in a massive release of bacteria into the environment without any adverse effects having been observed. Indeed, because of the bright orange colour of B. globigii colonies, and the resistance of their spores, this organism is used as a biological tracer to monitor air and water currents (A. Atkinson, personal communication). Also, B. subtilis var natto is consumed in vast quantities in the orient in the form of

natto, a vegetable cheese produced by the fermentation of boiled soya beans. High levels of oral ingestion of this material by humans have been reported not to cause any ill effects (Ehrlich, 1978b).

A disadvantage of using the Bacilli as hosts is that they can persist in the environment for long periods of time in the form of spores (Roberts and Hitchins, 1969). If bacteria containing recombinant DNA which renders the host a biohazard were to escape into the environment, this could present a problem due to the persistence of spores. Asporogenic mutants of B. subtilis, which autolyse when the cells reach the stationary phase of growth, have been isolated (Brown and Young, 1970) and multiply auxotrophic strains have been constructed (Young, 1980). It is hoped that the use of such strains will overcome the problems of persistence in the environment of accidentally released organisms.

In addition to B. subtilis several other species of Bacillus may be useful for the cloning and expression of foreign genes. Transformation, by plasmid DNA, has been reported for B. megaterium (Vorobjeva *et al.*, 1980; Brown and Carlton, 1980); B. thuringiensis (Alikhanian *et al.*, 1981; Martin *et al.*, 1981; Miteva *et al.*, 1981); B. licheniformis (Imanaka *et al.*, 1981b) and B. stearothermophilus (Imanaka *et al.*, 1982). In addition plasmids have been transferred to B. megaterium, B. licheniformis and B. polymyxa by fusion of protoplasts of these species to B. subtilis protoplasts harbouring the S. aureus plasmid pC221 (Dancer, 1980). The ability to transfer plasmid DNA into the above mentioned strains will allow studies on the expression of cloned genes within these hosts. The commercial usefulness of these strains then may be improved by genetic manipulation techniques.

4.1 PLASMIDS IN BACILLI

Since the initial discovery of plasmid DNA in B. megaterium (Carlton and Helinski, 1969) many plasmids indigenous to the Bacilli have subsequently been discovered. They range in size from the 1.5 Md plasmid pIM13 (Mahler and

Halvorson, 1980) to a 180 Md plasmid isolated from B. thuringiensis (Lereclus et al., 1982). Most of the Bacillus plasmids isolated appear to be cryptic in that they confer no known phenotype on their host (e.g. Bernhard, et al., 1978) but phenotypic traits such as bacteriocin production (Bernhard et al., 1978) have been assigned to some plasmids. More recently antibiotic-resistance plasmids have been isolated from several species of Bacillus and been shown to replicate and express antibiotic-resistance in B. subtilis (e.g. Bingham et al., 1979). Table 1.1 lists some of the antibiotic-resistance plasmids isolated from species of Bacillus.

Although several of these indigenous Bacillus plasmids have been further developed as vectors for molecular cloning (e.g. Kreft et al., 1978), the use of antibiotic-resistance plasmids from the Gram-positive bacterium Staphylococcus aureus has proved more successful. Several small antibiotic-resistance plasmids from S. aureus have been shown to transform B. subtilis (e.g. Ehrlich, 1977), undergo autonomous replication and express their antibiotic-resistance markers. Table 1.2 summarises the properties of some such plasmids.

Not all the S. aureus plasmids transformed into B. subtilis have behaved in a similar manner. Gryczan et al. (1978) reported that pUB101 (Pen^r, Cad^r, Fus^r in S. aureus), pK545 (Km^r in S. aureus) and pSH2 (Km^r in S. aureus) could repeatedly transform competent B. subtilis cells to Fus^r, Km^r and Km^r respectively. However, extrachromosomal plasmid DNA could not be isolated from such transformants. It was suggested by these workers that chromosomal integration may have occurred in each case. In contrast Erlich (1977) reported that pK545 and pS177 (Sm^r in S. aureus) failed to produce Km^r and Sm^r B. subtilis transformants respectively. Also the plasmid pTP2 (Tc^r, Pc^r in S. aureus) fragmented when introduced into B. subtilis to give either Tc^r or Pc^r transformants (Kono et al., 1978).

Several of these natural plasmids isolated from S. aureus have proved to be useful vectors for molecular cloning directly in B. subtilis, particularly pUB110 (Keggins et al., 1978; McDonald and Burke, 1982) and pCI94 (Michel et al., 1980;

Plasmid	Size (Md)	Unique restriction endonuclease sites	Plasmid-borne markers	Reference
pPL576	30.0		Involved in sporulation	Lovett, 1973
pPL1	4.7		Cryptic	Lovett & Bramucci, 1975
pPL2	46.3		Cryptic	
pMB1	7.0		Cryptic	
pMB2	5.6		Cryptic	
pAB118A	4.9	<u>Bam</u> HI, <u>Sal</u> I, <u>Kpn</u> I, <u>Xba</u> I, <u>Xma</u> I	Cryptic	Bingham <i>et al.</i> , 1979
pAB118B	3.0	<u>Bam</u> HI, <u>Kpn</u> I, <u>Xba</u> I	Cryptic	
pAB124	2.9	<u>Xba</u> I, <u>Hpa</u> I, <u>Ca</u> uII	Tc-resistance	
pAB128	2.5	<u>Bgl</u> II, <u>Hpa</u> I, <u>Ca</u> uII	Cryptic	
pBC16	2.8	<u>Bam</u> HI	Tc-resistance	Bernhard <i>et al.</i> , 1978
pIM13	1.5	<u>Hha</u> I, <u>Sac</u> I	Em-resistance	Mahler and Halvorson, 1980
pTB11-pTB18			Cryptic	Imanaka <i>et al.</i> , 1981a
pTB19	17.2	<u>Bam</u> HI	Tc, Km-resistance	
pJP3623	3.3		Tc-resistance	Polak & Novick, 1982
pJP3633	3.1		Tc-resistance	

TABLE 1.1: Some natural plasmids isolated from species of Bacillus

Plasmid	Size (Md)	Unique restriction endonuclease sites	Plasmid-borne markers	Reference
pC194	1.8	<u>HindIII</u> , <u>HaeIII</u> , <u>HpaII</u> , <u>HhaI</u> , <u>BglII</u> , <u>MspI</u>	Cm-resistance	Ehrlich, 1977; Wilson & Baldwin, 1978; Horinouchi & Weisblum, 1982a
pC221	3.0	<u>HindIII</u> , <u>BstEII</u> , <u>EcoRI</u>	Cm-resistance	
pC223	3.0	<u>HindIII</u>	Cm-resistance	
pUB112	3.0	<u>HindIII</u>	Cm-resistance	
pT127	2.0		Tc-resistance	
pUB110	3.0	<u>BamHI</u> , <u>BglII</u> , <u>EcoRI</u> , <u>XbaI</u> , <u>AvaI</u> , <u>PvuII</u>	Neo-resistance	Gryczan <i>et al.</i> , 1978 Lofdahl <i>et al.</i> , 1978
pSA2100	4.7	<u>EcoRI</u> , <u>HaeIII</u> , <u>XbaI</u> , <u>HindIII</u>	Cm, Sm-resistance	
pSA0501	2.8	<u>EcoRI</u> , <u>HindII</u> , <u>HindIII</u> , <u>XbaI</u>	Sm-resistance	
pE194	2.4	<u>HpaI</u> , <u>PstI</u> , <u>BclI</u> , <u>XbaI</u> , <u>HaeIII</u>	Em-resistance	Horinouchi & Weisblum, 1982b
pTP2	2.8	<u>EcoRI</u>	Pc, Tc-resistance	Kono <i>et al.</i> , 1978
pTP4	2.8	<u>HindIII</u>	Cm-resistance	

TABLE 1.2: Some natural *S. aureus* plasmids introduced into *B. subtilis*

Rutberg et al., 1981). However, more versatile cloning vectors have been developed from naturally isolated plasmids by recombination in vitro. Table 1.4 lists some of those hybrid vectors that can replicate in both B. subtilis and E. coli and Table 1.3 lists some of those which cannot replicate in E. coli. Examples of the uses of some of these vectors will be given later. (section 1.8)

1.5 TRANSFORMATION AND TRANSFECTION OF COMPETENT CELLS OF BACILLI

Unlike E. coli for which competence is an artificially induced state, several species of Bacillus develop a natural physiological state in which they are capable of absorbing DNA from the surrounding medium (Spizizen, 1958). Our current understanding of the mechanism of transformation of competent Bacillus cells stems from work with B. subtilis using mainly chromosomal DNA as donor molecules. However, transfecting DNA and plasmid DNA, although intracellularly processed in a different manner, probably share at least part of the same binding, uptake and processing machinery.

Chromosomal DNA Transformation

The following is a summary of our understanding of chromosomal DNA transformation of competent B. subtilis cells (for review see Dubnau, 1976). Upon addition of double-stranded chromosomal DNA to competent cells the DNA becomes bound to the competent cell surface at a few points along the length of each DNA molecule (Dubnau and Cirigliano, 1972b). The DNA undergoes fragmentation at the cell surface to yield double-stranded molecules of 10 to 20 Md (Dubnau and Cirigliano, 1972a; Arwert and Venema, 1973) and a single strand of chromosomal DNA is taken up. There is concomitant hydrolysis of the homologous strand to a low molecular weight form and its release into the culture medium (Piechowska and Fox, 1971; Dubnau and Cirigliano, 1972a; Davidoff-Abelson and Dubnau, 1973). Either strand may be taken up (Chilton, 1967;

Plasmid	Size (Md)	Unique restriction endonuclease sites	Plasmid-borne markers	Reference
pBD6	5.8	<u>Bam</u> HI, <u>Tac</u> I, <u>Bgl</u> II, <u>Hind</u> III	Km, Sm-resistance	Gryczan <i>et al.</i> , 1978, 1980
pBD8	6.0	<u>Eco</u> RI, <u>Hind</u> III, <u>Bgl</u> II, <u>Bam</u> HI, <u>Xba</u> I	Km, Sm, Cm-resistance	
pBD9	5.4	<u>Eco</u> RI, <u>Bam</u> HI, <u>Tac</u> I, <u>Bgl</u> II, <u>Bcl</u> I, <u>Hpa</u> I, <u>Pst</u> I	Km, Em-resistance	
pBD10	4.4	<u>Bgl</u> II, <u>Bam</u> HI, <u>Xba</u> I, <u>Hpa</u> I, <u>Bcl</u> I	Km, Cm, Em-resistance	
pBD11	4.0	<u>Xba</u> I, <u>Bam</u> HI, <u>Bgl</u> II, <u>Hpa</u> I, <u>Bcl</u> I	Km, Em-resistance	
pBD12	4.5	<u>Eco</u> RI, <u>Xba</u> I, <u>Bam</u> HI, <u>Tac</u> I, <u>Bgl</u> II, <u>Hind</u> III	Km, Cm-resistance	
pBD64	3.2	<u>Eco</u> RI, <u>Xba</u> I, <u>Tac</u> I, <u>Bam</u> HI, <u>Bcl</u> I	Km, Cm-resistance	
pBC16-1	1.8	<u>Eco</u> RI	Tc-resistance	Kreft <i>et al.</i> , 1978
pBS161-1	2.5	<u>Eco</u> RI, <u>Pst</u> I, <u>Hind</u> III	Tc-resistance	
pAB224	2.0	<u>Eco</u> RI, <u>Bst</u> EII, <u>Ca</u> uII, <u>Hpa</u> I, <u>Hpa</u> II, <u>Tha</u> I, <u>Hha</u> I	Tc-resistance	Bingham <i>et al.</i> , 1980
pAB524	2.3	<u>Hpa</u> I, <u>Hpa</u> II, <u>Ca</u> uII, <u>Bst</u> EII	Tc-resistance	
PTL12	6.4	<u>Eco</u> RI, <u>Bgl</u> II, <u>Bam</u> HI, <u>Sma</u> I, <u>Xma</u> I	<u>leu</u> A, B T _p -resistance	Tanaka & Kawano, 1980
pTB53	11.2	<u>Bam</u> HI, <u>Hind</u> III	Km, Tc-resistance	Imanaka <i>et al.</i> , 1981a
pKO101	6.3	<u>Bam</u> HI, <u>Xba</u> I, <u>Bgl</u> II	Neo, S1-resistance	McDonald & Burke, 1982
pBD90	4.6	<u>Hind</u> III	Cm, Em-resistance	Docherty <i>et al.</i> , 1981

TABLE 1.3: Some hybrid plasmids for use in *B. subtilis*

Plasmid	Size (Md)	Unique restriction endonuclease sites	Plasmid-borne antibiotic-resistance markers		Reference
			<u>E. coli</u>	<u>B. subtilis</u>	
pHV12	7.9		Cm, Ap	Cm	Ehrlich, 1978a
pHV14, 15	4.6	<u>AvaI</u> , <u>BamHI</u> , <u>Sall</u> , <u>EcoRI</u> , <u>PstI</u> , <u>PvuII</u>	Cm, Ap	Cm	
pHV16	9.7		Cm, Ap	Cm	
pHV18			Cm, Ap, Km	Cm	
pJK3	5.1	<u>Sall</u> , <u>BamHI</u>	Tc, Ap	Tc	Kreft <u>et al.</u> , 1978
pJK201	2.6	<u>BamHI</u> , <u>HindIII</u> , <u>Sall</u> , <u>EcoRI</u>	Tc, Cm	Tc	Goebel <u>et al.</u> , 1979
pJK501	7.9	<u>PstI</u> , <u>Sall</u> , <u>BamHI</u>	Tc	Tc	
pJK502	4.5	<u>HindIII</u> , <u>Sall</u> , <u>PstI</u> , <u>BamHI</u>	Tc	Tc	
pHV23	6.1	<u>AvaI</u> , <u>BamHI</u> , <u>EcoRI</u> , <u>HpaI</u> , <u>KpnI</u> , <u>PstI</u> , <u>PvuII</u> , <u>Sall</u> , <u>XbaI</u>	Ap, Cm, Tc	Tc, Cm	Michel <u>et al.</u> , 1980
pTA1302	5.3	<u>HindIII</u> , <u>EcoRI</u>	Cm, Ap	Cm	Sakaguchi <u>et al.</u> , 1981
pHV33	4.6	<u>PstI</u> , <u>EcoRI</u> , <u>BamHI</u> , <u>Sall</u>	Tc, Cm, Ap	Cm	Primrose & Ehrlich, 1981
pJK3.1		<u>PstI</u> , <u>Sall</u> , <u>HindIII</u> , <u>BamHI</u> , <u>EcoRI</u>	Tc	Tc	Kreft & Hughes, 1981
pJK302		<u>HindIII</u> , <u>BamHI</u> , <u>Sall</u> , <u>PstI</u> ,	Tc, Ap	Tc	
pOG2165	5.0	<u>BglII</u> , <u>HindIII</u> , <u>PstI</u> , <u>SstI</u>	Cm, Ap	Cm, Ap	Gray & Chang, 1981

TABLE 1.0: Some E. coli - B. subtilis bifunctional plasmids

Strauss, 1970; Rudner et al., 1968) but some of the ends of the DNA molecule are lost or otherwise made unavailable for subsequent recombination events (Guild et al., 1968).

The single-stranded donor DNA rapidly interacts with a homologous segment of recipient DNA to form an unstable complex (Davidoff-Abelson and Dubnau, 1973) which then becomes stabilised by base-pairing (Buitenwerf and Venema, 1977; 1978). After formation of the nascent donor-recipient complex the donor DNA becomes entirely base-paired to the homologous recipient DNA and eventually becomes covalently attached, replacing the equivalent recipient segment (Arwert and Venema, 1973; Davidoff-Abelson and Dubnau 1973). Finally expression of the donor genetic material results following segregation of the heteroduplex.

The overall process of chromosomal DNA transformation requires a high degree of donor-recipient DNA sequence homology. Transformation of B. subtilis with chromosomal DNA from closely related species occurs, but at a much lower frequency for most genetic markers (Wilson and Young, 1972). A functional recE4 gene-product, which is involved in recombination events, is also essential (Dubnau et al., 1973; Prozorov et al., 1982).

Investigations into the mechanism of transformation of competent cells by chromosomal DNA have exclusively involved B. subtilis. However, competence transformation systems have been developed for other species of Bacillus, namely B. amyloliquefaciens (Coukoulis and Campbell, 1971); B. licheniformis (Gwinn and Thorne, 1964; Thorne and Stull, 1966); B. thuringiensis (cited in Martin et al., 1981) and B. caldotenax (M. Munster, personal communication) where similar mechanisms of transformation may operate.

Bacteriophage DNA Transfection

Transfection is the process whereby cells are infected by naked nucleic acid from a virus resulting in the production of a complete virus. The mechanism of

transfection has been widely investigated using several B. subtilis bacteriophages and has proved to be a useful system for studying the uptake and processing of DNA by competent B. subtilis cells (for review see Trautner and Spatz, 1973).

Competence for both chromosomal DNA transformation and transfection develop with a similar time course (Trautner and Spatz, 1973) and, as with chromosomal DNA, transfecting DNA is processed to single-stranded intermediates during uptake (Loveday and Fox, 1978; Dishman, 1972, cited in Contente and Dubnau, 1979a). Following uptake, repair of damaged transfecting DNA occurs by a recombination event (Trautner and Spatz, 1973) and depending on the bacteriophage DNA used, from 2 to 4 molecules are required to produce a successful infection. Therefore transfection exhibits a 2nd to 4th order dose-response curve. Unlike chromosomal DNA transformation, transfection is a very inefficient process which could result from heavily damaged DNA molecules being unable to participate in a successful repair event (Trautner and Spatz, 1973; Contente and Dubnau, 1979a).

Plasmid DNA Transformation

Investigations into the uptake and processing of DNA molecules by competent B. subtilis cells using chromosomal DNA have been hindered by the size and sequence heterogeneity of the transforming DNA and also the large size of the recipient chromosome. Therefore transformation using plasmid DNA has been investigated both as a model of chromosomal DNA transformation and also because an understanding of plasmid DNA transformation is advantageous for genetic manipulation in the Bacilli.

All plasmid DNA molecules are probably taken up into competent cells by the same mechanism. However, subsequent processing events may differ depending on the nature of the transforming plasmid and also on whether the recipient harbours a homologous resident plasmid.

Firstly consider the case where the recipient does not contain a homologous resident plasmid and the transforming plasmid has no homology with the recipient chromosome. The following observations have been made of plasmid DNA transformation of competent *B. subtilis* cells. Plasmid DNA transformation, like chromosomal DNA transformation is a first order process (Contente and Dubnau, 1979a) suggesting that a single plasmid molecule is sufficient for a successful transformation event or that the interaction of a cell with a single plasmid molecule is rate limiting. However, in contrast to chromosomal DNA transformation, plasmid DNA transformation does not require the *recE4* gene-product (Gryczan *et al.*, 1978) and is a very inefficient process (Contente and Dubnau, 1979a). The addition of 10^6 to 10^7 molecules per transformant is required with an average uptake of 10^3 to 10^4 molecules per competent cell. Linear and nicked plasmid DNAs, in addition to ccc monomeric plasmid DNA, are not active in the transformation of competent cells (Ehrlich, 1977; Canosi *et al.*, 1978; Contente and Dubnau, 1979a), multimeric forms of plasmid DNA alone are responsible for all the transforming activity of a plasmid preparation.

Although the exact mechanism of transformation of competent *B. subtilis* cells by plasmid DNA is not known, the following model has been put forward (de Vos *et al.*, 1981). The model proposes that the entry of plasmid DNA is analogous to the entry of chromosomal DNA. A double-stranded scission of the plasmid DNA occurs and one strand is taken up whereas the other strand is degraded to acid soluble products. There is extensive intracellular breakdown of entered DNA resulting in the release of acid-soluble material. The degree of polymerisation of the transforming plasmid affects subsequent processing steps. Whereas transformation with monomeric species of plasmid DNA does not lead to the production of a transformed cell, transformation with multimeric plasmid DNA does. It is assumed that complementary single strands produced by transformation with a multimeric plasmid molecule, form partially double-stranded molecules in a *recE4*-independent event. Finally, these molecules are

converted to fully double-stranded monomer-length plasmid molecules by an ill-defined process. A model of plasmid DNA transformation of *B. subtilis* competent cells is summarised in Figure 1.1.

Secondly, consider the case where the transforming plasmid contains a piece of DNA which has homology with a region of the recipient chromosome or even a resident prophage; such DNA can be processed by a different mechanism to that described above. However, multimers of such plasmids may be processed as described in Figure 1.1. Monomeric plasmid molecules of this type are active in the transformation of *B. subtilis* competent cells but only if the host cell is *recE4*-proficient (Bensi *et al.*, 1981; Canosi *et al.*, 1981). It has been suggested that repair of damaged plasmid DNA can occur by a *recE4*-dependant recombination event involving base-pairing of homologous regions of the transforming plasmid and host chromosome. This occurs providing that the damage is within the homologous region of the plasmid. Figure 1.2 is a model summarising this event. If additional DNA fragments are inserted into the homologous region of such plasmids then plasmid rearrangements may occur upon transformation of *recE4*-proficient competent cells (Iglesias *et al.*, 1981). "Corrected" plasmids are produced which have the configuration of the plasmid into which a given alteration was initially introduced.

Thirdly, if the recipient harbours a plasmid which has homology with the transforming plasmid, then plasmid DNA is processed by a different mechanism to those described above. Contente and Dubnau (1979b) observed a ten-fold increase in plasmid transformation frequency when the recipient contained a homologous resident plasmid compared with strains which were plasmid-free or contained a non-homologous plasmid. Also, markers on linear plasmid molecules could be "rescued" providing that the linearising cut was within the homologous region of the transforming plasmid and from 0.2 to 0.5 Md of the junction between the homologous and non-homologous regions of the plasmid. This process was found to be *recE4*-dependant suggesting an obligate requirement for recombination events.

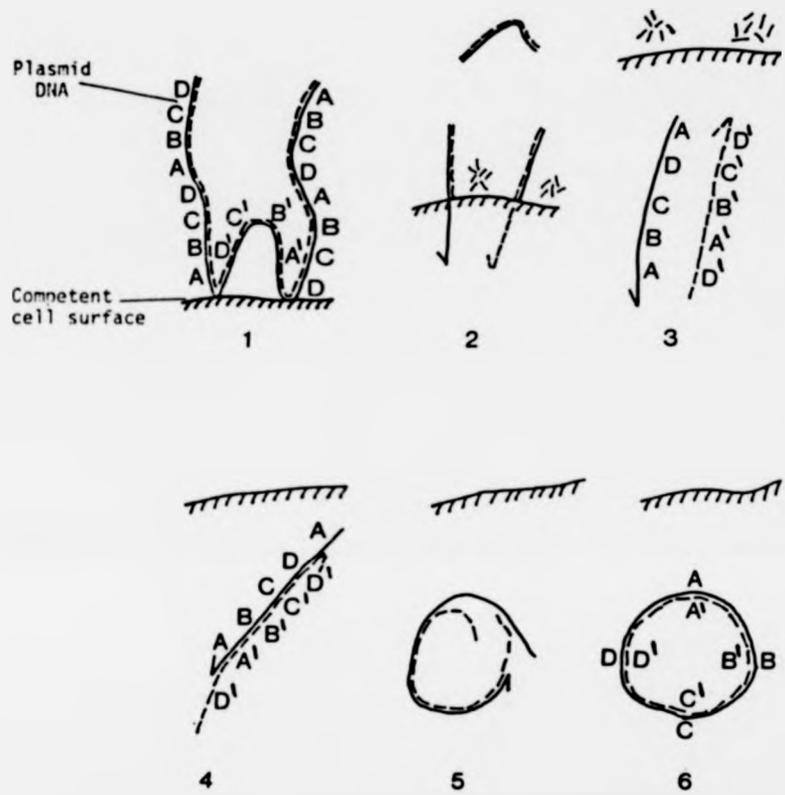


Figure 1.1: Transformation of competent *B. subtilis* cells by multimeric plasmid DNA (adapted from Canosi et al., 1981)

- 1 Plasmid DNA binds to the competent cell surface.
- 2, 3 Uptake of single-stranded plasmid DNA.
- 4 Base pairing of complementary single-stranded plasmid DNA strands.
- 5, 6 Circularisation to form fully double-stranded, monomer-length plasmid DNA.

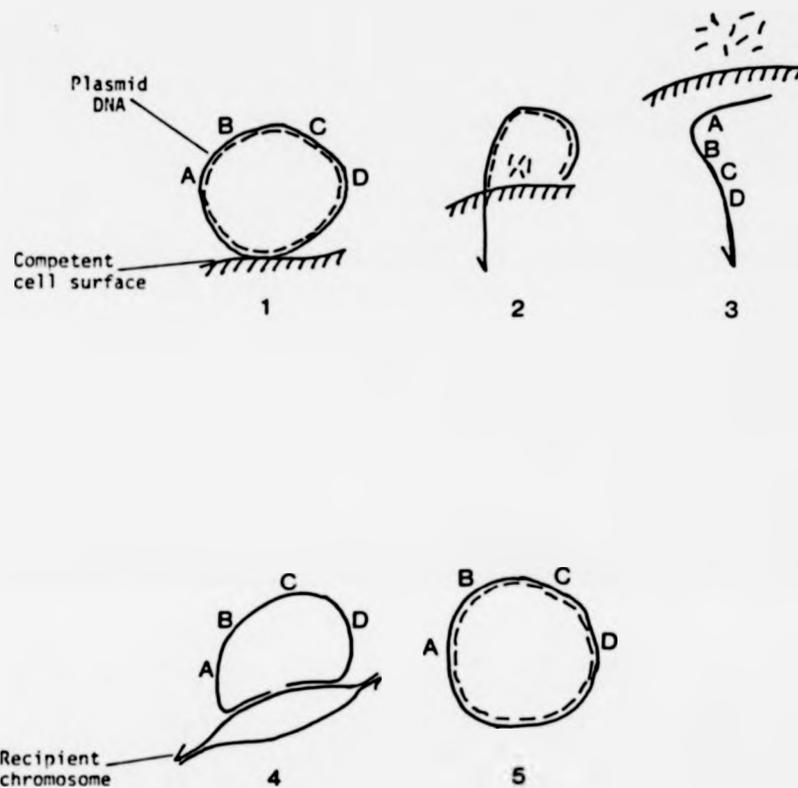


Figure 1.2: Transformation of competent *B. subtilis* cells by plasmid DNA which is partially homologous with the recipient chromosome (adapted from Canosi *et al.*, 1981)

- 1 Plasmid DNA binds to the competent cell surface.
- 2, 3 Uptake of single-stranded plasmid DNA.
- 4 Base pairing of plasmid DNA with complementary region of the recipient chromosome.
- 5 Trimming and DNA synthesis to produce fully double-stranded, monomer-length plasmid DNA.

The process of plasmid-rescue transformation has been modified to permit shotgun-cloning of heterologous chromosomal genes in B. subtilis (Gryczan et al., 1980a). This process relies on repair of damaged plasmid molecules by recombination between a homologous resident plasmid and the transforming hybrid plasmid. Figure 1.3 is a model showing how plasmid-rescue transformation probably occurs.

1.6 TRANSFORMATION OF PROTOPLASTS OF BACILLI

Following work on the regeneration and fusion of Bacillus protoplasts (e.g. Landman et al., 1968; Fodor and Alfoldi, 1976; Schaeffer et al., 1976), Chang and Cohen (1979) have developed a PEG-induced protoplast transformation system for B. subtilis. These workers reported that up to 80% of a population of protoplasts could be transformed with plasmid DNA with an efficiency of greater than 10^7 transformants per μg of DNA. Although Chang and Cohen (1979) could not detect transformation of B. subtilis protoplasts with chromosomal DNA, low frequency transformation with chromosomal DNA has subsequently been reported (Levi-Meyrueis et al., 1980). Also transformation of B. subtilis L-forms by bacteriophage DNA has been reported (White et al., 1981).

In contrast to the B. subtilis competence plasmid transformation procedure, the PEG-induced protoplast transformation procedure does not have a requirement for multimeric plasmid DNA; ccc, open circular and linear monomeric plasmid molecules also are active in transformation although open circular and linear molecules are one to three orders of magnitude less efficient in transformation (Chang and Cohen, 1979). de Vos and Venema (1981) demonstrated that plasmid DNA entered protoplasts in a double-stranded form and that the entered DNA was present predominantly as ccc DNA molecules. The efficiency of plasmid DNA transformation was found to be close to one indicating that each entered plasmid molecule could give rise to a transformed cell. This supported the observations made by these workers that little, if any, damage was

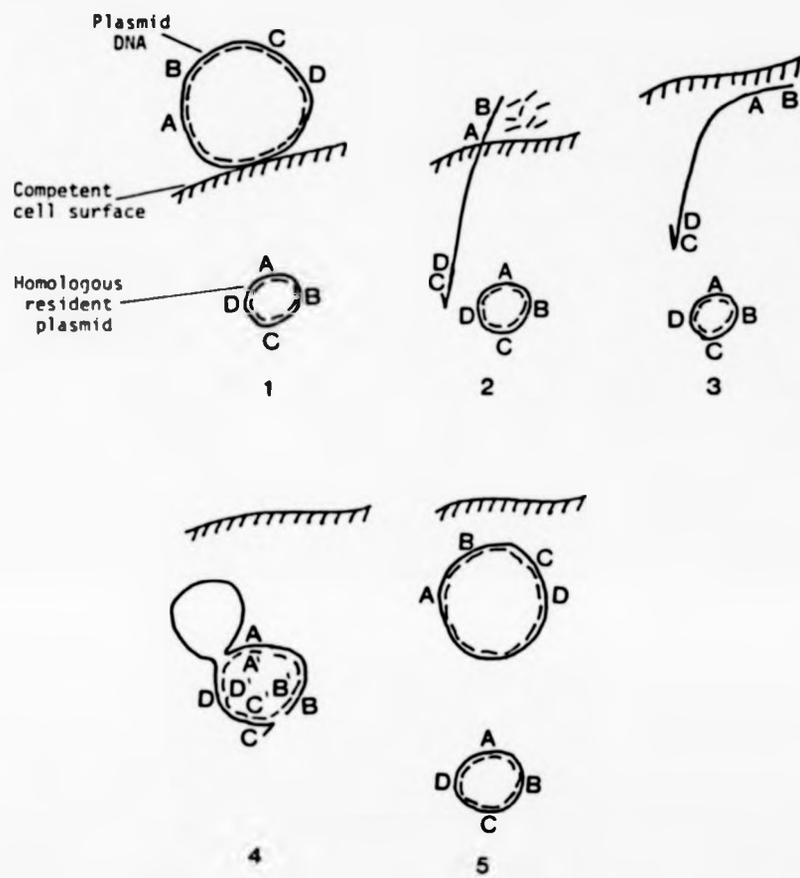


Figure 1.3: Plasmid-rescue transformation by competent *B. subtilis* cells (adapted from Contente and Dubnau, 1979b and Gryczan et al., 1980a).

- 1 Plasmid DNA binds to the competent cell surface.
- 2, 3 Uptake of single-stranded plasmid DNA.
- 4 Interaction of resident plasmid with homologous region of the transforming plasmid.
- 5 Repair to produce fully double-stranded, monomer-length plasmid DNA.

done to plasmid DNA upon entry into protoplasts. Thus, PEG-induced protoplast transformation has two major differences to simple competence plasmid transformation. Firstly, protoplast transformation is much more efficient and secondly, it does not have a requirement for multimeric plasmid DNA.

In summary, although naturally induced competence has been widely used to introduce plasmid DNA into B. subtilis cells, this method is not generally applicable to other species of Bacillus. Plasmid DNA transformation of competent cells has been reported for only one other species of Bacillus, B. licheniformis (Docherty et al., 1981). However, plasmid DNA has been introduced into protoplasts of several species of Bacillus including B. megaterium (Brown and Carlton, 1980), B. licheniformis (Bingham, 1980; Imanaka et al., 1981b), B. thuringiensis (Alikhanian et al., 1981; Martin et al., 1981; Miteva et al., 1981), and B. stearothermophilus (Imanaka et al., 1982) and therefore may be applicable to many other species of Bacillus.

1.7 RESTRICTION AND MODIFICATION IN BACILLI

Restriction endonucleases have been isolated from (see Roberts, 1981) and restriction and modification systems probably operate in many species of Bacillus. Bron et al., (1980a) and Trautner et al. (1974) have shown that in transformation with chromosomal DNA and in transfection of lysogenic recipients, restriction of donor DNA does not occur providing that it is homologous with the recipient DNA. Conversely, restriction of bacteriophage DNA does occur upon transfection of a non-lysogenic strain (Trautner et al., 1974; Bron et al., 1975; 1980a, 1980b). Plasmid DNA is restricted upon transformation of competent B. subtilis cells (Prozorov et al., 1980; Tanaka, 1979b) but if the transforming plasmid has homology with a resident plasmid in the recipient, restriction is not observed (Canosi et al., 1981). Also if a chromosomal DNA fragment is inserted into a plasmid, this fragment is not sensitive to restriction whereas the vector region of the plasmid is (Canosi et al., 1981).

Bron et al. (1980a) have postulated that in chromosomal DNA transformation and in systems where there is homology between the donor and recipient DNAs, pairing of the donor moiety with a modified recipient DNA strand occurs. This results in the formation of a restriction-resistant heteroduplex. In the absence of DNA homology single-stranded donor molecules can anneal upon themselves to yield restriction-sensitive homoduplexes. The above can be used to explain the observations made also with transfecting and plasmid DNAs, i.e. restriction is not observed if the transforming DNA has homology with host DNA.

Restriction of plasmid DNA upon transformation of Bacillus protoplasts has been reported for some species (Bingham, 1980; Vorobjeva et al., 1980; Imanaka et al., 1982) but not others (Chang and Cohen, 1979; Alikhanian et al., 1981). However, if restriction of plasmid DNA does prove to be a problem, it may be possible to isolate restriction-deficient mutants such as B. subtilis strain IG20 (trpC2, hsm⁻, hsr⁻).

1.8 CLONING IN BACILLI

For several years the Bacilli, particularly B. subtilis, have been used as hosts for gene cloning and although problems have been encountered, many successful cloning events have been reported in the literature (e.g. Gryczan and Dubnau, 1978). Suitable cloning vehicles are a prerequisite for DNA cloning in any organism and therefore many early reports detailed the construction of hybrid plasmids for use in B. subtilis (see Table 1.3). Plasmid cloning vehicles have been developed along similar lines to those developed for use in E. coli; mainly by the in vitro linkage of 2 or more plasmid molecules. A large number of hybrids have been constructed (e.g. Gryczan and Dubnau, 1978; Gryczan et al., 1980b) and some have been shown to possess several unique restriction endonuclease sites which can be used to clone foreign DNA. In addition, in some cases insertional inactivation of plasmid-borne markers has been demonstrated (e.g. Gryczan et al., 1980b).

Although the construction of hybrid plasmids has been a relatively simple process, shotgun-cloning of chromosomal genes has proved to be far more difficult. Using the naturally occurring *S. aureus* plasmid pUB110, Keggins *et al.* (1978) reported the first successful shotgun-cloning of chromosomal genes in *B. subtilis*. However, following this initial report, many workers have had great difficulty in shotgun-cloning chromosomal genes directly in *B. subtilis* employing competence transformation procedures (Gryczan *et al.*, 1980a; A.J.P. Docherty and A.H.A. Bingham, personal communications). The consensus of opinion now is that the failure to readily shotgun-clone chromosomal genes directly in *B. subtilis* is due to the inherent properties of the competence transformation process of this species. Gryczan *et al.* (1980a) reported that when foreign DNA was ligated to a plasmid vector, the yield of transformants was markedly reduced and that the extent of the reduction was greater than that expected solely from competition at the level of DNA uptake. This probably occurred because as the ratio of insert DNA to plasmid DNA increases, the number of vector oligomers decreases on subsequent ligation. Therefore, attempting to increase the number of hybrids decreases the number of plasmid oligomers produced. As reported by Canosi *et al.* (1978), only plasmid oligomers are active in the transformation of competent cells and hence, increasing the frequency of hybrid plasmids decreases the transformation frequency of a plasmid-chromosomal DNA mix.

Although it appeared that shotgun-cloning utilising competent *B. subtilis* cells was extremely difficult, recently several reports describing the successful shotgun-cloning of various chromosomal genes have appeared. Jayaraman *et al.* (1981) cloned the *B. subtilis* *spoOB* and *pheA* genes using pUB110. However, the *spoOB* and *pheA* genes, which were shown to reside on the same *Bam*HI fragment, were partially purified by agarose gel electrophoresis prior to cloning. Selection was made for *pheA*⁺ transformants, which were screened for a *spoOB*⁺ phenotype. McDonald and Burke (1982) have cloned a *B. subtilis* chromosomal sulfanilamide-resistance gene and Palva (1982) has cloned the *B. amyloliquefaciens* α -amylase

gene using pUB110. In the latter case no direct selection for α -amylase activity was applied, instead Neo^r transformants were screened for α -amylase production.

A systematic study of random-segment cloning in B. subtilis using competent cells was made by Michel et al. (1980). Random fragments of B. amyloliquefaciens chromosomal DNA were cloned in pHV33 and pC194. After selection for Cm^r transformants, clones were screened for the presence of chromosomal DNA fragments. Yields of 16% and 18% of transformants containing inserts were obtained respectively. The average size of the cloned DNA fragments was 1.1 and 0.9 Md respectively with sizes ranging from 0.4 to 2.0 Md. This compared to an average size of the starting insert DNA of 3 Md with sizes ranging from less than 0.4 Md to greater than 12 Md. Also, Michel et al. (1980) used the insertional inactivation vectors pHV11 and pHV23 to clone S. cerevisiae chromosomal DNA fragments. After initial selection for Cm-resistance the transformants were screened for Tc-resistance. 11.0% and 12.5% of the pHV11 and pHV23 Cm^r transformants respectively were found to be Tc^s . Two Cm^r , Tc^s clones examined were found to contain an insert indicating that these insertional inactivation vectors can be used for random-segment cloning. In summary, although a high frequency of insertion was found, the average size of the inserts was small compared to the size of the starting material. These workers suggested that the small size of the cloned segments may explain why other workers have had difficulties in isolating active genes by random-segment cloning in B. subtilis.

Although Michel et al. (1980) did not determine how and why small fragments of DNA were preferentially cloned, they suggested several reasons why this may occur. The hybrids detected may result from transformation by plasmid molecules having internal repeats of the cloning vector. Intracellular processing may then occur yielding hybrids with small inserts. Alternatively, fragments which do not contain recognition sites for the hosts restriction system may be preferentially cloned. As detailed previously (section 1.7), plasmid transformation

of competent cells is affected by the restriction system of the host. If restriction does prove to be a problem then restriction deficient hosts are available to circumvent this problem. A third explanation for the cloning of small DNA fragments is that large DNA fragments may be unstable and subject to deletion. If this is the case then it is difficult to imagine how such a problem could be overcome.

It is evident from the above that much more work needs to be done to determine the exact mechanism of, and the parameters that govern the cloning of random DNA fragments in B. subtilis before further progress can be made. Because of the difficulties encountered with cloning directly in B. subtilis employing competent cells, other approaches have been investigated and are detailed below.

A successful system for the shotgun-cloning of heterologous DNA fragments has been developed by Gryczan et al., (1980a). This stratagem was based on the findings of Contente and Dubnau (1979b) who showed that transformation of competent B. subtilis cells by linear, or nicked plasmid DNA occurred providing that the host carried a homologous resident plasmid and was recE4-proficient. Gryczan et al., (1980a) suggested that a damaged vector DNA-chromosomal DNA hybrid plasmid molecule could be rescued by recombination with a homologous resident plasmid if the damage to the transforming plasmid was within the homologous sequences. The method was demonstrated by cloning several B. licheniformis chromosomal DNA segments which complemented B. subtilis trp and his mutations. However, complementation of several other B. subtilis auxotrophic markers was not detected. This could have been because the equivalent B. licheniformis genes were unable to complement the B. subtilis mutations tested or the B. licheniformis genes may have been inactivated by the enzyme used to try and clone them. Alternatively, as was suggested by Michel et al. (1980) for the direct cloning system in B. subtilis, small fragments may be preferentially cloned by this method and markers not detected may have resided

on large DNA fragments. The sizes of the cloned fragments of Gryczan *et al.* (1980a) ranged from 0.6 to 4.7 Md, however the size of their starting material was not given.

Other workers also have successfully used the plasmid-rescue shotgun-cloning system to clone *B. licheniformis* chromosomal DNA genes in *B. subtilis* (Docherty *et al.*, 1981; Dubnau *et al.*, 1981). However, a major drawback of this system is that since the process is *recE4*-dependent, it cannot be used to clone homologous (i.e. *B. subtilis*) chromosomal DNA genes. The development of a similar system in other species of *Bacillus* would, however, allow the cloning of *B. subtilis* chromosomal genes.

The *B. subtilis* protoplast transformation system described by Chang and Cohen (1979) appears to be ideally suited to shotgun-cloning. Yields of 80% transformants with an efficiency of 4×10^7 transformants per μg of supercoiled DNA were obtained by these workers. The transformation procedure does not require oligomeric plasmid DNA and is *recE4*-independent. Chang and Cohen (1979) showed that hybrid plasmids constructed *in vitro* were active in transformation although at a frequency of one to three orders of magnitude lower than the frequency observed for ccc plasmid DNA.

The regeneration of *B. subtilis* protoplasts to the bacillary form requires a complex medium and hence a direct selection for complementation of auxotrophic markers cannot be applied. However, subsequently a minimal medium for the regeneration of *B. subtilis* protoplasts has been developed (Sanchez-Rivas, 1982). Therefore, using this regeneration medium it may be possible to apply direct selection for complementation of auxotrophic markers.

Although Goebel *et al.* (1979) and Chang and Cohen (1979) have used the protoplast transformation system to construct hybrid plasmids, there have been no reports in the literature of a successful shotgun-cloning of a chromosomal gene. Hence difficulties may have been encountered using this system. However, Williams *et al.* (1981a) using the protoplast transformation system, have shotgun-

cloned several fragments of DNA that promote expression of a chloramphenicol-resistance gene in B. subtilis. Therefore this system may be useful in certain cases.

Partly because of the difficulties encountered with cloning directly in B. subtilis, several workers have made use of E. coli as an alternative or intermediate host. Consequently E. coli has been used to clone DNA from Bacillus plasmids (Horinouchi et al., 1977; Kreft et al., 1978; Goebel et al., 1979;), Bacillus bacteriophages (Ehrlich et al., 1976; Duncan et al., 1977; Mellado and Salas, 1982) and Bacillus chromosomes (Mahler and Halvorson, 1977; Segall and Losick, 1977; Chi et al., 1978; Nagahari and Sakaguchi, 1978; Brammar et al., 1980; Gray and Chang, 1981; Imanaka et al., 1981b; Neugebauer et al., 1981; Winter et al., 1982). Also the construction of gene banks of B. subtilis has been reported (Rapoport et al., 1979; Ferrari et al., 1981).

The E. coli-B. subtilis bifunctional plasmids described in Table 1.4 are potentially very useful as cloning vectors. They can be used to shotgun-clone genes using the well developed E. coli cloning systems and subsequently the cloned genes can be introduced by transformation into B. subtilis. Rapoport et al., (1979) made use of the bifunctional vector pHV33 to construct a B. subtilis gene bank in E. coli. Cloned chromosomal genes then were introduced back into B. subtilis. If a recombination-deficient host was used, extrachromosomal maintenance of the cloned sequences was attained.

Bifunctional plasmids may not prove to be useful in every case since several Bacillus genes, such as those concerned with sporulation, may not be expressed in E. coli. However, alternative screening procedures such as the use of RNA or DNA probes could prove successful for detection of specific clones in E. coli.

In summary, a variety of strategies have been developed for cloning genes in B. subtilis. Direct shotgun-cloning in B. subtilis can be achieved but may not be

possible in every case. However, initial cloning in E. coli prior to introduction of the required cloned gene into B. subtilis may be advantageous.

1.9 EXPRESSION OF CLONED GENES IN BACILLI

With the development of techniques for introducing genetic material, particularly plasmids, into a given host cell coupled with the recent advances of in vitro genetic manipulation, it has become possible to study the expression of genes from one organism in a completely different organism. Although genes from many sources including prokaryotes and lower and higher eukaryotes have been functionally expressed in E. coli the situation in B. subtilis is very different.

B. subtilis chromosomal and bacteriophage genes have been expressed in B. subtilis when cloned in plasmid vectors. Examples of these are the B. subtilis leu genes (Tanaka and Sakaguchi, 1978) and the B. subtilis bacteriophage ϕ 3T-thyP3 gene (Galizzi et al., 1981). Chromosomal and plasmid genes isolated from other Bacilli also have been expressed in B. subtilis, e.g. the trp genes of B. pumilus and B. licheniformis (Keggins et al., 1978) and the tetracycline-resistance genes of the B. cereus plasmid pBC16 (Bernhard et al., 1978). Also S. aureus plasmid genes have been functionally expressed when introduced into B. subtilis (see Table 1.2).

Whereas Bacillus chromosomal and Bacillus bacteriophage genes usually are expressed when cloned in E. coli (Ehrlich et al., 1976; Duncan et al., 1977; Horinouchi et al., 1977; Mahler and Halvorson, 1977; Chi et al., 1978; Kreft et al., 1978; Nagahari and Sakaguchi, 1978; Goebel et al., 1979; Brammar et al., 1980; Gray and Chang, 1981; Imanaka et al., 1981b; Sakaguchi et al., 1981; Cornelis et al., 1982; Winter et al., 1982) the converse generally has not been observed. Lack of expression of several E. coli antibiotic-resistance genes has been reported including the pBR322, pSC122 and pWL7 ampicillin resistance genes (Kreft et al., 1978; Tanaka and Sakaguchi, 1978; Goebel et al., 1979; Kreft and Hughes, 1981; Mazza et al., 1982), the pACYC184 chloramphenicol resistance gene (Kreft et al.,

1978; Goebel et al., 1979) and the pWL7 and pSC105 kanamycin resistance genes (Ehrlich, 1977; Kreft et al., 1978) in addition to the E. coli recA (Mazza et al., 1982), hisG (Grandi et al., 1981), trpC (Ehrlich, cited in Grandi et al., 1981), and phoA (Kreft and Hughes, 1981) genes. In contrast Goebel et al. (1979) reported expression of the pBR322 tetracycline-resistance genes when cloned in the B. subtilis cryptic plasmid pBS1 and Rubin et al. (1981) reported expression of the E. coli thyA gene upon integration into the B. subtilis chromosome. However, in neither of the latter two cases was expression, utilising E. coli regulatory signals, demonstrated. Therefore it is likely that in general B. subtilis is unable to recognise E. coli regulatory signals.

To understand why E. coli genes usually are not expressed in B. subtilis, it is first essential to understand how regulation of gene expression occurs. There are basically three levels at which gene expression may be regulated: (i) transcriptional, (ii) translational and (iii) post-translational. In its simplest form a transcriptional unit (Fig. 1.4) consists of a start signal (promoter), the transcribed region, which contains the coding information for a polypeptide chain and a stop signal (terminator). In many cases, signals encoded around the transcriptional unit are targets for additional regulatory elements which permit the cell to respond to environmental changes. Fig. 1.5 shows the structure of a simplified E. coli promoter which shows the regions (boxed areas), where main DNA sequence homologies are found. The so called 'Pribnow box' at -10 (Pribnow, 1975) and the -35 region (Rosenberg and Court, 1979). These are specific sites on the DNA chain that are recognised by the E. coli DNA-dependant RNA polymerase. The σ - subunit of the polymerase is important for recognition of specific sequences although it alone does not bind to DNA (Burgess et al., 1969; Zillig et al., 1976; Lowe et al., 1979).

Termination of transcription by E. coli RNA polymerase generally occurs at specific sites on a DNA template and is modulated by the transcription termination factor ρ , in vivo. However, many terminators function in vitro in the

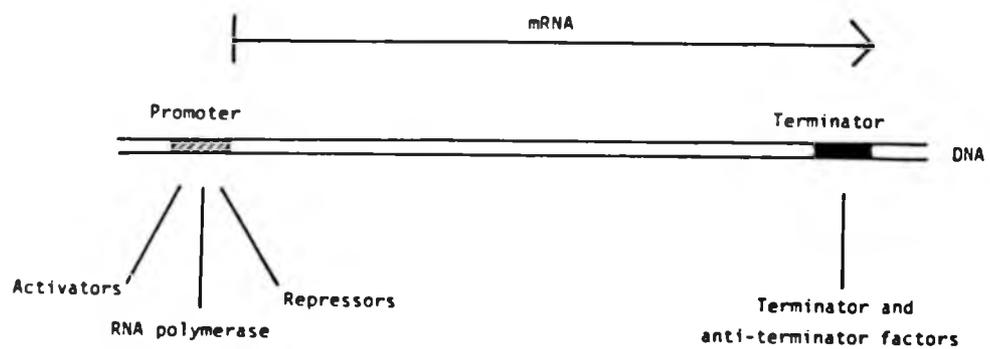


Figure 1.4: Properties of a generalised transcriptional unit
(adapted from Bujard, 1980)

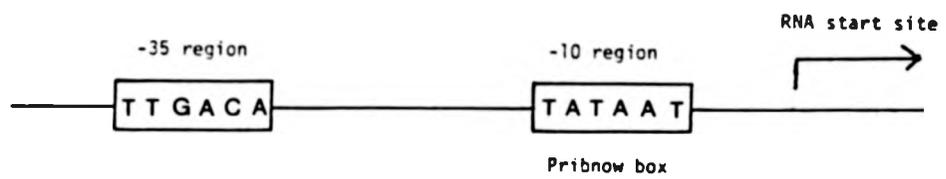


Figure 1.5: Major properties of a generalised *E. coli* promoter (adapted from Bujard, 1980) showing major regions of DNA sequence homology

absence of ρ . Sequence comparison of these ρ - independent termination sites reveals common features (Rosenberg and Court, 1979). These include a G-C-rich region of dyad symmetry and a series of terminal uridine residues in the transcript immediately 3' to the region of dyad symmetry.

Whereas the initiation of transcription is dictated by the existence of a promoter, the initiation of protein synthesis is governed at least in part by the availability of a ribosome binding site (Grunberg-Manago and Gross, 1977; Steitz, 1979) on the messenger RNA (mRNA) molecule. This region, about 3 to 11 bases upstream from the initiation codon, is complementary to the 3' end of the 16S ribosomal RNA (rRNA) (Shine and Dalgarno, 1975) and base-pairs with it during the initiation of protein synthesis (Steitz and Jakes, 1975). The ribosome binding site, or Shine and Dalgarno (S/D) sequence, precedes the translational start codon, AUG, by 3 to 12 bases (Steitz, 1979). Termination of translation is regulated by the presence of a stop codon, either UAG, UAA, or UGA or a combination of these (Kohli and Grosjean, 1981). Also additional signals flanking the stop codons may regulate termination of translation (Kohli and Grosjean, 1981).

Post-translational regulation probably plays a significant role in the expression of some proteins such as membrane-bound and periplasmic proteins of E. coli. Such proteins often require post-translational modifications to be functional.

Failure to recognise transcriptional, translational, or post-translational regulatory signals by B. subtilis cells could lead to non-expression of foreign genes. Goebel et al. (1979) have shown that the lack of expression of the pBR322 β -lactamase gene is due to a block at the post-translational level whereas expression of the pACYC184 chloramphenicol resistance gene is blocked at the level of transcription.

The differences in functional gene expression between E. coli and B. subtilis suggest that these two organisms possess different regulatory signals. What then are the common features of Bacillus regulatory signals? First let us

look at transcription, in particular the B. subtilis RNA polymerase and the nucleotide sequences which it recognises, since heterologous gene expression is probably restricted, at least in part, at this level.

In contrast to the E. coli RNA polymerase, the B. subtilis enzyme fails to efficiently transcribe the lacUV5 and coliphage T4 promoters (Shorenstein and Losick, 1973; Lee et al., 1980) and does not form stable, rapidly starting complexes with coliphage T7 promoters (Wiggs et al., 1979). Also, the E. coli and B. subtilis RNA polymerases do not bind to the same sites on the bifunctional plasmid pHV14 (Ehrlich, Jupp, Niaudet and Gore, cited in Ehrlich and Sgaramella, 1978). This suggests that the B. subtilis RNA polymerase may have a different consensus promoter recognition sequence to its E. coli counterpart. Several promoters which are recognised by the B. subtilis ⁵⁵-RNA polymerase, i.e. the normal vegetative σ -factor, have been sequenced (Moran et al., 1982) and unexpectedly the -10 and -35 regions are very similar to the canonical E. coli sequences in these regions. Also, the 17 to 18 base-pair gap between these regions is similar to that found in E. coli. However, it was observed by Moran et al. (1982) that the -10 and -35 region of the nine sequenced Bacillus promoters were more conserved than the corresponding E. coli sequences. Indeed, it was suggested by these workers that the failure of typical E. coli promoters to conform adequately to the B. subtilis canonical promoter could be the reason for lack of transcription of Gram-negative genes by B. subtilis.

This is unlikely to be the only reason for lack of transcription of E. coli genes in B. subtilis since additional features such as the stretch of A-T residues preceding the -35 regions of the veg promoter (Moran et al., 1982) and strong SP01 promoters (Lee et al., 1980) may also be important. In addition, the sequence PuTPuTG at position -18 to -14, which is present in some strong Bacillus promoters but is rarely found in E. coli promoters, may play an important role in transcription. In addition to the RNA polymerase σ factor, the δ factor also plays an important role in transcription (Pero et al., 1975; Tjian et al., 1977; Achberger

and Whitely, 1981) and recognition signals for this protein may be required for efficient transcription in B. subtilis.

Even if a gene is transcribed by B. subtilis, the mRNA may not be translated if it does not possess the correct translational regulatory signals. It has been demonstrated that B. subtilis ribosomes fail to translate effectively mRNAs from Gram-negative organisms (Legault-Demare and Chambliss, 1975). Conversely E. coli ribosomes can effectively translate Gram-positive mRNAs (Stallcup and Rabinowitz, 1973a, 1973b; Legault-Demare and Chambliss, 1975). A similar situation has been observed with B. stearothermophilus ribosomes which are unable to initiate translation of some coliphage DNAs (Lodish, 1970; Held et al., 1974).

From analysis of several sequenced Bacillus promoters, Moran et al. (1982) found that the ribosome-binding sites displayed extensive sequence complementarity to the 3' region of B. subtilis 16S rRNA. The calculated free energies of interaction (ΔG) for these sites was calculated to vary from -14 to -23 Kcal compared to -9.4 Kcal for the prototype E. coli ribosome-binding site AGGA. Indeed McLaughlin et al., (1981) proposed that the ribosome-binding site of Gram-positive mRNAs are able to form highly stable complexes with the 3' region of 16S rRNA, whereas the potential for base-pairing by E. coli ribosome-binding sites varies over a wide range. Despite these differences, a similarity in the distance between the ribosome-binding sites and the AUG initiation codon was observed between E. coli and Bacillus genes.

Translation of mRNA gives rise to a protein which in certain cases has to be modified to produce a fully active product. The failure of B. subtilis to express genes coding for membrane proteins such as the pBR322 β -lactamase might be due to the inability of B. subtilis to process the immature protein. Goebel et al. (1979) showed that although B. subtilis cells could transcribe and translate the pBR322 β -lactamase gene, it was not functionally expressed. By using B. subtilis minicells it was demonstrated that the inactive β -lactamase precursor was

produced but could not be processed to the mature, active β -lactamase. It is not yet known why the precursor β -lactamase protein is not processed by B. subtilis.

One of the main goals of recombinant DNA research is to produce large quantities of commercially important proteins from cells containing a desired cloned gene. Clearly then, in order to direct the expression of a cloned gene in B. subtilis, it is essential that the correct regulatory signals are present.

In E. coli, plasmids have been developed which can be used to clone transcriptional and translational regulatory signals (e.g. An and Friesen, 1979; Casadaban et al., 1980; Casadaban and Cohen, 1980) which have then been used to direct the synthesis of several authentic eukaryotic proteins (see Roberts et al., 1979; Goeddel et al., 1979; Guarente et al., 1980). In an attempt to develop a similar system in B. subtilis, Williams et al. (1981a) constructed the plasmid pPL603 which could be used to clone EcoRI-generated DNA fragments that promote gene expression in B. subtilis. Sequences from various sources that promoted expression of the Cm resistance gene of pPL603 were cloned in B. subtilis. The plasmid pL608, which is pPL603 containing a B. subtilis SP02 phage DNA fragment, was constructed and subsequently used (Williams et al., 1981b) to direct the expression of a mouse dihydrofolate reductase gene and E. coli trp genes in B. subtilis. The cloned mouse dihydrofolate reductase gene conferred trimethoprim-resistance on B. subtilis cells and the cloned trp genes were able to complement mutations in the B. subtilis trp C, D and F genes.

The expression of two other eukaryotic proteins in B. subtilis has been reported (Hardy et al., 1981). The hepatitis B core antigen and foot and mouth disease major antigen were placed under the control of the pBD9 erythromycin-resistance gene. Synthesis of both the above proteins could be detected in B. subtilis and was shown to be inducible by erythromycin, indicating that it was the erythromycin-resistance regulatory signals which were being utilised.

In summary, in contrast to E. coli, B. subtilis is stringent in its requirement for regulatory signals involved in gene expression. Although genes

from other species of Bacillus usually are expressed in B. subtilis, genes from Gram-negative organisms and from eukaryotes generally are not expressed. However, providing that the correct regulatory signals are provided, expression of any gene in B. subtilis may be possible.

1.10 PROTEIN SECRETION BY BACILLI

A large number of extracellular proteins, such as α -amylases produced by species of Bacillus, are of commercial importance per se (Priest, 1977). Therefore much attention has focussed on the physiological or environmental factors affecting exoenzyme production. Exoenzyme synthesis appears to be subject to control by both end-product inhibition and catabolite repression (for review see Glenn, 1976). Examples of the former are the repression of exoprotease synthesis by amino acids, and alkaline phosphatase synthesis by inorganic phosphate in B. subtilis. Examples of the latter are the α -amylases of B. subtilis and B. licheniformis which are subject to catabolite repression by glucose; the B. stearothermophilus α -amylase is repressed by fructose. The proteases of B. subtilis and B. licheniformis also are subject to catabolite repression by glucose.

In addition to investigations of the physiological conditions affecting exoprotein production, some investigations have focussed on the genetics of exoenzyme production. Dubnau and Pollock (1965) and Sherrat and Collins (1973) have investigated the B. licheniformis β -lactamase gene and Uehara et al. (1974), the B. subtilis protease gene. However, the majority of work has concerned the α -amylase gene of both B. licheniformis (Saito and Yamamoto, 1975) and B. subtilis (Sekiguchi et al., 1975; Yoneda and Maruo, 1975; Yoneda, 1980). The production of the latter enzyme has been found to be regulated by a number of different genes, the most significant being amyR, papM, and tmr (Yoneda, 1980). A 250-fold increase in α -amylase synthesis has been obtained (Yoneda, 1980) by introducing these and other genes into B. subtilis by transformation.

Recently much attention has focussed on the mechanism of enzyme secretion with the aim of understanding how cells specifically secrete certain proteins. Much of our understanding of protein secretion comes from work carried out with eukaryotic systems and the 'signal-sequence' hypothesis has been proposed (Blobel and Sabatini, 1971; Blobel and Doberstein, 1975) to explain how protein secretion occurs in eukaryotes. Basically the hypothesis suggests that proteins destined for secretion are synthesised on membrane-bound ribosomes and contain a N-terminal extension of amino acids, the so-called signal-sequence, or -peptide, which is thought to be responsible for initiating passage of the polypeptide through the membrane. This signal-peptide then is enzymatically removed either before or immediately after translation is completed.

A similar situation is thought to exist in prokaryotic cells. In Gram-negative bacteria membrane-bound and periplasmic proteins usually are synthesised as larger precursors containing at their N-terminus an extension of 15 to 30 predominantly non-polar, hydrophobic amino acids which are not found in the mature protein (e.g. Randall *et al.*, 1978). It is these signal-peptides which are responsible for membrane insertion or transport into the periplasm. The exact mechanisms by which secretion is accomplished are as yet unknown. However, it is likely that different signal-peptides determine the different cellular locations of secreted proteins.

It is assumed that secreted proteins produced by Gram-positive bacteria also are synthesised as precursor molecules containing a signal-peptide at their N-termini. This has been established for the *B. licheniformis* penicillinase protein (see Chapter V for further details) and also the *B. amyloliquefaciens* α -amylase protein (Palva *et al.*, 1981; Palva, 1982). It is now envisaged that proteins of commercial importance can be attached to the signal-peptides of such secreted proteins to promote the secretion of the protein of interest.

1.11 PLASMID STABILITY IN BACILLI

The stable maintenance of recombinant plasmids is a highly desirable property, particularly for the production of cloned gene-products on an industrial scale. In this respect, a major problem with using B. subtilis for genetic manipulation is that many plasmids, particularly recombinant plasmids, are remarkably unstable in Bacillus host cells. Two major types of instability are observed: the loss of the entire plasmid from the host cell, termed segregational instability and the rearrangement of plasmid sequences, termed structural instability. Although very little is known about factors which affect plasmid stability in Bacilli, the following are known to contribute to plasmid instability in other species.

Segregational instability occurs when there is no active segregation of plasmid molecules to daughter cells. Under such conditions the probability of generating plasmid-free cells increases with decreasing plasmid copy number and hence low copy-number plasmids are more likely to give rise to plasmid-free cells than high-copy number plasmids. Complete plasmid loss may be due to several factors (cited in Schukin, 1981); mutations in the host chromosome or the plasmid itself may affect the plasmid copy-number or inhibit plasmid replication.

Structural instability often results in the rearrangement of plasmid sequences via deletions. The presence of insertion sequences or transposable elements are known to alter plasmid structure including molecular size (Cohen, 1976; Bukhari et al., 1977) and in addition repetitive DNA sequences seem to be particularly prone to deletions (Cohen et al., 1978).

Very little has been reported in the literature of the stability of natural and hybrid plasmids in species of Bacillus making comparison of the stability of different plasmids extremely difficult. However, a brief summary is presented below.

Some natural plasmids (Bingham, 1980; Gryczan et al., 1978) and hybrid plasmids (Dubnau et al., 1981) can be stably maintained in B. subtilis in the absence of selection pressure for plasmid-borne markers. However, in general Bacillus plasmids are unstable. Firstly, consider natural plasmids which are

indigenous to species of Bacillus or have been introduced into B. subtilis by transformation. Both segregational instability (Lovett, 1973; Lovett et al., 1976; Ehrlich, 1977; Gryczan et al., 1978; Gryczan et al., 1980b; Grandi et al., 1981; Imanaka et al., 1981a) and structural instability (Kono et al., 1978; Grandi et al., 1981; Imanaka et al., 1981a) have been observed. Similarly, hybrid plasmids produced by in vitro genetic manipulation are in general unstable compared to the stability of the parental plasmids used in their construction (Goebel et al., 1979; Gryczan et al., 1978; Sakaguchi, 1981; Ehrlich et al., 1981; Fujii and Sakaguchi, 1980; Gryczan et al., 1980b; Dubnau et al., 1981; Grandi et al., 1981; Kreft et al., 1978; Kreft and Hughes, 1981; McDonald and Burke, 1981; Rutberg et al., 1981; Bingham et al., 1982; Mazza et al., 1982). Again, both segregational and structural instability have been observed.

Often hybrid plasmids constructed in vitro are both structurally and segregationally unstable and spontaneously give rise to more stable deletion derivatives (Gryczan et al., 1980b; Uhlen et al., 1981; Bingham et al., 1982). However, it is not clear whether it is the nature of certain plasmid sequences that makes plasmids unstable or merely the larger size of the hybrids compared to their parental plasmids. Small plasmids may be segregationally more stable than large plasmids and also cells containing small plasmids may have a growth advantage over those containing large plasmids (Uhlen et al., 1981).

Plasmid instability may also result in intramolecular recombination (e.g. Grandi et al., 1981). This may prove to be a problem in certain cases since the recombination event may occur by recE4-independent pathways (Tanaka, 1979a; Fujii and Sakaguchi, 1980; Uhlen et al., 1981).

In addition to segregational and structural instability, a third type of instability may occur. If the host contains a plasmid which has sequences homologous to the host chromosome, then part of, or the entire, plasmid may integrate into the host chromosome (Rapoport et al., 1979; Jayaraman et al., 1981). However, integration can be prevented by the use of host cells carrying

the recE4 mutation (Keggins et al., 1978; Rapoport et al., 1979; McDonald and Burke, 1982). Therefore this type of instability does not pose a major problem.

In summary, although very little is known about the stability of plasmids in the Bacilli, general observations suggest that both segregational and structural instability are major problems. Clearly, many more detailed investigations are needed before conclusions can be made about the stability of plasmids in the Bacilli.

Since it is envisaged that genetic manipulation in the Bacilli will play a much more important role in the future, the aim of the work detailed in this thesis was to investigate the usefulness of Bacillus subtilis and Bacillus licheniformis as hosts for genetic manipulation.

CHAPTER II

MATERIALS AND METHODS

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2.1 MATERIALS

2.1.1 Chemicals

Except where stated, all chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, England and were of Analytical reagent (Anala R) grade wherever possible.

The following were purchased from Sigma Chemical Company (U.K.) Ltd., Poole, Dorset, England. Ethidium bromide, chloramphenicol, tetracycline, ampicillin, neomycin sulphate, adenosine 5'-triphosphate (ATP), DL-dithiothreitol (DTT), Triton X-100, Ficoll, bromophenol blue, o-nitrophenyl- β -D-thiogalactopyranoside (ONPG), 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (BCIG), isopropyl β -D-thiogalactopyranoside (IPTG), isoamyl alcohol, polyoxyethylene 20 cetyl ether (Brij 58), sodium deoxycholate, and xanthine (sodium salt).

Polyethylene glycol (PEG) molecular weight 6000 was obtained from Koch-Light Laboratories, Coinbruck, Bucks., England.

Soya peptone and purified agar were obtained from Oxoid Ltd., London, England.

Tryptone, yeast extract, nutrient broth, casamino acids (vitamin assay) and penassay broth (antibiotic medium 3) were obtained from Difco Laboratories, West Holesey, Surrey, England, and were of Bacto grade.

Redistilled phenol (nucleic acid grade) and agarose (gel electrophoresis grade) were obtained from Bethesda Research Laboratories Ltd., Cambridge, England.

Sephadex G-50 was obtained from Pharmacia (GB) Ltd., Middlesex, England.

2.1.2 Enzymes

All restriction endonucleases and T₄ polynucleotide ligase were purchased from Bethesda Research Laboratories Ltd., Cambridge, England.

Proteinase K and E. coli DNA polymerase I, large fragment (Klenow) were obtained from Boehringer Mannheim GmbH, Mannheim, Germany.

Nick-translation enzymes were obtained from Amersham International Ltd., Amersham, Bucks, England.

Lysozyme (Grade I), bovine serum albumin (BSA) fraction V, xanthine oxidase (Grade I) and cytochrome c (horse heart, type III) were obtained from Sigma Chemical Company (U.K.) Ltd., Poole, Dorset, England.

2.1.3 Media

All the media listed below were made up in double-distilled water and autoclaved at 15 p.s.i. (121°C) for 15 min unless otherwise stated.

Tryptone Soya Broth Agar (TSBA)

This solid medium was routinely used for the cultivation of Bacillus strains and contained the following (g l⁻¹):

Tryptone	17.0
Soya peptone	3.0
NaCl	5.0
K ₂ HPO ₄	2.5
Glucose	2.5
Purified agar	30.0

Double-distilled water was added to 1000 ml and the pH adjusted to 7.3 with 2 M KCl.

Tryptone Yeast Extract Salt (TYS) Broth

This liquid medium was routinely used for the cultivation of Bacillus strains and contained the following (gl^{-1}):

Tryptone	20.0
Yeast extract	10.0
NaCl	10.0

Double-distilled water was added to 1000 ml, (natural pH of 7.2).

Luria (L) Broth

This medium was routinely used for the cultivation of E. coli strains and contained the following (gl^{-1}):

Tryptone	10.0
Yeast extract	5.0
NaCl	5.0

Double-distilled water was added to 1000 ml, (natural pH of 6.9). For solid media (L-agar), 2% (w/v) purified agar was added.

Nutrient Broth

This medium was routinely used for the cultivation of B. subtilis prior to protoplast formation and contained the following (gl^{-1}):

Nutrient broth	16.0
NaCl	5.0

Double-distilled water was added to 1000 ml, (natural pH of 7.5).

Subtilis Minimal Salts (SMS)

This defined medium was routinely used for the cultivation of Bacillus strains and contained the following (g l^{-1}):

$(\text{NH}_4)_2\text{SO}_4$	2.0
K_2HPO_4	14.0
KH_2PO_4	6.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
tri-Na citrate. $2\text{H}_2\text{O}$	1.0

Double-distilled water was added to 1000 ml, (natural pH of 7.3). After autoclaving, 10 ml of glucose (50%, w/v) was added. When necessary, amino acids (100 μg per ml final concentration) and purine and pyrimidine base supplements (50 μg per ml final concentration) were added. For solid media (SMS agar), 1.6% (w/v) purified agar was added.

TM Medium

This medium was used for the transformation of B. licheniformis strains and contained the following (g l^{-1}):

$(\text{NH}_4)_2\text{SO}_4$	2.0
K_2HPO_4	14.0
KH_2PO_4	6.0
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2
tri-Na citrate. $2\text{H}_2\text{O}$	3.0
Casamino acids	0.075
Yeast extract	0.2

Double-distilled water was added to 1000 ml, (natural pH of 7.3). After autoclaving, 10 ml of glucose (50%, w/v) was added. When necessary, amino acid supplements (100 µg per ml final concentration) were added.

M9 Medium

This defined medium was used for the cultivation of E. coli strains and was made as follows: 100 ml of M9 buffer was mixed with 10 ml of Ca/Mg solution and 10 ml of glucose (20%, w/v). Double-distilled water was added to 1000 ml.

For solid media (M9 agar), 2% (w/v) purified agar was added.

M9 buffer (gl⁻¹)

Na ₂ HPO ₄	60.0
KH ₂ PO ₄	30.0
NaCl	5.0
NH ₄ Cl	10.0

Ca/Mg solution (gl⁻¹)

CaCl ₂	1.1
MgSO ₄ ·7H ₂ O	24.6

DM3 Regeneration Medium

This medium was used for the regeneration of B. subtilis protoplasts.

Solution A:	Casamino acids	1.0g
	Yeast extract	1.0g
	MgCl ₂ ·6H ₂ O	0.81g
	Purified agar	1.6g

Double-distilled water was added to 90 ml.

Solution B: Sodium succinate 27.0g

Double-distilled water was added to 90 ml and the pH adjusted to 7.3 with 11.3 M HCl.

Solution C: K_2HPO_4 70.0 $g\ l^{-1}$
 KH_2PO_4 30.0 $g\ l^{-1}$

DM3 regeneration medium was made by adding 90 ml of solution A to 90 ml of solution B and 10 ml of solution C. Two ml of glucose, (50%, w/v) was added followed by 5 ml of BSA, (2%, w/v, filter sterilised).

DM4 Regeneration Medium

This medium was used for the regeneration of B. licheniformis protoplasts; it was essentially the same as DM3 regeneration medium except that solution B contained 32.4 g of sodium succinate.

2.1.4 Buffers

Double-distilled water was used for all buffers.

SMM, (SMM6)

0.5 M Sucrose (0.6 M sucrose for SMM6)
0.02 M Maleic acid
0.02 M $MgCl_2 \cdot 6H_2O$
0.2% (w/v) BSA

The pH was adjusted to 6.5 with 2 M NaOH.

SMMP, (SMM6P)

This was prepared by mixing equal volumes of 4 volume Penassay broth and 2 volume SMMP, or 2 volume SMM6 for SMM6P.

SMMP BSA, (SMM6P BSA)

These buffers were the same as SMMP and SMM6P except that they contained 1% (w/v) BSA final concentration. BSA was filter sterilised prior to addition.

TES

30 mM	Tris
50 mM	NaCl
5 mM	Na ₂ EDTA

The pH was adjusted to 8.0 with 11.3 M HCl.

TE

10 mM	Tris
0.1 mM	Na ₂ EDTA

The pH was adjusted to 8.0 with 11.3 M HCl.

Tris-borate

90 mM	Tris
90 mM	Boric acid
3 mM	Na ₂ EDTA

Natural pH of 8.0.

Tris-acetate

40 mM	Tris
20 mM	Na acetate
1 mM	Na ₂ EDTA

The pH was adjusted to 8.0 with glacial acetic acid.

Restriction endonuclease assay buffers

<u>BamHI:</u>	10 mM	Tris-HCl, pH 7.5
	10 mM	MgCl ₂
	10 mM	NaCl
	0.5 mM	DTT

This buffer was used also for BstEII and PstI.

<u>BglII:</u>	20 mM	Tris-HCl, pH 7.5
	7 mM	MgCl ₂
	0.5 mM	DTT

<u>EcoRI:</u>	100 mM	Tris-HCl, pH 7.5
	20 mM	MgCl ₂
	50 mM	NaCl
	0.5 mM	DTT

<u>HindIII:</u>	20 mM	Tris-HCl, pH 7.5
	20 mM	MgCl ₂
	50 mM	NaCl
	0.5 mM	DTT

<u>Sall:</u>	8 mM	Tris-HCl, pH 7.5
	6 mM	MgCl ₂
	150 mM	NaCl
	0.5 mM	DTT

All buffers were made up as 10 volume stock solutions and used appropriately.

Ligation Buffer

Solution A:	66 mM	Tris-HCl, pH 7.6
	66 mM	MgCl ₂
	10 mM	DTT
Solution B:	0.4 mM	ATP

Solutions A and B were made up as 10 volume stock solutions and used appropriately.

CAT Assay Buffer

One ml of Tris-HCl (0.1 M, pH 7.8), containing 4 mg per ml of 5-5'-dithiobis (2-nitrobenzoic acid) (DTNB) was added to 1 ml of Acetyl Coenzyme A (1 μM) and 8 ml of Distilled water.

SOD Assay Buffer

SOD assay buffer was prepared by adding 10 ml of potassium phosphate buffer, (pH 7.5), 0.2 mM Na₂ EDTA to 0.5 ml of cytochrome c solution (6 mg per ml in potassium phosphate buffer (pH 7.5), 0.1 mM Na₂ EDTA) and 0.5 ml of Xanthine solution (0.025%, w/v, in distilled H₂O).

2.1.5 Bacterial Strains and Plasmids

These are described in Tables 2.1 and 2.2.

2.2 METHODS

2.2.1 Storage and Growth of Bacterial Strains

All bacterial strains were stored on Dorset egg-agar slopes (Oxoid Ltd., England) at ambient temperature and also at -70°C in glycerol-containing medium. For storage at -70°C , *Bacillus* strains were streaked onto TSBA plates, incubated overnight at 37°C and the cells resuspended in 10 ml of TYS broth. Glycerol was added to 10% (v/v) final concentration and 1 ml aliquots were frozen at -70°C . *E. coli* strains were grown overnight in 10 ml of L-broth at 37°C with aeration. Glycerol was added to 10% (v/v) final concentration and 1 ml aliquots frozen at -70°C .

When first obtained plasmid-encoded markers and auxotrophic markers were checked by streaking onto appropriate media.

2.2.2 Transformation of Competent *B.subtilis* Cells

B. subtilis cells were transformed essentially as detailed by Bingham (1980). A single colony from a TSBA plate was inoculated into 50 ml of SMS in a 1500 ml ribbed conical flask. After overnight incubation at 37°C with vigorous aeration, the culture was diluted to an $\text{OD}_{450\text{nm}}$ of 0.1 to 0.4 with fresh, prewarmed SMS. Aeration was continued and the $\text{OD}_{450\text{nm}}$ of the culture monitored at 30 to 60 min intervals. When the cells became competent (see Figure 2.1), 1 ml aliquots of culture fluid were removed and added to the transforming DNA (1 to 50 μl) containing 50 μl of PEG 6000 (50%, w/v), in a 1 oz bottle. After incubation with vigorous aeration at 37°C for 1 hr, 5 ml of prewarmed TYS broth was added. Incubation was continued for a further 1 hr before plating onto selective media. Prior to plating on minimal medium, cells were first pelleted by centrifugation

Strain	Genotype/phenotype	Source
<u>B. subtilis</u>		
IG20	<u>trpC2</u> , <u>hsm</u> , <u>hsr</u>	A.H.A. Bingham
BD170	<u>trpC2</u> , <u>thr-5</u>	A.J.P. Docherty
BD224	<u>trpC2</u> , <u>thr-5</u> , <u>recE4</u>	A.J.P. Docherty
BD393	<u>trpC2</u> , <u>lys⁻</u> , <u>thyA</u> , <u>thyB</u>	A.J.P. Docherty
QB943	<u>trpC2</u> , <u>ilvA1</u> , <u>thyA</u> , <u>thyB</u>	R.J. Sharp
Marburg	Prototroph (wild type 168 strain)	R.J. Sharp
<u>B. licheniformis</u>		
LO2	Prototroph	A.H.A. Bingham
749/C	Constitutive penicillinase producing mutant	W. Brammar
<u>E. coli</u>		
MC1061	<u>araD139</u> , Δ (<u>ara</u> , <u>leu</u>) 7697, Δ <u>lacX74</u> , <u>galU</u> , <u>galK</u> , <u>hsr</u> , <u>hsm⁺</u> , <u>strA</u>	M.C. Casabadian
W3110	<u>thy⁻</u>	N.P. Minton

TABLE 2.1: Bacterial strains

Plasmid	Size (Md)	Plasmid-borne Markers	Comments	Source
pAB224	2.0	Tc	pAB124 deletion derivative	A.H.A. Bingham
pCD1	5.4	Tc, <u>thyP3</u>	Ø3T- <u>thyP3</u> gene + pMB9	F. Young
pBD64	3.2	Km, Cm	pUB110-pC194 deletion derivative	D. Dubnau
pHV14	4.4	Tc, Cm	pC194-pBR322 hybrid	N.P. Minton
pUB1660	4.4	Tc, Ap	pACYC184 + <u>B. licheniformis</u> 749/C <u>penP</u> gene	A.J.P. Docherty
pMC1871	4.4	Tc	Contains <u>lacZ</u> gene	M.C. Casabadian
pMC1396	6.6	Ap	Contains <u>lacY</u> and <u>lacZ</u> genes	M.C. Casabadian

TABLE 2.2: Plasmids

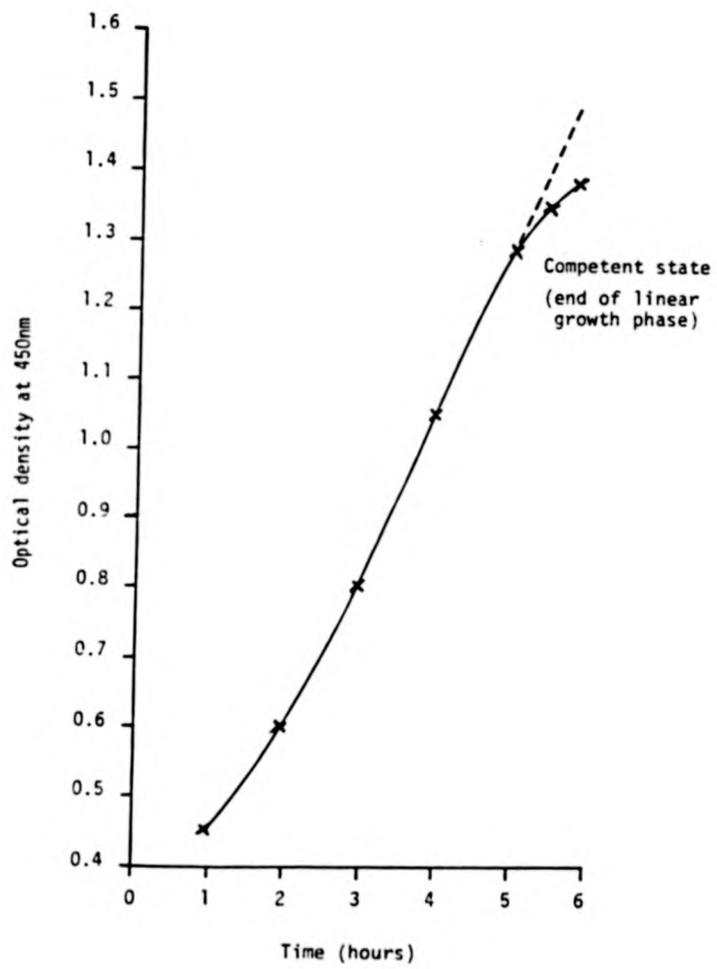


Figure 2.1: Growth of a culture of *B. subtilis* strain IG20 to competence

(5000 rpm for 10 min in an MSE Minor S bench-top centrifuge) and resuspended in 1 ml of SMS (containing no added supplements).

2.2.3 Transformation of B.subtilis Protoplasts

B. subtilis protoplasts were transformed essentially as detailed by Chang and Cohen, 1979 (Bingham, 1980). A single colony from a TSBA plate was used to inoculate 100 ml of nutrient broth. The cells were grown, with vigorous aeration, at 37°C overnight, in a 1500 ml ribbed conical flask. The culture was diluted with fresh, prewarmed nutrient broth to an OD_{450nm} of 0.1 to 0.4 and incubation was continued. When the OD_{450nm} of the culture reached 1.0 to 1.2, 40 ml of culture was centrifuged (5000 rpm for 10 min in a bench-top centrifuge) to pellet the cells. After gentle resuspension in 2 ml of SMM, 100 µl of lysozyme solution (4 mg per ml of lysozyme in SMM) was added. Protoplasts were formed by incubation at 42°C for 30 min.

Transforming DNA (1 to 50 µl containing an equal volume of 2 volume SMM) was added to 1.5 ml of PEG 6000 (40%, w/v in SMM) immediately followed by 0.5 ml of protoplast suspension. After mixing and incubation at ambient temperature for 2 min, 5 ml of SMMP was added. Protoplasts were pelleted by centrifugation (5000 rpm for 10 min in a bench-top centrifuge), resuspended in 1 ml of SMMP BSA and incubated at 37°C for 2 hrs with occasional mixing. Protoplasts were then diluted in SMMP BSA, spread on appropriate media and incubated at 37°C for up to 4 days.

2.2.4 Transformation of B. licheniformis Competent Cells

B. licheniformis strain LO2 mutants were transformed essentially as described by Dubnau and Pollock (1965). A single colony from a SMS agar plate was used to inoculate 3 ml of TM medium in a loz bottle and incubated at 35°C for 20 hrs with aeration. 150 µl of culture fluid was removed and added to 1.35 ml of TM medium containing 7×10^{-3} M NaCl and 2 µg of chromosomal DNA. Incubation

was continued at 40°C for 2 hrs. Prior to plating, the cells were pelleted by centrifugation (5,000 rpm for 10 min in a bench-top centrifuge) and resuspended in 1 ml of SMS. Duplicate SMS agar plates were incubated at 37°C and 55°C for up to 5 days to allow for growth of any transformants.

2.2.5 Transformation of B.licheniformis LO2 Protoplasts

Protoplast transformation of B. licheniformis strains was done as described by Bingham (1980). A single colony from a TSBA plate was used to inoculate 100 ml of TYS broth. The culture was grown, with vigorous aeration, in a 1500 ml ribbed flask at 55°C. This culture was then used to inoculate 200 ml of prewarmed TYS broth to give an OD_{550nm} of 0.3 to 0.4, and incubation continued. The OD_{550nm} was monitored at 15 to 30 min intervals and when it reached 0.7 to 0.9, 40 ml of culture fluid was pelleted by centrifugation (5000 rpm for 10 min in a bench-top centrifuge). The cells were resuspended in 2 ml of SMM6; 200 µl of lysozyme solution (20 mg per ml in SMM6) was added, and protoplasts were formed by incubation at 42°C for 30 min. Transforming DNA (1 to 50 µl, containing an equal volume of 2 volume SMM6) was added to 1.5 ml of PEG 6000 (40% w/v in SMM6) immediately followed by 0.5 ml of protoplast suspension. After mixing and incubation at 42°C for 2 min, 5 ml of prewarmed SMM6P was added. Protoplasts were pelleted by centrifugation (5000 rpm for 10 min in a bench top centrifuge), and resuspended in 2 ml of SMM6P BSA. After incubating for 2 hrs at 55°C with occasional mixing, protoplasts were diluted in SMM6P BSA and plated on DM4 plates containing antibiotics where appropriate. The inoculated petri dishes were sealed with plastic tape to reduce evaporation and incubated at 55°C for up to 4 days.

2.2.6 Transformation of E. coli Competent Cells

Strains of E. coli were transformed essentially as described by Cohen et al. (1972), (C.J. Duggleby, personal communication).

An overnight culture, in L-broth, was used to inoculate 50 ml of pre-warmed L-broth to an OD_{450nm} of 0.1 to 0.2, in a 250 ml conical flask. The culture was incubated at $37^{\circ}C$ with vigorous aeration and the OD_{450nm} of the culture monitored at 15 to 30 min intervals. When the OD_{450nm} reached 0.6 to 0.7, the flask was placed on ice for 20 min. 40 ml of culture was centrifuged (15,000 g for 10 min at $4^{\circ}C$) and the pellet resuspended in 25 ml of chilled 0.1 M $MgCl_2$. After a further centrifugation step, the cells were resuspended in 2.5 ml of chilled 0.1 M $CaCl_2$ and placed on ice for at least 2 hrs.

100 μ l of competent cells was added to the transforming DNA (10 μ l or less), in a sterile 1.5 ml Eppendorf tube and left on ice for 30 min. Cells were heat shocked at $42^{\circ}C$ for 2 to 3 min then placed on ice for a further 60 min. Transformed cells were brought to room temperature, diluted in L-broth and inoculated onto appropriate media. Plates were incubated overnight at $37^{\circ}C$.

2.2.7 Large Scale Isolation of Plasmid DNA from Bacillus Strains

Plasmid DNA was isolated as detailed by Bingham (1980). A single colony from a fresh TSBA plate was used to inoculate 1000 ml of TYS broth containing appropriate antibiotics. After incubation with vigorous aeration overnight, at $37^{\circ}C$, cells were harvested by centrifugation (15,000 g for 15 min at $4^{\circ}C$), washed twice in 200 ml of TES buffer and stored for 5-7 hrs at $4^{\circ}C$.

The cells were resuspended in 20 ml of TES buffer and 10 ml of Na_2 EDTA (200 mM, pH 8.0) was added followed by 10 ml of lysozyme solution (10 mg per ml in TES buffer). After 10 min on ice the cells were incubated at $37^{\circ}C$ with occasional agitation. When cell lysis commenced, as indicated by an increase in viscosity of the culture, 10 ml of SDS (10% w/v) was added followed by 20 ml of 5 M NaCl. The lysate was then well mixed and left overnight at $4^{\circ}C$. Cell debris was removed by centrifugation (45,000 g for 45 min at $4^{\circ}C$). The supernatant fluid was extracted twice with an equal volume of chloroform: isoamyl alcohol (24:1, v/v). Centrifugations were carried out at 35,000 g for 5 min at $4^{\circ}C$.

A major proportion of the chromosomal DNA present in the supernatant fluid was removed by alkali denaturation. The pH of the cleaved lysate was adjusted to 12.2 with 400 μ l additions of 3 M NaOH. After 10 min on ice the pH was re-adjusted to 8.5 with 1 ml additions of Tris-HCl (2 M, pH 7.0). Then, after 1 min on ice, the lysate was extracted twice with an equal volume of chloroform:isoamyl alcohol (24:1 v/v).

Plasmid DNA was precipitated by the addition of PEG 6000 which was added, as a solid, to a final concentration of 10% (w/v). After leaving on ice for 2 to 3 hrs, the DNA was pelleted by centrifugation (30,000 g for 10 min at 4°C) and resuspended in 8 ml of TES buffer. After three extractions with an equal volume of chloroform:isoamyl alcohol (24:1 v/v), a clearing spin was done (35,000 g for 10 min at 4°C) and the lysate was subjected to isopycnic centrifugation.

2.2.8 Small Scale Isolation of Plasmid DNA from Bacillus Strains

In order to rapidly screen *B. subtilis* cells for the presence of extrachromosomal DNA, mini-plasmid preparations were performed as follows. 10 ml cultures were grown overnight in 1 oz bottles. Cells were pelleted by centrifugation (5,000 rpm for 10 min in a bench-top centrifuge), washed twice in 5 ml of TES buffer, and resuspended in 200 μ l of TES buffer. 100 μ l of Na₂ EDTA (200 mM, pH 8.0) was added followed by 100 μ l of lysozyme solution (10 mg per ml in TES buffer). After 10 min on ice, cells were incubated at 37°C for 6 min with occasional mixing. 100 μ l of SDS (10% w/v) was added followed by 200 μ l of NaCl (5 M). After leaving on ice for 1-2 hrs, cell debris was removed by centrifugation for 5 min in a microcentrifuge (Quickfit Instrumentation, Staffs, England). The supernatant fluid was extracted 3 to 4 times with chloroform:isoamyl alcohol (24:1 v/v) and 100 μ l aliquots of the supernatant fluid were used for analysis on agarose gels.

2.2.9 Large Scale Isolation of Plasmid DNA from E. coli Strains

Plasmid DNA was purified from Escherichia coli strains essentially as described by Clewell and Helinski (1969), (C.J. Duggleby, personal communication).

A single colony from a fresh L-agar plate was inoculated into 150 ml of pre-warmed L-broth, in a 500 ml flask. Cells were grown with vigorous aeration at 37°C until the OD_{650nm} reached 0.9. Chloramphenicol was added (170 µg per ml final concentration) and the cultures were left shaking for a further 12-18 hrs.

Cells were harvested by centrifugation (15,000 g for 15 min at 4°C) and resuspended in 2 ml of sucrose (25% w/v) in Tris-HCl (0.05 M, pH 8.0). Cells were transferred to a 30 ml polypropylene centrifuge tube and kept on ice. 0.3 ml of lysozyme solution (20 mg per ml in 0.25 M Na₂ EDTA, pH 8.0) was added and swirled, on ice, for 2 min. 2 ml of Na₂ EDTA (0.25 M, pH 8.0) was added and the tubes swirled intermittently for 2 min. 3 ml of Brij/DOC solution (1%, w/v, Brij 58; 0.4%, w/v sodium deoxycholate in 0.01 M Tris-HCl; 0.001 M Na₂ EDTA, pH 8.0) was added and mixed well. Cells were left on ice for 20-30 min until lysis had occurred.

Cell debris, and the bulk of the chromosomal DNA, was pelleted by centrifugation (45,000 g for 45 min at 4°C). The supernatant fluid was decanted into a fresh tube and subjected to isopycnic centrifugation.

2.2.10 Isopycnic Centrifugation

Plasmid and chromosomal DNAs were separated by isopycnic centrifugation as follows. One g of solid caesium chloride and 0.1 ml of ethidium bromide solution (20 mg per ml in TE buffer) were added to each 1 ml volume of cleared lysate. Centrifugation was carried out at 15°C and 40,000 rpm either for 16 to 20 hrs using a Sorvall TV865 B rotor or 40 to 50 hrs using a Sorvall T-865 I rotor.

After centrifugation, the plasmid (lower) and chromosomal (upper) DNA bands were visualised with a UV transilluminator (Blak-ray, Ultra-Violet Products Inc., San Gabriel, California, U.S.A.), and the plasmid DNA band removed. This was done by puncturing the side of the centrifuge tube with a hypodermic needle

and withdrawing 1 to 2 ml into a 2 ml syringe. Ethidium bromide was removed by extracting 3 to 6 times with an equal volume of isoamyl alcohol. Caesium chloride was removed by dialysis against three changes of TE buffer at 4°C. At least 1000 volumes of dialysis buffer were used and the buffer changed after 1, 2 and 4 hours of dialysis.

The protein contamination and the concentration of the purified plasmid DNA was determined by measuring the optical density at 260 and 280 nm in quartz cuvettes. Protein contamination was considered negligible if the $OD_{260/280\text{nm}}$ was 1.7 or greater. When necessary contaminating protein was removed by extraction with phenol.

An optical density of 1.0 at 260 nm was taken to be equivalent to a DNA concentration of 50 µg per ml.

2.2.11 Isolation of Chromosomal DNA from Bacillus Strains

A single colony from a TSBA plate was used to inoculate 200 ml of TYS broth. After overnight incubation at 37°C (55°C for *B. licheniformis*), with vigorous aeration, cells were harvested by centrifugation (15,000 g for 15 min at 4°C), washed twice in 20 ml of TES buffer and resuspended in 10 ml of TES buffer. 1 ml of lysozyme solution (10 mg per ml in TES buffer) and 2 ml of Na₂ EDTA (200 mM, pH 8.0) were added and the mixture left on ice for 10 min. Cells were placed at 37°C and when lysis commenced, as indicated by an increase in viscosity of the culture, 2 ml of Triton X-100 (10% v/v), and 0.5 ml of proteinase K (10 mg per ml in distilled water, pretreated at 60°C for 10 min), were added. After 2 to 3 hrs on ice, the lysate was extracted with phenol and the DNA spooled onto a glass rod after precipitation with 2 volumes of ethanol (-20°C) and 0.1 volumes of sodium acetate (3 M, pH 5.5). The DNA was resuspended in TE buffer and the phenol extraction-spooling procedure repeated 2 to 6 times. The chromosomal DNA was then dried by passing a stream of nitrogen over the DNA and finally the DNA was resuspended in TE buffer (A.H.A. Bingham, personal communication).

2.2.12 Precipitation of DNA

DNA was precipitated by the addition of 0.1 volume of sodium acetate (3 M, pH 5.5) and 2 volumes of ethanol (95% v/v, pre-cooled to -20°C). After 10 to 15 min at -70°C or 1 to 16 hrs at -20°C , the DNA was pelleted by centrifugation for 10 min in a micro-centrifuge at 4°C . The supernatant fluid was poured off and the pellet vacuum-dried and resuspended in an appropriate volume of TE buffer.

2.2.13 Phenol Extraction of DNA

Phenol (redistilled) was melted by heating to 55°C , and equilibrated with TE buffer. An equal volume of equilibrated phenol was added to the DNA of interest, mixed, centrifuged in a microcentrifuge for 2 min and the phenol layer removed. This was repeated 2 to 4 times. Traces of phenol were then removed by extraction 2 to 4 times with ether. Traces of ether were then removed by passing a stream of nitrogen over the sample.

2.2.14 Treatment of DNA with Enzymes

2.2.14.1 Restriction endonucleases

An appropriate amount of DNA was mixed with the required assay buffer and from 1 to 5 units of the required restriction endonuclease per μg of DNA added. After incubation at 37°C for 1 to 2 hrs the restriction endonuclease was inactivated by heating at 70°C for 10 min. If BstEII was used, incubation was carried out at 60°C and the enzyme was not heat inactivated. If necessary, digestion with a second enzyme followed. If the second enzyme required a different assay buffer the concentration of the buffer constituents was altered accordingly.

2.2.14.2 T4 polynucleotide ligase

Appropriate amounts of DNA to be ligated were mixed and heated at 70°C for 10 min. However, if EcoRI-digested DNA samples were to be ligated, the DNA was first precipitated with ethanol and resuspended in TE buffer. After cooling to room temperature the ligase assay buffers were added followed by T4 polynucleotide ligase; usually 0.02 to 0.1 units of ligase per µg of DNA.

The ligation mixtures were incubated overnight at 4°C. Samples were analysed on mini-gels for the presence of new molecular species which was an indication that ligation had occurred. Unless otherwise stated, such ligation mixtures were used for transformation without further treatment.

2.2.14.3 DNA polymerase I, large fragment (Klenow)

Bacteriophage λ DNA was end-labelled by a procedure modified from Downing et al. (1979) (C.J. Duggleby, personal communication). 1 µg of HindIII-digested λ cl₈₅₇ DNA (Bethesda Research Laboratories, Cambridge, England) was treated at 70°C for 15 min to inactivate the restriction endonuclease. The volume of the HindIII-digested λ cl₈₅₇ DNA solution was made to 20 µl with distilled H₂O and 1 µl of No buffer was added followed by approximately 20 µCi of (α³²P)-labelled dTTP (Amersham International Ltd., Amersham, Bucks, England) and 0.2 units of E. coli DNA polymerase I, large fragment (Klenow). After mixing, incubation was carried out at ambient temperature for 30 min.

2.2.14.4 Nick-translation

Nick-translation of plasmid DNA was carried out using an Amersham International nick-translation kit and was carried out according to the instructions supplied by the manufacturer. Unincorporated deoxynucleotide triphosphates were removed from the labelled DNA by gel filtration through a (20 x 1cm) Sephadex G-50 column.

2.2.15 Agarose Gel Electrophoresis

1. Vertical, 20cm x 20cm, slab gels were routinely used for the analysis and sizing of DNA fragments. 1% (w/v) agarose was used with either Tris-acetate or Tris-borate buffer. The DNA was electrophoresed at a constant 125 V for 3 to 4 hrs or a constant 8 to 10 V for 16 to 20 hrs.
2. 'Mini-gels' were routinely used for the rapid analysis of DNA samples. 1% (w/v) agarose was used with Tris-borate buffer. Electrophoresis was carried out at a constant 100 mA for 15 to 30 min.

If the size of the DNA fragments was to be estimated, λ cl_{857} DNA, digested with HindIII (Bethesda Research Laboratories, Cambridge, England), was used. This produced DNA fragments with sizes of 14.47, 6.28, 4.18, 2.82, 1.58, 1.39 and 0.31 Mdal (Murray and Murray, 1975; Bingham, 1980) (see Fig. 2.2).

Tracking Dye

Prior to electrophoresis of DNA samples, 0.3 volume of tracking dye was added to each DNA sample. Tracking dye had the following composition:

20% (w/v)	Ficoll
10% (w/v)	Sucrose
0.1 mM	Na ₂ EDTA
0.15 mg/ml	Bromophenol blue

After electrophoresis, gels were stained using electrophoresis buffer containing ethidium bromide (about 10 μ g per ml final concentration), and unless otherwise stated, were photographed using a UV transilluminator (Chromato-vue

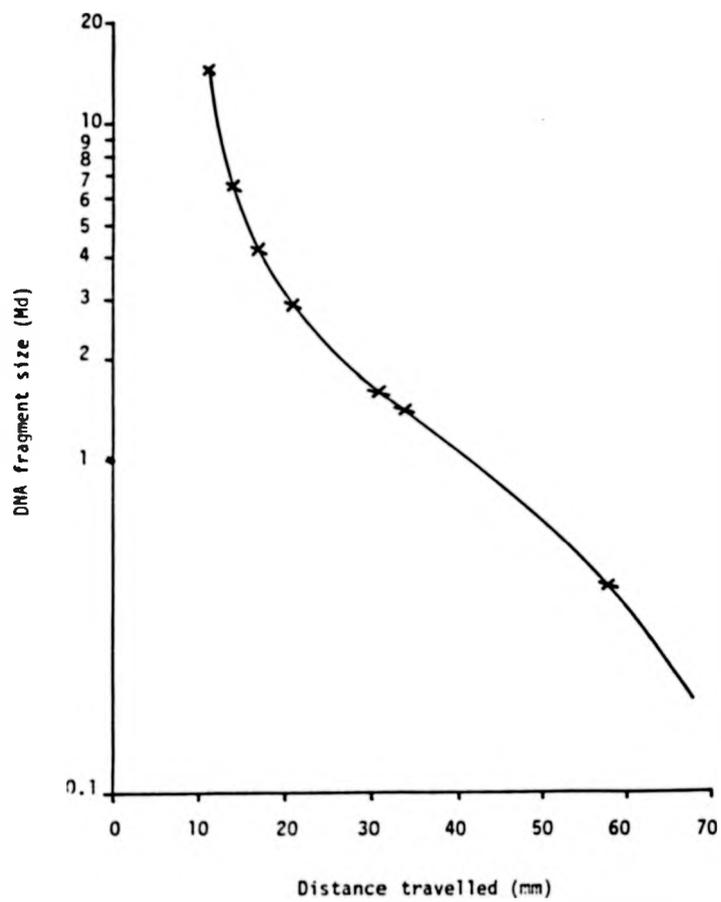


Figure 2.2: Standard curve for the determination of DNA fragment sizes

HindIII-digested λ DNA gives fragments of 14.47, 6.23, 4.18, 2.82, 1.58, 1.39 and 0.31 (Murray and Murray, 1975; Bingham 1980).

Transilluminator, model C-61, Ultra-Violet Products Inc., San Gabriel, California, U.S.A.) with a Polaroid MP4 land camera using a Wratten 2B ultraviolet filter and a Polaroid Type 667 Coaterless Land Pack Film or a Praktica LT camera using a Wratten 2B ultraviolet filter and Ilford FP4 film.

2.2.16 Recovery of DNA from Agarose Gels

DNA was extracted from agarose gels essentially as described by McDonell *et al.* (1977). After agarose gel electrophoresis and staining with ethidium bromide, the required DNA fragment was cut from the gel with a scalpel using a UV transilluminator (Blak-ray, Ultra-Violet Products Inc., San Gabriel, California, U.S.A.) to visualise the DNA bands. The gel slice was placed in a length of 1 cm³ diameter dialysis tubing and submerged in 0.05 volume Tris-borate buffer. The tubing was placed in an electrophoresis tray and just covered with 0.05 volume Tris-borate buffer. Electroelution was carried out at 300 V, 12 mA for 1-2 hrs. After electroelution the Tris-borate buffer, containing the eluted DNA, was removed from the dialysis tubing, extracted twice with isoamyl alcohol, twice with phenol, ethanol precipitated and resuspended in TE buffer.

2.2.17 Southern Transfer

After electrophoresis through a 1% (w/v) agarose gel, employing Tris-acetate buffer, the gel was stained with ethidium bromide and placed on a U.V. transilluminator (Chromato-vue, Transilluminator model C-61) for about 5 min to nick large fragments of DNA. The gel was placed in denaturing solution (0.5 M NaOH, 1.5 M NaCl) at ambient temperature for 30 min, washed once in distilled water for 10 min and then placed in neutralising solution (1 M Tris-HCl, 3 M NaCl pH 5.5) at ambient temperature for 60 min. After washing in 2 volume SSC (0.2 M NaCl, 0.03 M Na citrate), the denatured DNA was transferred to a nitrocellulose filter sheet (Schleicher and Schull, ex. Anderman & Co. Ltd., Surrey, England) essentially as described by Southern (1975). After leaving for 24 to 40 hrs the

nitrocellulose membrane was removed, washed in 2 volume SSC, dried at ambient temperature and then baked at 80°C for 2 hrs.

2.2.18 DNA-DNA Hybridisation and Autoradiography

Nick-translated probe DNA was first denatured by adding 1 µl of NaOH (12.4 M) and heating at 100°C for 10 min. The probe was placed on ice and neutralised with 50 µl of Tris-HCl (1 M, pH 7.5) and 2 µl of HCl (11.3 M). After soaking the membrane in 50% (v/v) formamide/2 volume SSC, it was placed in a polythene bag and sealed. The bag was pierced and 5 ml of 50% (v/v) formamide/2 volume SSC was pipetted into the bag followed by the DNA probe. Hybridisation was then carried out overnight at 37°C.

After hybridisation, the nitrocellulose membrane was removed from the polythene bag, washed for 30 min three times, in a mixture of 50% (v/v) formamide/2 volume SSC, and three times in 2 volume SSC.

The membrane was then dried at 37°C and subjected to autoradiography. This was carried out at -70°C using Kodak X-ray film and a Dupont Cronex Xtra Life intensifying screen, for up to 3 days.

2.2.19 β-Galactosidase Assays

β-galactosidase assays were done essentially as described by Miller (1972). An overnight culture in minimal or complex broth, as required, was used to assay for β-galactosidase activity. After leaving on ice for 20 min, cultures were treated as follows:

E. coli:- The OD_{600nm} of the culture was measured prior to lysis. 1 ml aliquots of culture were lysed by vortexing for 10 s after the addition of 20 µl of chloroform and 20 µl of SDS (0.1% w/v).

B. subtilis:- 1 ml aliquots were centrifuged (2 min at 4°C in a microcentrifuge) and the supernatant fluid was decanted, centrifuged for a further 2 min, and finally decanted into a fresh Eppendorf tube and placed on ice. The cell pellet was resuspended in 1 ml of fresh medium, centrifuged for 2 min, resuspended in 1 ml of fresh medium and placed on ice. The OD_{600nm} of the cell suspension was then measured.

β-galactosidase activity was measured as follows: 1 to 500 μl (v), of enzyme solution was made up to 1 ml with Z-buffer in a 1.5 ml Eppendorf tube. The reaction was then commenced by the addition of 200 μl of ONPG (4 mg per ml in 0.1 M phosphate buffer, pH 7.0). Incubation was carried out at 28°C and the time of incubation (t), monitored. The reaction was stopped by the addition of 0.5 ml of Na₂CO₃ (1 M). The OD_{550nm} and OD_{420nm} of each sample was then measured.

For E. coli the units of β-galactosidase were calculated from:

$$\text{units} = \frac{\text{OD}_{420\text{nm}} - (1.75 \times \text{OD}_{550\text{nm}})}{1000 \times t \times v \times \text{OD}_{600\text{nm}}}$$

t = time of reaction (min)

v = sample volume (μl)

For B. subtilis the units of β -galactosidase were calculated from:

$$\text{units} = \frac{\text{OD}_{420\text{nm}} - (1.65 \times \text{OD}_{550\text{nm}})}{1000 \times t \times v \times \text{OD}_{600\text{nm}}}$$

t = time of reaction (min)

v = sample volume (μl)

The figure of 1.65 for B. subtilis was calculated by measuring the absorbance at 420nm and 550nm of B. subtilis cultures and is detailed further in Fig. 2.3.

The units are proportional to the increase in o-nitrophenol per minute per bacterium. Units per ml of culture were calculated by using an $\text{OD}_{600\text{nm}} = 1.0$, in the above equations.

2.2.20 Superoxide Dismutase Assays

Enzyme solutions of B. subtilis cultures were prepared as described in Section 2.2.18 above. Superoxide dismutase (SOD) activity was measured using a modified procedure of McCord and Fridovich (1969), (A. Atkinson and C.J. Bruton, personal communication).

1.0 ml of SOD assay buffer was incubated at 30°C and the $\text{OD}_{550\text{nm}}$ monitored on a chart recorder using a Pye Unicam SP8-100 ultraviolet spectrophotometer. Sufficient xanthine oxidase was added to catalyse the reduction of cytochrome c by xanthine to give an absorbance change of 0.2 to 0.4 units per min at 550 nm.

From 1 to 250 μl (v), of SOD-containing solution was added to the above solution and the inhibition of the rate of reduction of cytochrome c was monitored.

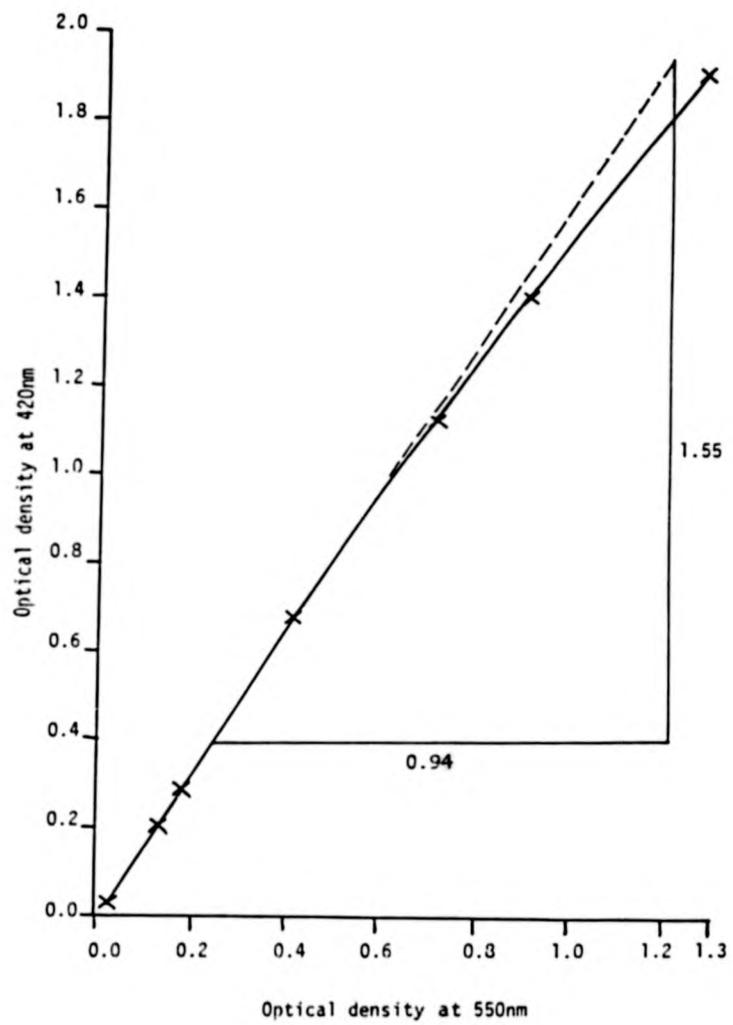


Figure 2.3: Absorbance of *B. subtilis* cultures at 550nm and 420nm.

The value of 1.65 calculated for *B. subtilis* was the gradient ($1.55/0.94$) of the linear part of the graph as shown.

One unit of SOD was defined as the amount required to cause a 50% inhibition of the rate of reduction of cytochrome c in the above reaction.

Units were calculated from:

$$\frac{\% \text{ inhibition} \times \text{dilution factor} \times 1000}{v \times 50} = \text{units per ml}$$

v = sample volume (μl)

Z-buffer

0.06 M	Na_2HPO_4
0.04 M	$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$
0.01 M	KCl
0.001 M	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$
0.05 M	β -mercaptoethanol

No-buffer

1 μl	10 mM dCTP
1 μl	10 mM dGTP
1 μl	10 mM dATP
8 μl	<u>HindIII</u> restriction endonuclease assay buffer
70 μl	Double-distilled H_2O

2.2.21 Chloramphenicol Acetyltransferase Assays

B. subtilis cells were grown with aeration, at 37°C , overnight in 10 ml of TYS broth, in 1 oz bottles. Cells were pelleted by centrifugation (5000 rpm for 10 min in a bench-top centrifuge), washed once with 5 ml of Tris-HCl (0.1 M, pH 7.8) and resuspended in 3 ml of Tris-HCl (0.1 M, pH 7.8). The cells were then disrupted by

sonication on ice (three 20 s sonications using a MSE 150 watt Ultrasonic Disintegrator at 20 Hz per s, 5 amps). The cell lysates were assayed for chloramphenicol acetyl transferase (CAT) activity using a modified procedure of Shaw (1975) (M.D. Scawen, personal communication). 1 ml of CAT assay buffer was added to a 1 ml cuvette and equilibrated to 30°C. Either 5 or 50 µl of lysate was added followed by 25 µl of chloramphenicol (5 mM). After mixing, the optical density at 412 nm was monitored on a chart recorder using a Pye Unicam SP8-100 ultraviolet spectrophotometer. The units of enzyme activity were calculated from:

$$\text{units per ml} = \frac{A_{412} \text{ per min} \times 73.5 \times \text{dilution factor}}{\text{sample volume } (\mu\text{l})}$$

using E_{412} for DTNB equivalent to 13.6×10^3 M.

2.2.22 B. licheniformis Mutagenesis

200 ml of prewarmed (55°C) TYS broth in a 1500 ml conical flask, was inoculated with a single colony from a TSBA plate and incubated at 55°C with vigorous aeration. The $OD_{450\text{nm}}$ of the culture was monitored at 15 to 30 min intervals and when it reached about 1.2, 10 ml of culture fluid was centrifuged (5000 rpm for 10 min in a bench top centrifuge) to pellet the cells. After resuspending in 10 ml of saline (0.9%, w/v, NaCl at 55°C), 100 µl of the suspension was added to 10 ml of saline (0.9%, w/v, NaCl at 55°C), poured into a sterile glass petri-dish, and UV irradiated. A Phillips TUV 15 W Germicidal lamp was used at a height of 20 cm above the petri-dish.

Unless otherwise stated, 1 ml aliquots of cell suspension were removed, added to 100 ml of pre-warmed TYS broth in a 1500 ml flask, and incubated at 55°C with vigorous aeration for 3 hrs, in the dark, to allow for mutant segregation.

After incubation the culture was centrifuged (5,000 rpm for 10 min in a bench-top centrifuge), washed in 10 ml of saline (0.9%, w/v, NaCl at 55°C), diluted in TYS broth and inoculated onto solid media. Plates were incubated for up to 5 days at 55°C.

2.2.23 B. licheniformis Plate U.V. Mutagenesis

A single colony from a TSBA plate was used to inoculate 100 ml of pre-warmed (55°C) TYS broth in a 1500 ml conical flask. Incubation was carried out at 55°C with vigorous aeration. The OD_{450nm} of the culture was monitored at 15 to 30 min intervals and when it reached 1.0 to 1.4, 100 µl aliquots were spread on pre-warmed TSBA plates. The plates were exposed to UV light (a Phillips TUV 15 W Germicidal lamp was used at a height of 20 cm above the petri-dish) for various lengths of time and then immediately placed in a 55°C incubator. After overnight incubation, mutants amongst the survivors were detected either by replica-plating or tooth-picking onto appropriate media. Presumptive mutants were further checked to determine their phenotype by streaking onto appropriate media.

2.2.24 Determination of Reversion Frequency of B. licheniformis Mutants

100 ml of SMS in a 1500 ml conical flask was inoculated with a single colony from an SMS agar plate. Incubation was carried out at 55°C and the OD_{450nm} monitored at 30 min intervals. When the OD_{450nm} reached 1.2 to 1.6, 20 ml of culture fluid was centrifuged (5000 rpm for 10 min in a bench top centrifuge) and the cells washed twice with 20 ml of SMS before resuspending in 1 ml of SMS. A sample of the original culture was also diluted in SMS and plated on TSBA plates to determine the cell density. The cell suspension, in SMS, was diluted in SMS, plated on minimal agar plates lacking the particular requirement under investigation, and incubated for up to 5 days at 55°C. The phenotype of any revertants which appeared was checked by streaking onto appropriate media.

2.2.25 Analysis of Plasmid Stability

200 ml of TYS broth, in a 1500 ml conical flask, was inoculated with a single colony from a TSBA plate and incubated at 37°C with vigorous aeration, overnight. A cell count of each culture was made, on selective media as necessary and 100 colonies from these plates were tooth-picked onto further selective media to determine their phenotypes.

CHAPTER III

SHOTGUN-CLONING IN *B. subtilis*

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3.1 INTRODUCTION

The development of a useful shotgun-cloning system in B. subtilis has been the subject of much attention over the past few years. The systems which have been or are being developed can be divided into 3 classes. Firstly, there is the direct cloning system using plasmid vectors, e.g. pUB110 (Keggins et al., 1978) which is analagous to the plasmid cloning systems available for use in E. coli, e.g. pBR322 (Bolivar et al., 1977). Secondly, there is the plasmid-rescue shotgun-cloning system developed by Gryczan et al. (1980a). This system can be used only for the cloning of heterologous chromosomal genes since an intact recipient recombination system is required and homologous genes cloned using this system would recombine with the recipient chromosome. There is no analagous E. coli system but a similar system has been developed for use in Streptococcus (Behnke, 1982). The third cloning strategy makes use of B. subtilis bacteriophages which have been developed as cloning vectors. Several genes such as the B. amyloliquefaciens α -amylase gene (Yoneda et al., 1979) have been cloned successfully using bacteriophage vectors. However, a disadvantage of bacteriophage systems is that sub-cloning into plasmid vectors is often required to permit further investigations of the cloned gene. This may prove difficult as has been demonstrated for the α -amylase gene cloned in the B. subtilis bacteriophage Φ 3T (Y. Yoneda and E. Kenny, personal communications).

Although the construction of plasmid-plasmid hybrids has proved to be a relatively simple procedure in B. subtilis (see Table 1.3 for a list of hybrid plasmids constructed), the shotgun-cloning of chromosomal DNA fragments directly in B. subtilis has proved to be more difficult (e.g Gryczan et al., 1980a). Keggins et al. (1978) reported the first successful shotgun-cloning of chromosomal genes in B. subtilis. The trp genes of B. licheniformis, B. pumilus and B. subtilis were cloned into pUB110 and introduced into competent B. subtilis cells. Following this initial report, pUB110 has been used to shotgun-clone a

B. subtilis sulfanilamide-resistance gene (McDonald and Burke, 1982); pheA and spoOH genes (Jayaraman et al., 1981) and the B. amyloliquefaciens α -amylase gene (Paiva, 1982). Also, the plasmid vectors pBD9 and pHV33 have been used to shotgun-clone B. licheniformis and S. cerevisiae chromosomal genes respectively, directly in competent B. subtilis cells (Michel et al., 1980). To date there have been no reports of the shotgun-cloning of a chromosomal gene employing the protoplast transformation system developed by Chang and Cohen (1979). However, Goebel et al. (1979) have constructed hybrid plasmids *in vitro* and introduced them into B. subtilis protoplasts and Williams et al. (1981a) have cloned many chromosomal DNA fragments that promote expression of a Cm-resistance gene in B. subtilis, using the protoplast transformation procedure.

Several physical properties of the plasmid pAB224 suggest that it may be useful as a vector for the cloning and expression of foreign genes in B. subtilis and B. licheniformis. It is similar to the widely used vector pUB110 in several respects; it is a small, multi-copy, antibiotic-resistance plasmid, which has a unique EcoRI site, and is stably maintained in B. subtilis. However, in contrast to pUB110, pAB224 can be stably maintained and express antibiotic-resistance in B. licheniformis strain LO2 (Bingham, 1980). Furthermore, the plasmid pH1 (Bingham, 1980) is a hybrid plasmid consisting of pAB224 ligated to the plasmid pUB1654 (Docherty, 1981). The insertion was at the single EcoRI site of pAB224 suggesting that this site can be used to clone foreign DNA without inactivating essential plasmid functions. For these reasons, pAB224 was chosen as a vector to shotgun-clone genes into its unique EcoRI site. Restriction endonuclease cleavage maps of pAB224 and the parental plasmid pAB124 are shown in Figure 3.1.

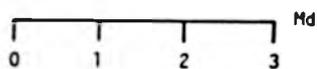
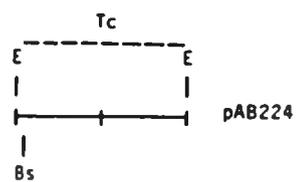
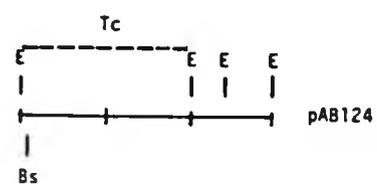


Figure 3.1: Restriction endonuclease cleavage maps of the plasmid pAB124 and the deletion derivative pAB224 (adapted from Bingham *et al.*, 1980)

3.2 RESULTS

3.2.1 Attempted Shotgun-Cloning of the *B. subtilis* trpC Gene

The *B. subtilis* *trpC* gene previously has been shotgun-cloned directly in *B. subtilis* (Keggins *et al.*, 1978). These workers used the 2.9 Md *S. aureus* neomycin-resistance plasmid pUB110 (Gryczan *et al.*, 1978) as a cloning vector. *EcoRI*-digested chromosomal DNA from a wild-type (*trpC*⁺) *B. subtilis* 168 strain was inserted into the *EcoRI* site of pUB110 and used to transform competent *B. subtilis* BD224 cells. This latter strain, in addition to the *trpC2* and *thr-5* mutations, carries the *recE4* mutation which prevents integration of homologous sequences into the host chromosome (Dubnau and Cirigliano, 1974). *Trp*⁺ transformants were selected for directly by their ability to grow in a minimal medium lacking tryptophan. Using this procedure a hybrid plasmid pSL106 was isolated which contained a 1.6 Md insert into pUB110 and which could complement *B. subtilis* mutations in the *trpC* and *trpF* genes. This experiment demonstrated for the first time that it was possible to shotgun-clone chromosomal genes directly in *B. subtilis* using a plasmid vector and a competence transformation procedure.

To investigate the usefulness of pAB224 as a cloning vector, attempts were made to clone the *B. subtilis* *trpC* gene into the unique *EcoRI* site of this plasmid using a similar strategy to that employed by Keggins *et al.* (1978). The *B. subtilis* *trpC* gene cloned by Keggins *et al.* (1978), present in pSL106, could not be sub-cloned into the *EcoRI* site of pAB224 since the insert of pSL106 is not bounded by *EcoRI* sites.

Plasmid pAB224 was isolated from *B. subtilis* strain BD170 (*trpC2*, *thr-5*) and chromosomal DNA was isolated from *B. subtilis* strain 168 (Marburg). This latter strain is a wild-type prototrophic strain and hence has a wild-type *trpC* gene. Transformation of competent *B. subtilis* BD224 cells with pAB224 DNA routinely gave rise to 10³ to greater than 10⁵ Tc^r transformants per µg of DNA

after selection on TSBA containing 12 µg per ml of Tc; transformation with Marburg chromosomal DNA gave rise to greater than 10^6 Trp⁺ transformants per µg of DNA after selection on SMS agar lacking tryptophan. Transformation with EcoRI-digested Marburg chromosomal DNA gave rise to Trp⁺ transformants but at a frequency 10 to 50 fold lower than with undigested chromosomal DNA. This lower frequency could have been the result of non-specific nuclease damage to the DNA during digestion with EcoRI but alternatively, and a more likely explanation, is that the lower transformation frequency was due to the smaller molecular size of the EcoRI-digested chromosomal DNA. Harris-Warick *et al.* (1975) observed a similar phenomenon upon EcoRI-digestion of B. subtilis chromosomal DNA.

The reversion frequency of the trpC2 mutation of the recipient strain, B. subtilis BD224 was determined to estimate the background level of Trp⁺ revertants produced. This was done as described in section 2.2.24 except that cells were grown at 37°C. No Trp⁺ revertants were detected when 1.7×10^9 cells were inoculated onto SMS agar lacking tryptophan. Therefore spontaneous reversion of the trpC2 mutation was unlikely to cause any problem in the shotgun-cloning experiments.

For the shotgun-cloning of the trpC gene, complete EcoRI digests of B. subtilis Marburg chromosomal DNA were mixed with EcoRI-digests of pAB224 DNA at a ratio of 6 to 1 respectively. This ratio was chosen because it was successfully used by Keggins *et al.* (1978) to shotgun-clone several trp genes. After ligation of the vector and insert DNA at a final DNA concentration of 50 µg per ml, 4 µg aliquots of the ligation-mix were used to transform competent B. subtilis BD224 cells. Transformed cells were spread onto TSBA containing 12 µg per ml of Tc and SMS agar lacking tryptophan and the plates were incubated at 37°C for up to two days.

The results showed that B. subtilis Marburg chromosomal DNA could not transform competent B. subtilis BD224 cells to Trp⁺, the recE4 mutation of this

strain preventing recombination of homologous transforming DNA with the recipient chromosome. Whereas 1 μg of pAB224 DNA gave rise to 2.3×10^5 Tc^r transformants, 4 μg of ligation-mix DNA containing approximately 0.6 μg of pAB224 DNA, gave rise to only 4.8×10^2 Tc^r transformants; an equivalent of 8.4×10^2 Tc^r transformants per μg of plasmid DNA. The reason for the large decrease in transformation frequency after ligation of the vector and insert DNA is unknown, but similar findings have been reported (Gryczan *et al.*, 1980a). Since in the above transformation an equivalent of 48 Tc^r transformants were spread onto minimal agar, Trp^+ transformants were not expected. Clearly, to increase the chances of shotgun cloning the *trpC* gene, many more Tc^r transformants need to be screened for a Trp^+ phenotype.

The number of Tc^r transformants spread onto minimal agar was increased by transforming 2 ml of competent cells with 8 μg of ligation-mix DNA and the transformed cells were pelleted by centrifugation (5000 rpm for 10 min in a benchtop centrifuge), resuspended in 200 μl of SMS, and spread directly onto minimal plates without dilution. Also, to check the frequency of transformation to Tc-resistance cells from one batch, in each experiment, were diluted and plated onto Tc-containing media. In several experiments, values ranging from 8.0×10^1 to 2.1×10^3 Tc^r transformants per 8 μg of ligation-mix were obtained and in total an equivalent of greater than 30,000 Tc^r transformants were spread onto SMS agar lacking tryptophan. However, Trp^+ transformants were never detected. Possible reasons for the failure to clone the *B. subtilis trpC* gene are given below.

The insert DNA to vector DNA ratio, ligation DNA concentration, and transformation frequencies in this present investigation were essentially the same as those of Keggins *et al.* (1978). The selection procedure was for Trp^+ in both cases but Keggins *et al.* (1978) used a minimal broth whereas minimal agar was used in this case. It was thought unlikely that any of these factors markedly affected the cloning experiments and therefore alternative reasons leading to the failure to clone the *trpC* gene were sought.

Although a total of over 30,000 Tc^r transformants were spread onto minimal agar, not all of these transformants would have harboured a hybrid plasmid containing a chromosomal DNA insert. The actual insertion frequency was not determined but it may have been too low to reasonably have expected to clone the trpC gene. Other possible explanations for the failure to clone the trpC gene can be made by examining the results obtained by Keggins *et al.* (1978, 1979).

The recombinant plasmid pSL106, containing the cloned *B. subtilis* trp genes, was examined subsequently (Keggins *et al.*, 1979). These workers found that the level of the plasmid-specified trpC gene-product was equal to or below that of the derepressed chromosomally specified enzyme. Since pUB110 is present at 30 copies per cell (Keggins *et al.*, 1978) the level of expression of each pSL103-borne trpC gene must be lower than that of the chromosomal trpC gene. Therefore, if the trpC gene had been shotgun-cloned using pAB224, only low level synthesis of the trpC gene-product may have occurred since the copy number of pAB224 is only 5 to 7 copies per cell (Bingham, 1980), compared to 30 copies per cell for pUB110. However, it is more likely that such cells would merely have a reduced growth rate. Plates were incubated for 5 days at 37°C but Trp⁺ clones were not detected.

Keggins *et al.* (1979) found that the level of the trp enzymes present in pSL106 were not affected by the presence of tryptophan, suggesting that the cloned trp fragment had been separated from its normal genetic regulatory elements. Indeed, the cloned fragment present in pSL106, which could complement only trpC and trpF *B. subtilis* mutations, was derived from a central portion of the *B. subtilis* tryptophan gene cluster. The entire gene cluster trpE-D-C-F-B-A, appears to constitute a transcriptional unit (Carlton and Whitt, 1969), with transcription apparently occurring in the direction trpE → trpA (Roth and Nester, 1971). These observations could offer an explanation for the failure to clone the trpC gene in this present investigation.

Expression of the trpC gene present in pSL106 was likely to occur from genetic regulatory signals present in the pUB110 region of pSL106. Indeed, since pSL106 surprisingly had only one EcoRI site, genetic alterations may have occurred which placed the trpC-containing fragment under the control of pUB110 genetic regulatory signals. If the trpC-containing fragment was cloned into pAB224, expression may not have occurred due to a lack of suitable regulatory signals to promote expression of the trpC gene.

Harris-Warrick *et al.* (1975) estimated the size of the trpC-containing EcoRI fragment of B. subtilis chromosomal DNA to be about 13 Md, again suggesting that the fragment cloned by Keggins *et al.* (1978) had undergone a massive deletion. The large size of the trpC-containing EcoRI fragment of B. subtilis chromosomal DNA may indeed have hindered the cloning of the gene in this present investigation. Selective cloning of small DNA fragments has in fact been reported (Michel *et al.*, 1980).

No further attempts were made to shotgun-clone the B. subtilis trpC gene because of several factors. Namely: (i) the inherent problems associated with the competence transformation system employed, i.e. the obligate requirement for oligomeric plasmid DNA; (ii) the large size of the B. subtilis chromosomal trpC-containing EcoRI fragment and (iii) the possible requirement for genetic rearrangements of trpC-containing fragments to promote expression of the trpC gene devoid of its normal regulatory elements. Instead efforts were directed to the cloning of the B. licheniformis 749/C penP gene. As mentioned later, possible problems associated with (ii) and (iii), detailed above for the trpC gene, are not present in the case of the penP gene.

3.2.2 Attempted Shotgun-Cloning of the B. licheniformis 749/C penP Gene

B. licheniformis strain 749/C, is a penicillinase-producing mutant isolated by Dubnau and Pollock (1965), which constitutively produces a 2 to 5 fold higher enzyme level than the induced wild-type strain, B. licheniformis strain 749. The

penicillinase gene (penP) from the constitutive mutant has been cloned into the bacteriophage vector λ NM574 in E. coli (Brammar et al., 1980) and the structural penP gene, and its associated regulatory signals have been shown to be present in the recombinant bacteriophage λ -pen, and to lie on a 2.9 Md EcoRI fragment. Therefore, this gene apparently was an ideal candidate for shotgun-cloning into the EcoRI site of the plasmid vector pAB224.

3.2.2.1 Employing competent B. subtilis cells

Initial attempts to clone the B. licheniformis 749/C penP gene in B. subtilis were made using the competence transformation procedure. Chromosomal DNA isolated from B. licheniformis 749/C, was digested with EcoRI and ligated to EcoRI-digested pAB224. Similar conditions to those for the attempted cloning of the B. subtilis trpC gene were used. A ratio of insert DNA to vector DNA of 6 to 1 respectively was used, with a final concentration of DNA in the ligation-mix of 50 μ g per ml.

B. subtilis strain IG20 (trpC2, hsm, hsr) was used as a host in this case because it is restriction and modification deficient. This genotype renders the host incapable of restricting incoming non-modified foreign DNA and since B. licheniformis chromosomal DNA was used, the use of strain IG20 prevented restriction of any cloned DNA fragments. A recE4 mutation was not required since the B. licheniformis 749/C penP gene has no known homology with the B. subtilis chromosome.

After transformation of B. subtilis IG20, cells were pelleted by centrifugation (5,000 rpm for 10 min in a benchtop centrifuge), resuspended in 200 μ l of TYS broth and spread onto TSBA containing either Tc or Ap at a concentration of 12 μ g per ml. Plates were incubated for up to 4 days at 37°C.

One μ g of pAB224 DNA produced 4.2×10^4 Tc^r but 0 Ap^r transformants whereas 8 μ g of ligation-mix DNA produced 7.2×10^2 Tc^r but no Ap^r transformants. As was seen in the attempted shotgun-cloning of the B. subtilis

trpC gene (Section 3.2.1), a large reduction in transformation frequency upon transformation with the ligation-mix compared to vector DNA alone, occurred also in this case. In other experiments from 1.9×10^1 to 1.1×10^3 Tc^r transformants per 8 µg of ligation-mix, were obtained and in total, an equivalent of 24,000 Tc^r transformants were spread onto Ap-containing medium. However, Ap^r transformants were never detected. Possible explanations for the failure to clone the penP gene are given below.

Non-expression of the B. licheniformis 749/C penP gene in B. subtilis, although a possibility, is unlikely. B. subtilis and B. licheniformis probably have similar genetic regulatory signals and therefore the penP signals, which are present on the 2.9 Md EcoRI fragment of λ pen, along with the penP structural gene (Brammar *et al.*, 1980) are likely to be recognised by B. subtilis. Indeed, the plasmid pAB224 originally is of B. licheniformis origin (Bingham *et al.*, 1980) and the fact that this plasmid replicates and expresses Tc-resistance in B. subtilis indicates the similarity of the genetic regulatory signals of these two species. Also, other workers have reported the expression of B. licheniformis genes in B. subtilis (Keggins *et al.*, 1978; Michel *et al.*, 1980) when cloned in plasmid vectors.

A possible explanation for the failure to clone the penP gene was that the insertion frequency was not high enough, i.e. only a small proportion of the Tc^r transformants harboured a plasmid containing a chromosomal DNA insert. A similar explanation was mentioned previously for the attempted cloning of the B. subtilis trpC gene (Section 3.2.1). If this was the reason why the shotgun-cloning met with repeated failure, it is difficult to envisage how this problem could be overcome. A low insertion frequency would require the screening of hundreds of thousands of clones, clearly a non-ideal situation.

Since attempts at shotgun-cloning the B. licheniformis 749/C penP gene, employing the competence transformation procedure, met with repeated failure, attempts were made to shotgun-clone the penP gene using the protoplast transformation procedure developed by Chang and Cohen (1979).

3.2.2.2 Employing *B. subtilis* Protoplasts

Chang and Cohen (1979) reported that between 10^6 and 10^7 transformants per μg of plasmid DNA could be obtained by PEG-induced transformation of *B. subtilis* protoplasts. In addition, linear plasmid DNA molecules and hybrid plasmids constructed *in vitro* were active in transformation although at a frequency of 1 to 3 orders of magnitude lower than that observed for ccc plasmid DNA. Thus the protoplast transformation system appears to be ideally suited to the shotgun-cloning of genes in *B. subtilis*. Therefore attempts were made to shotgun-clone the *B. licheniformis* 749/C *penP* gene using this system.

Initially the minimum inhibitory concentration to Tc and Ap of *B. subtilis* on DM3 medium was determined because Chang and Cohen (1979) found that the resistance of *B. subtilis* to Cm and Km increased when using DM3 medium. This was done by toothpicking colonies onto antibiotic-containing DM3 medium and incubating overnight at 37°C . It was found that growth was inhibited by Tc or Ap at a concentration of $25 \mu\text{g}$ per ml. On DM3 medium *B. licheniformis* 749/C grew in the presence of $200 \mu\text{g}$ per ml of Ap, and *B. subtilis* IG20 harbouring pAB224 grew in the presence of $50 \mu\text{g}$ per ml of Tc. Therefore a concentration of $25 \mu\text{g}$ per ml of Tc or Ap was used for the selection of *B. subtilis* transformants on DM3 medium.

Using the protoplast transformation procedure, transformation of *B. subtilis* IG20 protoplasts routinely gave 10^5 to greater than 10^6 Tc^r transformants per μg of pAB224 DNA with regeneration frequencies ranging from 10% to 100% of the plated protoplasts. Although the frequency of transformation was an order of magnitude lower than that obtained by Chang and Cohen (1979), it was one to several orders of magnitude higher than that obtained upon transformation of competent *B. subtilis* cells with pAB224 DNA (section 3.2.1).

For shotgun-cloning of the *penP* gene using the protoplast transformation procedure, the transforming DNA was prepared as previously described (section 3.2.2.1), i.e. complete *EcoRI* digests of *B. licheniformis* 749/C chromosomal DNA

and pAB224 DNA were mixed at a ratio of 6 to 1 respectively and ligation carried out at a final DNA concentration of 50 μg per ml. After transformation and spreading onto DM3 medium containing either Tc or Ap at a concentration of 25 μg per ml, plates were incubated for up to 4 days at 37°C.

Whereas pAB224 produced 3.7×10^5 Tc^r and 0 Ap^r transformants, chromosomal DNA isolated from B. licheniformis 749/C did not transform to either Ap- or Tc-resistance, as expected. Transformation with either 1 or 5 μg of ligation-mix gave 1.4×10^2 and 2.0×10^2 Tc^r transformants; Ap^r transformants were not detected. This experiment was repeated several times and similar results were obtained, i.e. although pAB224 routinely gave rise to 10^5 to 10^6 Tc^r transformants per μg of DNA, the ligation-mix DNA gave only 10^1 to 10^2 transformants, even when 5 μg of DNA was used for transformation.

The reasons for the large decrease in transformation frequency upon ligation of pAB224 DNA to B. licheniformis 749/C chromosomal DNA are unknown. It cannot be attributed to restriction of heterologous (B. licheniformis) transforming DNA since the host strain used, IG20, was restriction and modification deficient. Also, Chang and Cohen (1979) reported that restriction of heterologous transforming DNA did not occur upon transformation of B. subtilis protoplasts. Although these same workers observed a decrease in transformation frequency when open-circular DNA molecules constructed in vitro were introduced into B. subtilis protoplasts, the decrease was of only one to two orders of magnitude. This compares with a decrease of between 10^3 and 10^4 orders of magnitude observed in this investigation. The reason for this large decrease is not clear but it may be related to the different ligation conditions used or the source of the insert DNA; Chang and Cohen (1979) used plasmid DNA whereas chromosomal DNA of heterogeneous size was used in this investigation.

An observation made during transformation of B. subtilis protoplasts with plasmid DNA which may have affected the usefulness of the protoplast transformation system was that as the density of protoplasts inoculated per

regeneration plate increased, the protoplast regeneration and transformation frequencies did not increase proportionally. This phenomenon (Table 3.1) cannot be the result of inhibition by a toxin present in the transforming DNA solution inhibiting regeneration at lower dilutions; inhibition of regeneration of non-transformed protoplasts also occurred in a similar fashion. Also, inhibition by lysozyme is unlikely because most of the lysozyme was probably removed during the transformation procedure. The regeneration inhibition was probably not due to the presence of a reversion inhibitory factor (De Castro-Costa and Landman, 1977); this factor was reported to inhibit protoplast reversion only when a density of greater than 5×10^5 colony forming units per ml was used; inhibition at low protoplast densities was not observed.

Since low transformation frequencies were repeatedly obtained when ligation-mix DNA was used to transform *B. subtilis* IG20 protoplasts, no further attempts at shotgun-cloning the *B. licheniformis* 749/C *penP* gene were made. However, subsequently, the *penP* gene was sub-cloned from the plasmid pUB1660 (Docherty, 1981) into the *EcoRI* site of pAB224 (see Chapter V). Transformants harbouring the hybrid *penP*-containing plasmid pTAH1 could not be selected for directly on Ap-containing media and therefore even if the *penP* gene was shotgun-cloned in this investigation, a *penP*-containing clone would probably not have been detected since direct selection on Ap-containing media was made.

Dilution factor	Colony Count on DM3	Regeneration frequency (%)	Colony count on DM3-Tc	Transformation frequency (transformants per μg DNA)
10^{-3}	-	-	79	7.9×10^4
10^{-4}	381	16	17	1.7×10^5
10^{-5}	66	28	5	5.0×10^5
10^{-6}	15	63	0	-
10^{-7}	2	84	0	-

TABLE 3.1: Regeneration and transformation of *B. subtilis* IG20 protoplasts

B. subtilis IG20 protoplasts were transformed with 1 μg of pAB224 DNA. Tc was added to 25 μg per ml where necessary and incubated for 3 days at 37°C.

3.3 DISCUSSION

The work reported in this chapter details attempts to shotgun-clone the B. subtilis trpC gene and the B. licheniformis 749/C penP gene into the unique EcoRI site of the plasmid vector pAB224. Attempts met with repeated failure for apparently unknown reasons.

Although at the outset of this work there had been only one report of a successful shotgun-cloning of chromosomal genes in B. subtilis (Keggins et al., 1978) several other successes have been recently reported (e.g. Palva, 1982). Why then have some workers been able to shotgun-clone chromosomal genes in B. subtilis whereas others have been unsuccessful? It is likely that several factors such as the plasmid vector used, the particular gene to be cloned, and the size of the DNA fragment required, are important.

The failure to shotgun-clone chromosomal genes is often attributed to the inherent properties of the competence transformation system employed, i.e. only plasmid oligomers are active in transformation of competent cells and attempts to increase the number of recombinant plasmids produced after ligation decreases the transforming efficiency of the DNA. The rationale behind this is that, as reported by Canosi et al. (1978), monomeric plasmid DNA is inactive in the transformation of competent B. subtilis cells. Also, as the ratio of insert DNA to vector DNA increases, the likelihood of forming oligomeric vector decreases. Therefore one would predict that attempts to shotgun-clone chromosomal genes directly in competent B. subtilis cells would result in (i) a low transformation frequency and (ii) a low insertion frequency. These two factors probably contributed to the failure to shotgun-clone chromosomal genes using competent B. subtilis cells in this work. However, this explanation cannot be applied to the transformation of B. subtilis protoplasts since, as stated previously, monomeric plasmid DNA and linear plasmid DNA are active in the transformation of B. subtilis protoplasts.

Very little is known of the actual plasmid species arising after ligation of chromosomal and plasmid DNAs and also how variations in the vector to insert DNA ratio and DNA concentration affect the final composition of plasmid species produced after ligation. These factors are clearly important and probably markedly affect the outcome of a shotgun-cloning experiment. Also, nothing is known about the structure of plasmid species, containing chromosomal DNA inserts, as in a shotgun-cloning, which give rise to transformants when introduced into competent B. subtilis cells. Are contiguous vector molecules required or can two or more vector molecules be separated by a length of chromosomal DNA? Also, are linear hybrids containing contiguous vector molecules in addition to a chromosomal DNA fragment active in transformation? The answer to these questions are not yet known but are clearly important to an understanding of plasmid DNA transformation of B. subtilis.

It has been demonstrated that it is possible to shotgun-clone chromosomal genes using competent B. subtilis cells, e.g. Palva (1982) obtained a transformation frequency of 10^5 transformants per 2.5 μg of DNA with an insertion frequency of 13%. Thus the B. amyloliquefaciens α -amylase gene was successfully cloned. A systematic study of random-segment cloning (Michel et al., 1980) also demonstrated that under the correct conditions, a high transformation and insertion frequency can be obtained. One important finding of Michel et al. (1980) and Palva (1982), was that the average size of the cloned DNA fragments was smaller than the average size of the original chromosomal DNA fragments used for ligation to vector DNA. Michel et al. (1980) suggested that the selective cloning of small DNA fragments may explain the failure of several workers to shotgun-clone genes in B. subtilis. The reasons for the selective cloning of small DNA fragments reported by Michel et al. (1980) is unknown. These workers suggested that it could be due to the instability of large DNA fragments or, alternatively, small hybrid plasmids, produced from intracellular processing of an oligomeric vector-insert DNA molecule may

predominate and displace any large plasmid molecules present. The latter explanation is generally more acceptable since it may not be so much the size of a cloned DNA fragment that is important to its stability as the nature of the particular cloned sequence itself. This would apply to both the competence and protoplast transformation systems.

If the shotgun-cloning of large DNA fragments cannot be overcome, it may still be possible to clone many genes by cloning relatively small fragments produced by digestion with enzymes such as Sau3A or MboI. Palva (1982) used MboI-generated fragments of 1.4 to 3.4 Md to clone the B. amyloliquefaciens α -amylase gene. However in this work from 90,000 to 150,000 clones had to be screened and only one desired clone was found. The use of insertional inactivation vectors, such as those described by Michel *et al.* (1980), may be useful to rapidly and simply determine the insertion frequency. This can be done by screening transformants for inactivation of an antibiotic-resistance marker carried on the vectors.

In summary, it has been demonstrated in several cases that direct shotgun-cloning in B. subtilis employing plasmid vectors is possible. However, for unknown reasons, the shotgun-cloning of various genes has proved to be extremely difficult. Therefore, unless there is a specific reason for wanting to shotgun-clone directly in B. subtilis, it is probably simpler to perform the initial shotgun-cloning in E. coli and subsequently introduce the cloned gene into B. subtilis. The use of bifunctional plasmid vectors capable of autonomous replication in both E. coli and B. subtilis may prove to be particularly useful in this respect.

CHAPTER IV

ISOLATION AND CHARACTERISATION OF THY P3-CONTAINING

PLASMIDS IN B.subtilis

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4.1 INTRODUCTION

Infection of B. subtilis with $\Phi 3T$, a temperate bacteriophage of B. subtilis (Tucker 1969), results in the conversion of thymine-requiring mutants to prototrophy. The $\Phi 3T$ gene responsible for this conversion has been designated thyP3. At the outset of this work, this gene had been cloned in E. coli by two groups of workers. Ehrlich et al. (1976) cloned the thyP3 gene into the EcoRI site of pSC101 and a series of hybrids were isolated, "pFT-plasmids", which could transform E. coli thymine auxotrophs to both Tc-resistance and thymine prototrophy (Thy⁺). Transformation of B. subtilis thymine auxotrophs with the pFT plasmids gave rise to thymine prototrophs which resulted from integration into the B. subtilis chromosome of only the thyP3 region of the hybrids; pSC101 sequences were not integrated. Ehrlich et al. (1976) suggested that the thyP3 gene carried its own promoter since expression in E. coli of the thyP3 gene of the hybrid plasmids, was found to be independent of the orientation of the thyP3 gene.

Duncan et al. (1977, 1978) cloned EcoRI digests of $\Phi 3T$ DNA into the EcoRI site of the E. coli vector pMB9. A hybrid plasmid, pCD1, was isolated which could transform E. coli thymine auxotrophs to Tc-resistance and Thy⁺. The same plasmid could transform B. subtilis thymine auxotrophs to Thy⁺; transformation to Tc-resistance was not observed even though the entire recombinant plasmid became integrated into the B. subtilis chromosome. The site of integration was in fact determined to be near the chromosomal thyA gene (cited in Duncan et al., 1978). In addition to the structural thyP3 gene, which was shown to be present in pCD1 on a 1.5 Md BglIII fragment, pCD1 also contains a 0.47 Md DNA fragment, derived from $\Phi 3T$. Duncan et al. (1978) showed that the presence of this latter fragment was sufficient to promote integration into the B. subtilis chromosome, of a hybrid plasmid carrying this fragment. A hybrid plasmid, pCD2, was constructed which had the BglIII, thyP3-containing fragment of pCD1

removed but which retained the 0.47 Md BglII-EcoRI fragment. The $\beta 22$ thy gene was then inserted into this plasmid to produce pCD4, which could transform B. subtilis thymine auxotrophs to Thy⁺. The site of integration into the B. subtilis chromosome of pCD4 was determined to be near the ilvA gene; greater than 150 map units from the chromosomal thyA gene (Phillips *et al.*, 1980). The necessity of the 0.47 Md BglII-EcoRI fragment for integration of the $\beta 22$ thy gene was shown by cloning this thy gene into pMB9. The recombinant plasmid, pCD5, could not transform B. subtilis thymine auxotrophs to Thy⁺. If the 0.47 Md BglII-EcoRI fragment was removed from pCD1, the resulting plasmid pCD3 could still transform B. subtilis thymine auxotrophs to Thy⁺ but at a 100-fold lower frequency compared to pCD1. Therefore the BglII, thyP3-containing fragment of pCD1 alone was sufficient to promote integration of pCD3.

Genetic studies in B. subtilis (e.g. Harris-Warrick and Lederberg, 1978) have established that nucleotide sequence homology in the region of recombination is important in regulating transformation by heterologous DNA. Thus, since pCD1 and some of its derivatives are capable of integration into the B. subtilis chromosome, one would predict that pCD1 contained DNA sequences homologous to the B. subtilis chromosome. In fact hybridisation analysis has established that the 0.47 Md BglII-EcoRI fragment of pCD1 has homology with a discrete EcoRI-generated fragment of the B. subtilis chromosome (E.M. Rubin, cited in Duncan *et al.*, 1978). Also the purified thyP3 gene-product is similar to the B. subtilis thymidylate synthetase (thyA) enzyme (Williams and Young, 1977, cited in Duncan *et al.*, 1978), and the thyP3 gene has nucleotide sequence homology with the B. subtilis chromosomal thyA gene (E.M. Rubin, cited in Duncan *et al.*, 1978).

The reason why pCD1, the thyP3-containing hybrid plasmid of Duncan *et al.* (1977, 1978), integrated in entirety into the B. subtilis chromosome whereas the thyP3-containing plasmids of Ehrlich *et al.* (1976) did not, is unclear. The difference in behaviour of the plasmids may be due to the nature of the sequences flanking the thyP3 gene, or even the different cloning vectors used by

these workers. It must be stressed that in both these cases, chromosomal integration was essential for expression of the thyP3 gene since neither pSC101 nor pMB9 are functional replicons in B. subtilis (A.H.A. Bingham, personal communication).

The plasmid pAB224, a small (1.95 Md) Tc-resistance plasmid isolated by Bingham *et al.* (1980), is potentially a useful vector for investigating the expression of cloned genes in both B. subtilis and B. licheniformis. Attempts to use this vector to shotgun-clone chromosomal genes directly in B. subtilis, as described in the previous chapter were unsuccessful. Therefore it was decided to sub-clone a gene into pAB224 and investigate the expression and properties of pAB224-containing hybrids. The gene chosen for study was the thyP3 gene of the B. subtilis bacteriophage, $\phi 3T$, for three reasons. Firstly, the gene had already been cloned in E. coli plasmids (Ehrlich *et al.*, 1976; Duncan *et al.*, 1977). Secondly, the cloned thyP3 gene of Duncan *et al.* (1977) was shown to be present on an EcoRI fragment and therefore this fragment could be inserted into the unique EcoRI site of pAB224. Thirdly, B. subtilis and B. licheniformis strain LO2 mutants (see Chapter VI) were available which had mutations in their chromosomal thy genes. This enables expression of the thyP3 gene to be investigated in both these hosts. It was also of interest to determine whether chromosomal integration would occur if the thyP3 gene was present in a hybrid plasmid that is capable of autonomous replication in B. subtilis.

This chapter details the construction and characterisation in B. subtilis of pAB224 derivatives carrying the thyP3 gene.

4.2 RESULTS

The hybrid plasmid, pCD1, has been shown to contain the thyP3 gene of the B. subtilis bacteriophage $\phi 3T$ (Duncan *et al.*, 1977) which is located on a 2.0 Md EcoRI fragment. This plasmid was purified from an E. coli strain and used to transform competent B. subtilis QB943 cells. After transformation and

spreading onto SMS-agar plates lacking thymine and incubation for 2 days at 37°C, 3.1×10^4 Thy⁺ transformants per µg of pCD1 were detected. After tooth-picking onto TSBA plates containing 12 µg per ml of Tc, all the Thy⁺ transformants were found to be Tc-sensitive (Tc^S), as expected from the results of Duncan *et al.* (1977). Also, pCD1 was digested with EcoRI to confirm the presence of the 2.0 Md fragment, a photograph of which is shown in Figure 4.1.

4.2.1 Construction and Characterisation of thyP3-containing Plasmids

The sub-cloning of the thyP3-containing fragment of pCD1 into the unique EcoRI site of pAB224 was done as follows. One µg of EcoRI-digested pCD1 DNA was mixed with 1 µg of EcoRI-digested pAB224 DNA and ligated in a final reaction volume of 20 µl. The ligation-mix was then used to transform competent B. subtilis QB943 cells. Transformed cells were spread onto SMS-agar plates lacking thymine and onto the same media but also containing 12 µg per ml of Tc. After 2 days incubation at 37°C, no Tc^r, Thy⁺ transformants were detected on the latter plates but 2.3×10^5 Thy⁺ transformants were detected on the former plates. These Thy⁺ transformants were replica-plated onto TSBA plates containing 25 µg per ml of Tc and three Tc^r clones appeared after overnight incubation at 37°C. The Thy⁺, Tc^r phenotype of these clones was then checked by streaking onto appropriate selection media.

Duncan *et al.* (1977) showed that EcoRI digestion of pCD1 did not destroy the Thy⁺ transforming activity of the DNA upon transformation of competent B. subtilis cells. Therefore the Tc^r, Thy⁺ transformants, isolated as described above, could have resulted from transformation with, and integration of, the thyP3 gene and simultaneous transformation with pAB224 DNA. Therefore it was necessary to determine whether the Tc^r, Thy⁺ transformants each contained a hybrid plasmid consisting of the thyP3 gene covalently linked to pAB224 sequences. Mini-plasmid preparations were done on the 3 Tc^r, Thy⁺ transformants to screen for the presence of extrachromosomal plasmid DNA.

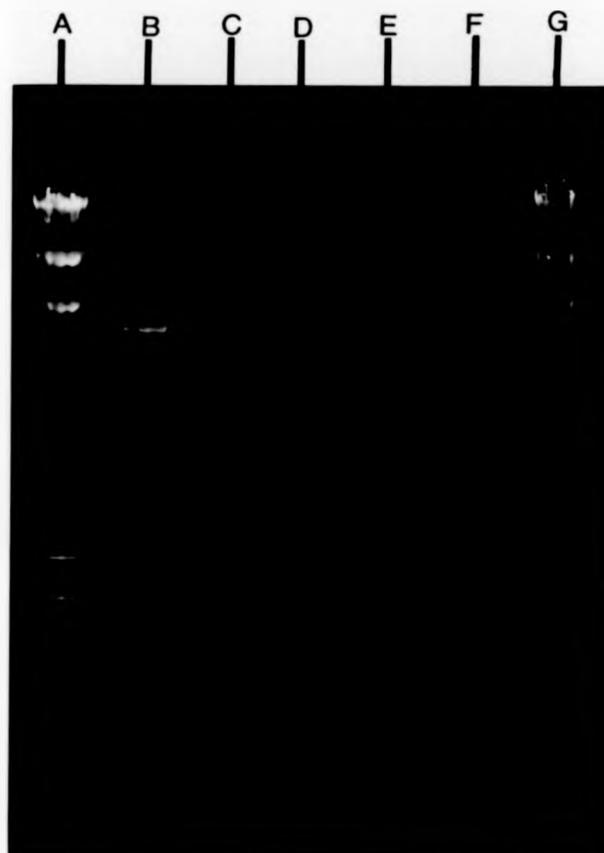


Figure 4.1: Photograph showing EcoRI-digested plasmid DNAs after agarose gel electrophoresis

Lanes A and G HindIII-digested λ DNA

Lane B, pCD1 (3.40, 2.10Md)

Lane C, nTT3 (3.70, 2.00Md)

Lane D, nTT2 (4.60, 2.00Md)

Lane E, nTT1 (5.10, 3.10, 2.00Md)

Lane F, pAB224 (2.00Md)

The 5.1Md band in Lane C is linearised nTT1.

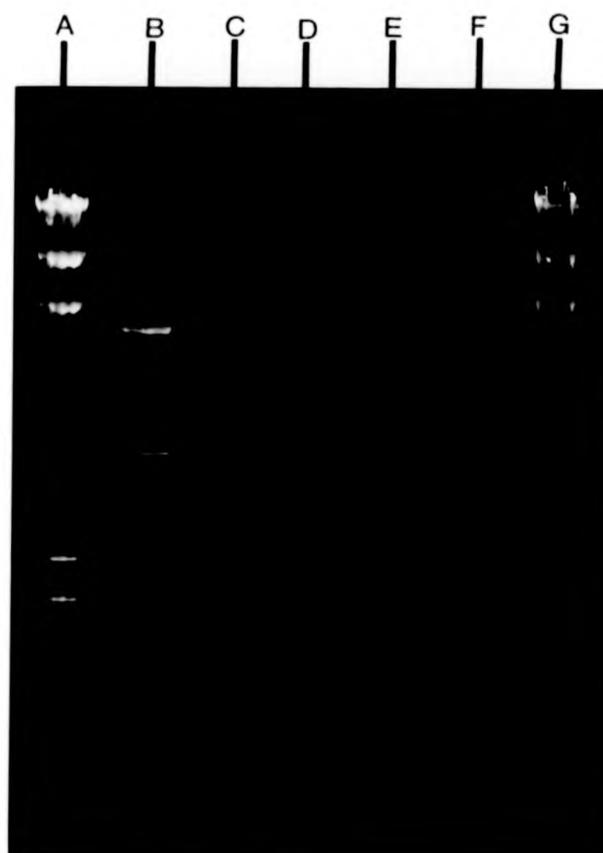


Figure 4.1: Photograph showing *EcoRI*-digested plasmid DNAs after agarose gel electrophoresis

Lanes A and G *HindIII*-digested λ DNA

Lane B, pCD1 (3.40, 2.10Md)

Lane C, nTT3 (3.70, 2.00Md)

Lane D, nTT2 (4.60, 2.00Md)

Lane E, nTT1 (5.10, 3.10, 2.00Md)

Lane F, pAB224 (2.00Md)

The 5.1Md band in Lane C is linearised nTT1.

After agarose gel electrophoresis, the presence, in each case, of a plasmid with a higher molecular weight than pAB224 DNA, was confirmed. The three plasmids were named pTT1, pTT2 and pTT3.

Large scale plasmid isolations were done on *B. subtilis* QB943 cells harbouring pTT1, pTT2 and pTT3; in each case cells were grown in TYS broth containing 25 µg per ml of Tc. Further characterisation of these plasmids, and also of pCD1, was done by analysis on agarose gels of single and double restriction endonuclease digests. Details of the construction of restriction endonuclease cleavage maps of each of these plasmids is given below.

Restriction endonuclease cleavage mapping of pTT1: single and double enzyme digests of pTT1 using the enzymes EcoRI, BstEII, BglII and HindIII are shown in Table 4.1. From single enzyme digests with the restriction endonucleases HpaI, BglII, HindIII and BstEII, which gave only 1 fragment upon digestion of pTT1 with sizes of 5.00, 5.20, 5.20 and 5.10 Md respectively, the size of linear pTT1 was estimated to be approximately 5.10 Md. pTT1 did not contain any recognition sites for the restriction endonuclease BamHI.

A restriction endonuclease cleavage map of pTT1 for the enzymes EcoRI, BstEII, BglII and HindIII was constructed as follows. Digestion with EcoRI gave two fragments with sizes of 3.10 and 2.00 Md. The 2.00 Md band corresponds in size to linear pAB224, the parental vector used in the construction of pTT1. pAB224 has a BstEII site 0.05 Md from the EcoRI site (Bingham *et al.*, 1980); therefore a BstEII-EcoRI double-digest of pTT1 was done. This produced fragments of 3.10 and 1.90 Md. The 2.0 Md EcoRI fragment was reduced in size to 1.90 Md suggesting that this fragment contained a BstEII site approximately 0.10 Md from one end. It was assumed that a small (0.10 Md) fragment also was produced but this was not seen on an agarose gel. Therefore the 2.00 Md EcoRI fragment of pTT1 is probably equivalent to pAB224.

A BglII-EcoRI double-digest of pTT1 suggested that the BglII site was present in the 3.10 Md EcoRI fragment and approximately 0.50 Md from one end.

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)			
	pTT1	pTT2	pTT3	pCD1
<u>EcoRI</u>	3.10, 2.00	4.60, 2.00	3.70, 2.00	3.40, 2.10
<u>BglII</u>	5.20	6.60	-	3.75, 1.60
<u>BamHI</u>	-	-	5.70	5.50
<u>BstEII</u>	5.10	3.50, 3.10	3.60, 2.20	5.60
<u>HindIII</u>	5.20	6.70	5.80	3.15, 1.85, 0.20
<u>EcoRI-BglII</u>	2.55, 2.00, 0.50	4.25, 2.00, 0.55	-	-
<u>EcoRI-BamHI</u>	-	-	3.35, 2.05, 0.50	-
<u>EcoRI-BstEII</u>	3.10, 1.90, (0.10)	3.20, 2.10, 1.60, (0.10)	2.20, 2.00, 1.50, (0.10)	2.05, 1.90, 1.55
<u>EcoRI-HindIII</u>	2.10, 1.95, 1.10	2.85, 2.05, 1.90	3.55, 2.10 (0.15)	3.10, 1.70, 0.20*, 0.15
<u>BglII-BstEII</u>	4.30, 0.50	3.40, 2.50, 0.60	-	2.40, 1.65, 1.55
<u>BglII-HindIII</u>	3.70, 1.45	5.60, 1.25	-	3.15, 1.25, 0.70, 0.20, (0.05)
<u>BamHI-BstEII</u>	-	-	3.50, 1.75, 0.50	4.00, 1.55
<u>BamHI-HindIII</u>	-	-	5.25, 0.30	3.05, 1.90, 0.25, 0.20
<u>BstEII-HindIII</u>	2.90, 2.10	3.50, 1.80, 1.35	3.50, 2.00, 0.25	1.90, 1.65, 1.60, 0.15

TABLE 4.1: DNA fragment sizes of pTT1, pTT2, pTT3 and pCD1 obtained after restriction endonuclease digestions

Plasmid DNA fragment sizes shown in brackets are estimated sizes. These fragments were not seen on agarose gels.

* represents 2 fragments of the same size.

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)			
	pTT1	pTT2	pTT3	pCD1
<u>EcoRI</u>	3.10, 2.00	4.60, 2.00	3.70, 2.00	3.40, 2.10
<u>BglII</u>	5.20	6.60	-	3.75, 1.60
<u>BamHI</u>	-	-	5.70	5.50
<u>BstEII</u>	5.10	3.50, 3.10	3.60, 2.20	5.60
<u>HindIII</u>	5.20	6.70	5.80	3.15, 1.85, 0.20
<u>EcoRI-BglII</u>	2.55, 2.00, 0.50	4.25, 2.00, 0.55	-	-
<u>EcoRI-BamHI</u>	-	-	3.35, 2.05, 0.50	-
<u>EcoRI-BstEII</u>	3.10, 1.90, (0.10)	3.20, 2.10, 1.60, (0.10)	2.20, 2.00, 1.50, (0.10)	2.05, 1.90, 1.55
<u>EcoRI-HindIII</u>	2.10, 1.95, 1.10	2.85, 2.05, 1.90	3.55, 2.10 (0.15)	3.10, 1.70, 0.20*, 0.15
<u>BglII-BstEII</u>	4.30, 0.50	3.40, 2.50, 0.60	-	2.40, 1.65, 1.55
<u>BglII-HindIII</u>	3.70, 1.45	5.60, 1.25	-	3.15, 1.25, 0.70, 0.20, (0.05)
<u>BamHI-BstEII</u>	-	-	3.50, 1.75, 0.50	4.00, 1.55
<u>BamHI-HindIII</u>	-	-	5.25, 0.30	3.05, 1.90, 0.25, 0.20
<u>BstEII-HindIII</u>	2.90, 2.10	3.50, 1.80, 1.35	3.50, 2.00, 0.25	1.90, 1.65, 1.60, 0.15

TABLE 4.1: DNA fragment sizes of pTT1, pTT2, pTT3 and pCD1 obtained after restriction endonuclease digestions

Plasmid DNA fragment sizes shown in brackets are estimated sizes. These fragments were not seen on agarose gels.

* represents 2 fragments of the same size.

Similarly a HindIII-EcoRI double-digest suggested that the HindIII site was approximately 1.10 Md from one end of the 3.10 Md EcoRI fragment. The actual position of the HindIII and BglII sites on the 3.10 Md EcoRI fragments was determined by a BglII-HindIII double-digest. This produced fragments of 3.70 and 1.45 Md. Since both the HindIII and BglII sites lie within the 3.10 Md EcoRI fragment, they must be 1.45 Md apart on this fragment. If the BglII site is 0.50 Md from one end of the 3.10 Md EcoRI fragment then the HindIII site must be 1.95 Md from the same end and therefore 1.15 Md from the other end. This is in agreement with the actual values obtained from a HindIII-EcoRI double-digest.

The position of the BstEII site relative to the BglII and HindIII sites was determined as follows. A BstEII-BglII double-digest produced fragments of 4.50 and 0.50 Md which suggested that the BglII site was 0.50 Md from the BstEII site. Therefore a BstEII-HindIII double-digest should produce fragments of approximately 3.00 and 2.10 Md; the actual sizes were 2.90 and 2.10 Md confirming the above. From this data the restriction endonuclease cleavage map shown in Figure 4.2 was constructed.

Restriction endonuclease cleavage mapping of pTT2: single and double enzyme digests of pTT2 with the enzymes EcoRI, BglII, BstEII and HindIII are shown in Table 4.1. From single enzyme digests with HindIII and BglII, which each have one recognition site within pTT2, the size of linear pTT2 was estimated to be 6.70 Md. pTT2 did not contain any recognition sites for the restriction endonuclease BamHI.

A restriction endonuclease cleavage map for the enzymes EcoRI, BglII, BstEII and HindIII was constructed as follows. Digestion with EcoRI produced two fragments with sizes of 4.60 and 2.00 Md. Double-digestion with BglII and EcoRI produced fragments of 4.25, 2.00 and 0.55 Md. Therefore the BglII site lies within the 4.60 Md EcoRI fragment and 0.55 Md from one end. Similarly, a HindIII-EcoRI double-digest produced fragments of 2.85, 2.05 and 1.90 Md which suggested that the HindIII site also lies within the 4.60 Md EcoRI fragment and is

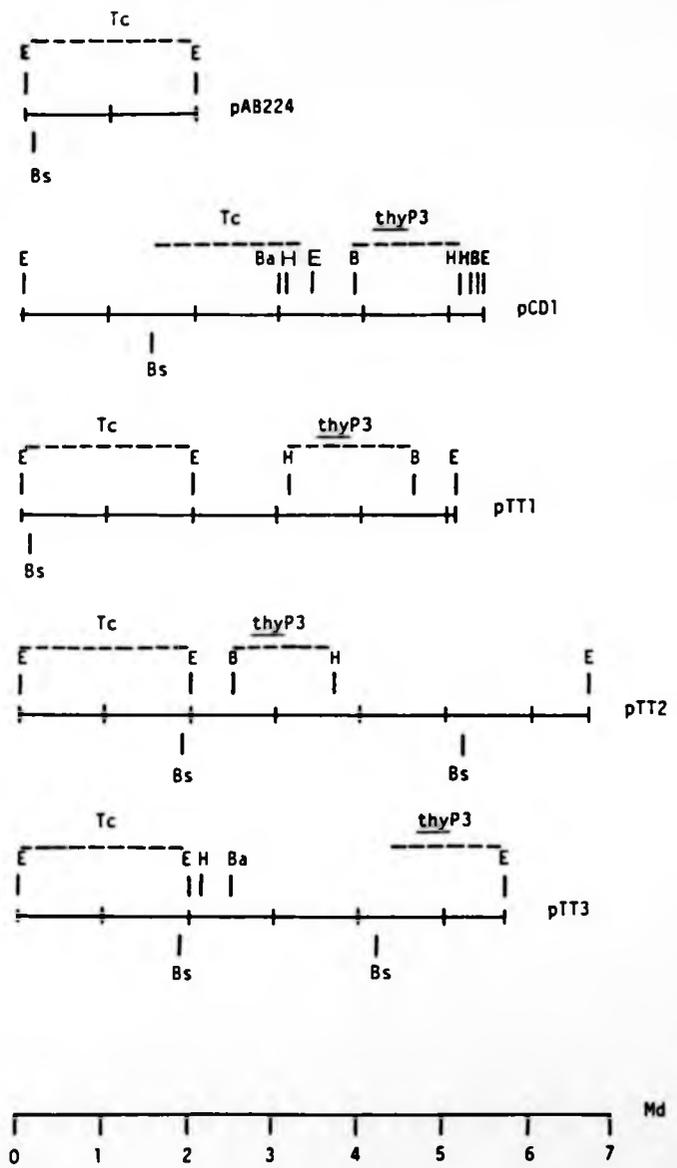


Figure 4.2: Restriction endonuclease cleavage maps of pTT1, pTT2 and pTT3 and the parental plasmids used in their construction

approximately 1.90 Md from one end. A BglIII-HindIII double-digest produced fragments of 5.60 and 1.25 Md. Therefore the HindIII and BglIII sites are approximately 1.25 Md apart and are within the 4.60 Md EcoRI fragment.

The 2.00 Md EcoRI fragment corresponds in size to linear pAB224. A BstEII-EcoRI double-digest was done to determine whether the 2.00 Md EcoRI fragment contained a restriction site for BstEII. This double-digest produced 3 fragments of 3.20, 2.00 and 1.60 Md. The 4.60 Md EcoRI fragment was cleaved by BstEII to produce fragments of 3.20 and 1.60 Md but the 2.00 Md EcoRI fragment did not appear to be cleaved by BstEII. Since BstEII alone produced 2 fragments after digestion of pTT2, a BstEII-EcoRI double-digest should have produced 4 fragments. The fact that only 3 fragments were detected suggested that another small fragment was produced which could not be seen on the agarose gel. Since the BstEII sites are greater than 3.0 Md apart, one of these BstEII sites must lie in close proximity to an EcoRI site. The actual position of this BstEII site was determined by a double-digest with BstEII and BglIII or HindIII. A BstEII-BglIII double-digest produced fragments of 3.40, 2.50 and 0.60 Md. Therefore the BglIII site must lie on the 3.10 Md BstEII fragment and be 0.60 Md from one end. A BstEII-HindIII double-digest produced fragments of 3.50, 1.80 and 1.35 Md and therefore the HindIII site also must lie on the 3.10 Md BstEII fragment and be 1.35 Md from one end. Since the BglIII and HindIII sites were determined to be 1.25 Md apart, if the BglIII fragment is 0.6 Md from one end of the 3.10 Md BstEII fragment then the HindIII site must be 1.85 Md away from that same end. The BglIII and HindIII sites are 0.55 Md and 1.90 Md away from an EcoRI site. Therefore the BstEII site must be very close to this EcoRI site. Although the actual position of the BstEII site could not be determined, it is likely to be on the 2.00 Md EcoRI fragment. If this is in fact the case then the 2.00 Md EcoRI fragment is equivalent to pAB224. From this data the restriction endonuclease cleavage map shown in Figure 4.2 was constructed.

Restriction endonuclease cleavage mapping of pTT3: single and double enzyme digests of pTT3 with the enzymes EcoRI, BamHI, BstEII and HindIII are shown in Table 4.1. From single enzyme digests with HindIII and BamHI, which each have 1 recognition site within pTT3, the size of linear pTT3 was estimated to be 5.70 Md. pTT3 did not contain any recognition site for the restriction endonuclease BglII.

A restriction endonuclease cleavage map of pTT3, for the enzymes EcoRI, BamHI, HindIII and BstEII, was constructed as follows. Digestion with EcoRI produced 2 fragments of 3.70 and 2.00 Md. The positions of the unique recognition sites of BamHI and HindIII, relative to the EcoRI sites, was determined by double-digests. A BamHI-EcoRI double-digest produced fragments of 3.35, 2.05 and 0.50 Md and therefore the BamHI site must lie approximately 0.50 Md from one end of the 3.70 Md EcoRI fragment. A HindIII-EcoRI double digest produced fragments of 3.55 and 2.10 Md. The existence of a third fragment was expected since digestion with EcoRI alone produced 2 fragments and digestion with HindIII alone produced 1 fragment. Therefore, as only 2 fragments were detected, the HindIII site must lie close to an EcoRI site. The 3.70 Md EcoRI fragment was reduced in size to 3.55 Md after cleavage with HindIII. This suggested that the HindIII site is approximately 0.15 Md from one end of the 3.70 Md EcoRI fragment. This 0.15 Md fragment was not detected on agarose gels.

The BamHI site is 0.50 Md from one end of the 3.70 Md EcoRI fragment. If the HindIII site is 0.15 Md from the same EcoRI site then fragments of approximately 5.35 and 0.35 Md should be produced after double-digestion of pTT3 with BamHI and HindIII. A BamHI-HindIII double-digest produced fragments of 5.25 and 0.30 Md confirming the above.

To determine whether the 2.00 Md band produced after EcoRI digestion is equivalent to pAB224, a BstEII-EcoRI double-digest was done. Digestion with BstEII alone produced 2 fragments of 3.60 and 2.20 Md whereas double-digestion

with BstEII and EcoRI produced 3 fragments of 2.20, 2.00 and 1.50 Md. The 3.70 Md EcoRI fragment was cleaved by BstEII to produce fragments of 2.20 and 1.50 Md but the 2.00 Md EcoRI fragment did not appear to be cleaved by this enzyme. The fact that only 3 fragments were detected after a BstEII-EcoRI double-digest suggested that a BstEII and EcoRI site lie in close proximity. To determine the position of this BstEII site, double digests of BstEII with HindIII and BamHI were done. A BstEII-HindIII double-digest produced fragments of 3.50, 2.00 and 0.25 Md which suggested that the HindIII site was 0.25 Md from one end of the 3.60 Md BstEII fragment. Similarly, a BstEII-BamHI double-digest produced fragments of 3.50, 1.75 and 0.50 Md suggesting that the BamHI site lies 0.50 Md from the same end of the 3.60 Md BstEII fragment. Since the BamHI and HindIII sites also lie 0.50 Md and 0.15 Md respectively, from one end of the 3.70 Md EcoRI fragment, this BstEII site must be near the same EcoRI site. Although it was not determined, the BstEII site probably lies just within the 2.00 Md EcoRI fragment. Therefore this EcoRI fragment is probably equivalent to pAB224. From this data the restriction endonuclease cleavage map of pTT3, shown in Figure 4.2, was constructed.

Restriction endonuclease cleavage mapping of pCD1: The positions of the restriction endonuclease sites of HindIII and BstEII on the pCD1 restriction endonuclease cleavage map of Duncan *et al.* (1977), was determined by double-digests with each of EcoRI, BamHI and BglII. Restriction endonuclease digestions are shown in Table 4.1.

pCD1 contains one recognition site for the enzyme BstEII since a single fragment of 5.5 Md was produced after digestion with this enzyme. The position of this site relative to the EcoRI, BglII and BamHI sites was determined by double-digests. A BstEII-EcoRI double-digest produced fragments of 2.05, 1.90 and 1.55 Md. Therefore, the BstEII site lies within the 3.40 Md EcoRI fragment of pCD1. A BstEII-BamHI double-digest produced fragments of 4.00 and 1.55 Md and since the BstEII fragment lies within the 3.40 Md EcoRI fragment, it must be

approximately 1.55 Md to the left of the BamHI site, as shown in Figure 4.2. To confirm this, a BstEII-BglIII double-digest was done which, as expected, produced fragments of 2.40, 1.65 and 1.55 Md. Therefore the BstEII site was positioned within the 3.40 Md fragment of pCD1 as shown in Figure 4.2.

A HindIII single digest of pCD1 produced 3 fragments of 3.15, 1.85 and 0.20 Md. To determine the relative positions of these sites with respect to EcoRI, BglIII, BamHI and BstEII sites, double-digests were done. A BamHI-HindIII double-digest produced fragments of 3.05, 1.90, 0.25 and 0.20 Md. Since HindIII alone produced fragments of 3.15, 1.85 and 0.20 Md, it is likely that a HindIII site lies approximately 0.25 Md from the BamHI site. A BglIII-HindIII double-digest produced fragments of 3.15, 1.25, 0.70 and 0.20 Md. The 3.75 Md BglIII fragment was cleaved by HindIII to produce fragments of 3.15 and 0.70 Md and therefore this HindIII site can be positioned to the right of the BamHI site as shown in Figure 4.2. The 1.55 Md BglIII fragment was cleaved by HindIII to produce fragments of 1.25 and 0.20 Md. A third fragment also must have been produced but was probably too small to be detected on the agarose gels used. The relative positions of the HindIII sites within the 1.50 Md BglIII fragment was determined by a BstEII-HindIII double-digest. This produced fragments of 1.90, 1.65, 1.60 and 0.15 Md and therefore the 3.15 Md HindIII fragment must contain the BstEII site approximately 1.60 Md from each end. Thus the HindIII site must lie to the right of the 1.55 Md BglIII fragment as shown in Figure 4.2. This was also confirmed by a HindIII-EcoRI double-digest which produced fragments of 3.10, 1.70, 0.20 and 0.15 Md. The 0.20 Md fragment in this case was assumed to be a double fragment because of its intensity relative to other bands. From this data the restriction endonuclease cleavage map shown in Figure 4.2 was constructed.

A comparison of the restriction endonuclease cleavage maps of the parental plasmids pAB224 and pCD1, with the hybrid plasmids pTT1, pTT2 and pTT3 (Figure 4.2) showed that the hybrids cannot have been produced by the simple insertion of the 2.0 Md, thyP3-containing EcoRI fragment of pCD1 into

the unique EcoRI site of pAB224. Therefore all 3 hybrids must have been produced by genetic rearrangements at some stage. This phenomenon is very common in *B. subtilis* and there have been many reports of similar findings (e.g. Gryczan and Dubnau, 1978).

The thyP3 gene present in pCD1 is bounded by BglII sites. However, pTT1 and pTT2 each have only one BglII site and therefore a deletion of one of these sites has occurred in each case. Also pTT3 has no BglII sites suggesting that thyP3 gene is unlikely to contain any BglII sites. Since all the thyP3-containing hybrid plasmids are larger than the expected hybrid of 4.0 Md they must each contain sequences not present in the 2.0 Md thyP3-containing EcoRI fragment of pCD1. To determine more precisely what sequences were present in the hybrid pTT1, radioactively labelled pTT1 was hybridised to EcoRI-digested pCD1 and EcoRI-digested pAB224 DNA. A photograph of an autoradiograph of this is shown in Figure 4.3. This indicated that pMB9 sequences, i.e. the 3.4 Md EcoRI fragment of pCD1, in addition to thyP3 sequences, the 2.0 Md EcoRI fragment of pCD1, and also pAB224, are present in pTT1. The presence of pMB9 sequences in the hybrids pTT2 and pTT3, although not directly demonstrated by hybridisation studies, can be inferred from their restriction endonuclease cleavage maps. Both pTT2 and pTT3 contain 2 BstEII sites. One of these is probably derived from pAB224 but the other may be from the pMB9 region of pCD1. Also, pTT3 has a BamHI site which is probably of similar origin.

The 2.0 Md EcoRI fragment present in each of the hybrids probably corresponds to pAB224. This was confirmed by the presence of a BstEII site either at, or near, an EcoRI site; pAB224 has a BstEII site 0.05 Md from the EcoRI site (Bingham *et al.*, 1980).

The position of the thyP3 gene on each of the hybrids was inferred as follows. The 0.5 Md BglII-EcoRI fragment present in pCD1 is also present in pTT1 and pTT2. The thyP3 gene is adjacent and linked to this fragment in pCD1 and therefore presumably also in pTT1 and pTT2. It was noted that the thyP3



Figure 4.3: Photograph of an autoradiograph showing hybridisation of a pTT1 probe to restriction endonuclease-digested plasmid DNAs

Lane A undigested pAB224 (2.0Md)

Lane B BnlII-digested pCD1 (3.75, 1.60Md)

Lane C EcoRI-digested nCD1 (3.40, 2.10Md)



Figure 4.3: Photograph of an autoradiograph showing hybridisation of a pTT1 probe to restriction endonuclease-digested plasmid DNAs

Lane A undigested pAB224 (2.0Md)

Lane B *Bgl*II-digested pCD1 (3.75, 1.60Md)

Lane C *Eco*RI-digested pCD1 (3.40, 2.10Md)

region of pTT1 is arranged in an opposite orientation to that of pTT2, relative to the pAB224 region of the hybrids. This suggests that expression of the thyP3 gene is not initiated from within the pAB224 region of the hybrids. Ehrlich *et al.* (1976) suggested that the thyP3 gene contained its own transcriptional promoter, and recently the thyP3 promoter has in fact been isolated and sequenced (E. Kenny, Personal Communication). The thyP3 gene of pTT3 could not be positioned relative to the 0.5 Md BglIII-EcoRI fragment since this fragment is not present in pTT3. It was positioned between the BstEII and EcoRI fragments as shown in Figure 4.2 because it was thought likely that the BstEII-BamHI fragment, present on the largest EcoRI fragment of pTT3, was derived from pMB9 sequence. Therefore the thyP3 gene cannot reside on this segment.

The presence of the thyP3 gene on each of the hybrid plasmids was further demonstrated by their ability to transform thymine auxotrophs of B. subtilis to prototrophy, as detailed below.

4.2.2 Transformation of Competent Cells with the Hybrid pTT Plasmids

A comparison of the transforming activity of the 3 hybrid plasmids pTT1, pTT2 and pTT3 with the parental plasmids pCD1 and pAB224 was made by transforming competent B. subtilis QB943 cells with 1 μ g aliquots of each plasmid (Table 4.2). As expected, the parental plasmids pCD1 and pAB224 only produced Tc^S, Thy⁺ and Tc^r, Thy⁻ transformants respectively whereas the 3 hybrid plasmids each produced Tc^r and Thy⁺ transformants. With the pTT plasmids, if selection was made on Tc-containing media, the transformation frequency was about ten-fold lower than when selection was made for Thy⁺ transformants. The reasons for this are unknown but it could be due simply to the type of selection applied.

An unusual phenomenon was observed in the above transformations. If selection was made for Tc-resistance after transformation with the pTT plasmids, then all the transformants were found to be also Thy⁺ after tooth-

Transforming plasmid	Initial selection	Transformants per μg of DNA	Phenotype of transformants (%)
pCD1	Thy ⁺	1.4×10^5	100 Tc ^S , Thy ⁺
	Tc ^r	0.0	-
pAB224	Thy ⁺	0.0	-
	Tc ^r	1.4×10^6	100 Tc ^r , Thy ⁻
pTT1	Thy ⁺	5.2×10^6	50 Tc ^r , Thy ⁺ 50 Tc ^S , Thy ⁺
	Tc ^r	2.1×10^3	100 Tc ^r , Thy ⁺
pTT2	Thy ⁺	3.0×10^5	53 Tc ^r , Thy ⁺ 47 Tc ^S , Thy ⁺
	Tc ^r	3.2×10^6	100 Tc ^r , Thy ⁺
pTT3	Thy ⁺	6.4×10^3	54 Tc ^r , Thy ⁺ 46 Tc ^S , Thy ⁺
	Tc ^r	4.1×10^2	100 Tc ^r , Thy ⁺

TABLE 4.2: Transformation of competent *B. subtilis* QB943 cells

Transformants were spread onto TSBA containing 12 μg per ml of Tc and SMS agar lacking thymine and incubated for up to 2 days at 37°C.

picking onto minimal agar lacking thymine. However, if initial selection was made for Thy⁺ transformants, some of these were found to be Tc^S after tooth-picking onto TSBA plates containing 25 µg per ml of Tc. As shown in Table 4.2, 50 out of 100 Thy⁺ pTT1 transformants were found to be Tc^R, the remaining 50 being Tc^S. Similarly for pTT2 and pTT3, 53 and 54 out of 100 Thy⁺ transformants respectively, were Tc^R. In other experiments values were obtained ranging from 20 to 90 out of 100 for the Thy⁺ transformants which were also Tc^R. The reasons for the variation in the relative frequencies of the Tc^R and Tc^S Thy⁺ transformants is unknown.

The above results showed that the pTT plasmids can each give rise to two different types of transformant, the first type being Tc^R, Thy⁺ and the second type Tc^S, Thy⁺. Further analysis of these transformants was done to determine why two types of transformant were produced after initial selection for Thy⁺ transformants whereas only one type was produced after initial selection for Tc^R transformants.

Mini-plasmid preparations were done on both types of transformant to screen for the presence of extrachromosomal plasmid DNA. Analysis of supernatant fluid from each type of pTT transformant revealed that Tc^S, Thy⁺ transformants did not contain extrachromosomal plasmid DNA; gel analysis revealed the presence of only a chromosomal DNA band. Conversely, Tc^R, Thy⁺ transformants always contained extrachromosomal plasmid DNA as evidenced by the presence of additional bands on agarose gels. The plasmid DNA of each Tc^R, Thy⁺ transformant had an electrophoretic mobility equivalent to that of the transforming plasmid suggesting that such transformed cells contained an equivalent of the parental plasmid. As expected, pAB224 transformants which were always Tc^R, Thy⁻ always contained extrachromosomal plasmid DNA and pCD1 transformants, which were always Tc^S, Thy⁺, never contained extrachromosomal plasmid DNA.

The most plausible explanation for the Tc^S, Thy⁺ type of pTT transformants, is integration of plasmid DNA sequences into the host chromosome. The entire transforming plasmid could have integrated, as is the case with pCD1 (Duncan *et al.*, 1977), or selective integration of only the thyP3 gene may have occurred, as reported by Ehrlich *et al.* (1976). If the entire pTT plasmids become integrated into the host chromosome, then low level resistance to Tc might result from expression of the integrated Tc-resistance genes. Therefore the level of resistance to Tc of the Thy⁺ transformants not containing extrachromosomal plasmid DNA was determined. Analysis of 12 independently isolated pTT1 Thy⁺ transformants, of the type described above, failed to detect resistance to Tc even at a concentration of 2 µg per ml. Also, pTT2 and pTT3 transformants of this type were found to be sensitive to low levels of Tc. Therefore if integration of the entire plasmid does occur, with either pTT1, pTT2 or pTT3, functional expression of the Tc-resistance genes does not occur. As detailed above, the alternative to total plasmid integration is selective integration of only the thyP3 region of the hybrids. To determine whether the thyP3 gene of pTT1 could integrate independently of pAB224 sequences, the effect of EcoRI-digestion of pTT1 upon transformation was investigated.

4.2.3 Effects of Restriction Endonuclease Digestion on Plasmid Transformation

Duncan *et al.* (1977) demonstrated that the hybrid plasmid pCD1 retained its Thy⁺ transforming activity of competent B. subtilis cells, even after digestion with EcoRI. This demonstrated that neither an intact plasmid, nor pMB9 sequences, were required for integration of the thyP3 gene. Therefore, EcoRI digested pTT1 DNA was used to transform competent B. subtilis QB943 cells to determine whether the thyP3 gene of pTT1 had retained the integration properties of the pCD1 thyP3 gene. The results of such an experiment are shown in Table 4.3.

Transforming plasmid	Restriction endonuclease treatment	Initial selection	Transformants per μg of DNA	Phenotype of transformants (%)
pCD1	None	Thy ⁺ Tc ^r	4.6×10^5 0.0	100 Tc ^s , Thy ⁺ -
	<u>EcoRI</u>	Thy ⁺ Tc ^r	6.0×10^3 0.0	100 Tc ^s , Thy ⁺ -
pAB224	None	Thy ⁺ Tc ^r	0.0 4.2×10^6	- 100 Tc ^r , Thy ⁺
	<u>EcoRI</u>	Thy ⁺ Tc ^r	0.0 0.0	- -
pTT1	None	Thy ⁺	4.4×10^5	60 Tc ^r , Thy ⁺
		Tc ^r	1.2×10^5	40 Tc ^s , Thy ⁺ 100 Tc ^r , Thy ⁺
	<u>EcoRI</u>	Thy ⁺ Tc ^r	4.4×10^3 0.0	100 Tc ^s , Thy ⁺ -

TABLE 4.3: Transformation of competent *B. subtilis* QB943 cells

Transformants were spread onto TSBA containing 12 μg per ml of Tc and SMS agar lacking thymine and incubated for up to 2 days at 37°C.

As expected from the results of Duncan *et al.* (1977), EcoRI-digested pCD1 was capable of transforming cells to Thy⁺. Also, EcoRI-digested pAB224 DNA was unable to transform competent cells to Tc^r. EcoRI-digested pTT1 produced only Tc^s, Thy⁺ and not Tc^r, Thy⁺ transformants. The pTT1 Tc^s, Thy⁺ transformants were screened for the presence of extrachromosomal plasmid DNA, but were found not to contain any. Therefore these transformants probably resulted from integration into the host chromosome of the thyP3 gene present on the 3.1 Md EcoRI fragment of pTT1.

The 100-fold decrease in Thy⁺ transforming activity of pTT1 upon EcoRI-digestion was probably due to the smaller size of the thyP3-containing EcoRI fragment, compared to the size of the intact plasmid. A similar explanation was given by Duncan *et al.* (1977) to explain the decrease in transformation frequency of pCD1 upon EcoRI digestion. The fact that the thyP3 gene of pTT1 can integrate independently of pAB224 DNA sequences suggests that selective integration of intact pTT1 sequences could occur. However, this does not provide evidence for such an event since the thyP3 gene of pCD1 has similar integration properties and entire plasmid integration occurs with pCD1 (Duncan *et al.*, 1977).

Although the above experiment does not explain why the pTT plasmids gave rise to two types of transformant, it does suggest a possible explanation. The data show that a plasmid DNA fragment, containing an intact thyP3 gene, can give rise to Tc^s, Thy⁺ transformants. Therefore any pTT plasmid DNA fragment, or even a linear plasmid molecule, should be able to transform competent cells to Tc^s, Thy⁺ providing that the thyP3 gene is intact. A plasmid preparation normally contains damaged plasmid DNA such as open circular and linear molecules, but probably not in sufficient quantities to account for the large number, i.e. 10⁴ to 10⁶ Tc^s, Thy⁺ transformants produced after transformation with an unfractionated pTT plasmid preparation. Therefore there must be another explanation to account for these Tc^s, Thy⁺ transformants. From

the work of Canosi et al. (1978), Contente and Dubnau (1979a) and de Vos et al. (1981), it is known that monomeric, nicked or linear plasmid DNA is inactive in the transformation of competent B. subtilis cells and also that extensive processing of plasmid DNA occurs upon transformation of competent cells. Therefore, processing of pTT plasmid DNA, and particularly of pTT monomeric DNA, upon transformation, may destroy its Tc^r transforming activity. However, if damage does not occur within the thyP3 region of the plasmid, Tc^s, Thy⁺ transformants may be produced.

4.2.4 Transformation with ccc Monomeric Plasmid DNA

To determine whether the two types of transformants produced with the pTT plasmids could be the result of transformation by different molecular species of plasmid DNA, competent cells were transformed with ccc monomeric plasmid DNA. This molecular species was isolated as follows: after agarose gel electrophoresis of pTT1, pTT2 and pTT3 DNA, the fastest migrating band in each case was cut from the gel and isolated by electroelution. This purified ccc monomeric plasmid DNA was then used to transform competent B. subtilis QB943 cells.

From the data shown in Table 4.4 it can be seen that monomeric pTT1, pTT2 and pTT3 produced only Tc^s, Thy⁺ and not Tc^r, Thy⁺ transformants. A sample of the Thy⁺ transformants was screened for the presence of extrachromosomal plasmid DNA but was found not to contain any. Thus all or part of the transforming plasmids must have been integrated into the host chromosome. Therefore it is likely that upon transformation of competent B. subtilis cells with an unfractionated pTT plasmid preparation, some Tc^s, Thy⁺ transformants arise by the transformation of such cells with ccc monomeric plasmid DNA. Conversely, Tc^r, Thy⁺ transformants are produced by transformation with multimeric plasmid DNA. It is also possible that multimeric plasmid DNA could give rise to Tc^s, Thy⁺ transformants since a multimeric

Transforming plasmid monomer	Initial selection	Transformants per μg of DNA	Phenotype of transformants (%)
pTT1	Thy ⁺ Tc ^r	3.7×10^4 0.0	100 Tc ^s , Thy ⁺ -
pTT2	Thy ⁺ Tc ^r	3.9×10^4 0.0	100 Tc ^s , Thy ⁺
pTT3	Thy ⁺ Tc ^r	2.8×10^3 0.0	100 Tc ^s , Thy ⁺

TABLE 4.4: Transformation of competent *B. subtilis* QB943 cells by monomeric plasmid DNA

Transformants were spread onto TSBA containing 12 μg per ml of Tc and SMS agar lacking thymine and incubated for up to 2 days at 37°C.

plasmid molecule could be partially degraded during the transformation event producing a molecule incapable of autonomous replication. However, if the thyP3 gene was still intact, such a molecule might still be able to integrate into the host chromosome and produce Tc^S, Thy⁺ transformants.

4.2.5 Construction and Characterisation of CAT-containing Plasmids

There could be several reasons for the lack of expression of the Tc-resistance genes of the pTT plasmids when the thyP3 gene integrates into the host chromosome. Integration of damaged Tc-resistance genes, poor transcription if integrated, selective deletion from, or non-integration into the host chromosome are all possibilities. To try and determine which of these mechanisms actually occurs, two lines of investigation were followed. Firstly, integration of the pC194 chloramphenicol acetyl transferase (CAT) gene was investigated and, secondly, the chromosomal DNA of antibiotic-sensitive, Thy⁺ transformants was examined for the presence of plasmid DNA sequences.

The CAT gene of pC194 has been observed by several workers (Rapoport *et al.*, 1979; Haldenwang *et al.*, 1980), to integrate into the B. subtilis chromosome when present in hybrid plasmids also containing fragments of DNA homologous to the host chromosome. Furthermore, expression of the integrated CAT gene was shown to occur since cells harbouring a chromosomally integrated CAT gene were Cm^r. Therefore the pC194 CAT gene was cloned into pTT1 to investigate whether plasmid integration was total or limited to specific sequences.

pTT1 has a unique recognition site for the restriction endonuclease BglII, and thus was chosen as the site to insert the CAT gene of pC194. This latter plasmid does not contain a BglII recognition site (Ehrlich, 1977) and therefore, the hybrid plasmid pBD64 (Gryczan *et al.*, 1980b) was chosen as a source of the pC194 CAT gene. This plasmid has unique BglII and a BamHI recognition sites which do not lie within the CAT gene. These restriction endonucleases were

used to remove the CAT gene from pBD64 which was then inserted into BglII-digested pTT1. Although the BamHI DNA recognition sequence is different from that of BglII, the central four nucleotides, GATC, are the same in each case. Therefore a BamHI "sticky-end" is complementary to a BglII "sticky-end" and hence BamHI DNA fragments can be joined to BglII DNA fragments.

Plasmid pBD64 was purified from B. subtilis cells and its characteristics (Gryczan et al., 1980b) confirmed as shown in Table 4.5. Hybrid pTT1-pBD64 plasmids were constructed as follows. One μg of BglII-BamHI double-digested pBD64 was ligated to 1 μg of BglII-digested pTT1 in a final reaction volume of 20 μl . Competent B. subtilis QB943 cells were transformed with the ligation-mix DNA and spread onto selective media. The phenotype of 100 of each type of transformant was determined by tooth-picking onto selective media.

As can be seen from Table 4.6, many Tc^r , Cm^r , Thy^+ transformants were detected in addition to six other phenotypically different types of transformant. The plasmid content of four Tc^r , Cm^r , Thy^+ transformants, obtained after initial selection for Cm^r , was investigated further. Large-scale plasmid isolations were done on these transformants and the isolated plasmids pTTC1, pTTC2, pTTC3 and pTTC4 were examined by the analysis on agarose gels of single and double restriction endonuclease digests. Single and double enzyme digests of pTTC1, using the enzymes EcoRI, BstEII, BglII and HindIII are shown in Table 4.7 and a photograph of restriction endonuclease-digested plasmid DNAs is shown in Fig. 4.4. From single enzyme digests with the restriction endonucleases BglII, HindIII and BstEII, which each have only one recognition site within pTTC1, the size of linear pTTC1 was estimated to be 6.30 Md.

A restriction endonuclease cleavage map of pTTC1, for the enzymes EcoRI, BglII, BstEII and HindIII, was constructed as follows. Digestion with EcoRI produced 2 fragments of 4.20 and 2.10 Md. The position of the single BglII and HindIII sites relative to the EcoRI sites was determined by double-digests. A BglII-EcoRI double-digest produced fragments of 3.60, 2.10 and 0.45 Md.

used to remove the CAT gene from pBD64 which was then inserted into BglIII-digested pTT1. Although the BamHI DNA recognition sequence is different from that of BglIII, the central four nucleotides, GATC, are the same in each case. Therefore a BamHI "sticky-end" is complementary to a BglIII "sticky-end" and hence BamHI DNA fragments can be joined to BglIII DNA fragments.

Plasmid pBD64 was purified from B. subtilis cells and its characteristics (Gryczan et al., 1980b) confirmed as shown in Table 4.5. Hybrid pTT1-pBD64 plasmids were constructed as follows. One μ g of BglIII-BamHI double-digested pBD64 was ligated to 1 μ g of BglIII-digested pTT1 in a final reaction volume of 20 μ l. Competent B. subtilis QB943 cells were transformed with the ligation-mix DNA and spread onto selective media. The phenotype of 100 of each type of transformant was determined by tooth-picking onto selective media.

As can be seen from Table 4.6, many Tc^r , Cm^r , Thy^+ transformants were detected in addition to six other phenotypically different types of transformant. The plasmid content of four Tc^r , Cm^r , Thy^+ transformants, obtained after initial selection for Cm^r , was investigated further. Large-scale plasmid isolations were done on these transformants and the isolated plasmids pTTC1, pTTC2, pTTC3 and pTTC4 were examined by the analysis on agarose gels of single and double restriction endonuclease digests. Single and double enzyme digests of pTTC1, using the enzymes EcoRI, BstEII, BglIII and HindIII are shown in Table 4.7 and a photograph of restriction endonuclease-digested plasmid DNAs is shown in Fig. 4.4. From single enzyme digests with the restriction endonucleases BglIII, HindIII and BstEII, which each have only one recognition site within pTTC1, the size of linear pTTC1 was estimated to be 6.30 Md.

A restriction endonuclease cleavage map of pTTC1, for the enzymes EcoRI, BglIII, BstEII and HindIII, was constructed as follows. Digestion with EcoRI produced 2 fragments of 4.20 and 2.10 Md. The position of the single BglIII and HindIII sites relative to the EcoRI sites was determined by double-digests. A BglIII-EcoRI double-digest produced fragments of 3.60, 2.10 and 0.45 Md.

(A)

Restriction endonuclease treatment	DNA fragment sizes
<u>EcoRI</u>	3.10
<u>BglII</u>	3.10
<u>BamHI</u>	3.10
<u>BglII-BamHI</u>	1.95, 1.50

(B)

Transforming DNA	Selection	Transformants per μg of DNA
pBD64	Cm^r	1.4×10^3
	Km^r	3.7×10^3
No DNA	Cm^r	0.0
	Km^r	0.0

TABLE 4.5: Properties of the plasmid pBD64

- A) Restriction endonuclease analysis.
- B) Transformation of competent *B. subtilis* BD393 cells.
Transformants were spread onto TSBA containing either 10 μg per ml of Cm or 25 μg per ml of Km and incubated overnight at 37°C.

Transforming DNA	Initial selection	Transformants per μg of DNA	Phenotype of transformants (%)
pTT1-pBD64 ligation-mix	Tc ^r	1.7×10^4	37 Tc ^r , Cm ^r , Thy ⁺
			5 Tc ^r , Cm ^r , Thy ⁻
			47 Tc ^r , Cm ^s , Thy ⁺
			11 Tc ^r , Cm ^r , Thy ⁻
	Cm ^r	5.5×10^3	40 Tc ^r , Cm ^r , Thy ⁺
			10 Tc ^r , Cm ^r , Thy ⁺
			4 Tc ^r , Cm ^r , Thy ⁻
			46 Tc ^s , Cm ^r , Thy ⁻
	Thy ⁺	2.2×10^5	11 Tc ^r , Cm ^r , Thy ⁺
			3 Tc ^s , Cm ^r , Thy ⁺
			26 Tc ^r , Cm ^s , Thy ⁺
			60 Tc ^s , Cm ^s , Thy ⁺

TABLE 4.6: Construction of pTT1-pBD64 hybrid plasmids

Transformants were spread onto TSBA containing 12 μg per ml of either Tc or Cm and SMS agar lacking thymine and incubated for up to 2 days at 37°C.

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>EcoRI</u>	4.20, 2.10
<u>BstEII</u>	6.20
<u>BglII</u>	6.30
<u>HindIII</u>	6.30
<u>EcoRI-BstEII</u>	4.20, 2.10, 0.10*
<u>EcoRI-BglII</u>	3.60, 2.10, 0.45
<u>EcoRI-HindIII</u>	3.10, 2.10, 1.35
<u>BstEII-BglII</u>	5.40, 0.60
<u>BstEII-HindIII</u>	3.10, 3.00
<u>BglII-HindIII</u>	3.80, 2.55

TABLE 4.7: DNA fragment sizes of pTTC1 obtained after restriction endonuclease digestions

* This fragment was not seen on agarose gels and is an estimated size.

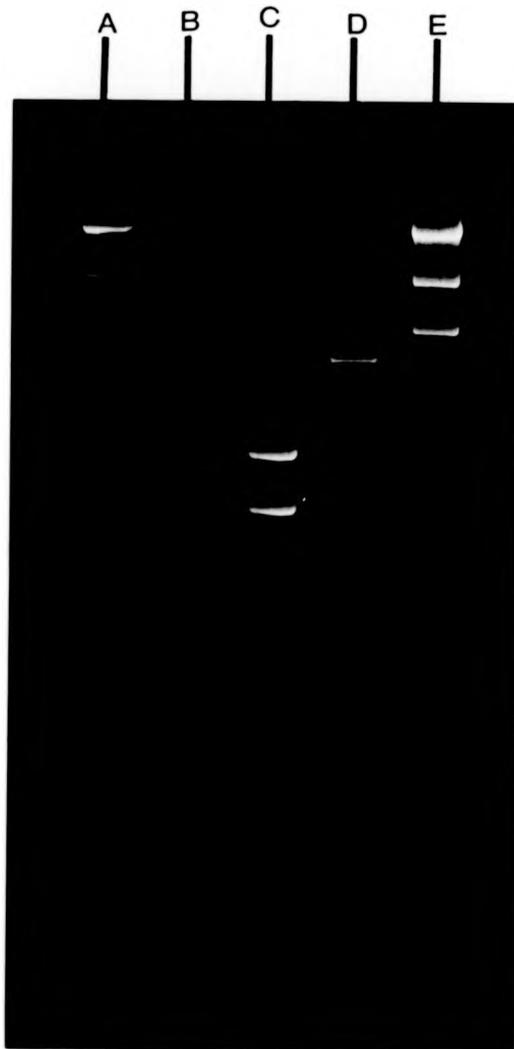


Figure 4.4: Photograph showing restriction endonuclease-digested plasmid DNAs after agarose gel electrophoresis

Lanes A and E HindIII-digested λ DNA
 Lane B BamHI-BglII-digested pBD64 (1.95, 1.50Md)
 Lane C BglII-EcoRI-digested pTT1 (2.55, 2.00, 0.50Md)
 Lane D BglII-EcoRI-digested pTTC1 (3.60, 2.10, 0.45Md)
 The 0.45Md fragment (Lane D) cannot be seen on this gel

Therefore the BglIII site must lie 0.45 Md from one end of the 4.20 Md EcoRI fragment. A HindIII-EcoRI double-digest produced fragments of 3.10, 2.10 and 1.35 Md and therefore the HindIII site must lie 1.35 Md from one end of the 4.20 Md EcoRI fragment. A BglIII-HindIII double-digest produced fragments of 3.80 and 2.55 Md. Since the HindIII and BglIII sites are both within the 4.20 Md EcoRI fragment, the HindIII site must be 1.35 Md from one end and the BglIII site 0.45 Md from the opposite end of this fragment. This gives a predicted BglIII-HindIII fragment of 2.30 Md; a 2.55 Md band was actually observed.

To determine the relative orientation of the pAB224 region of the plasmid, i.e. the 2.00 Md EcoRI fragment, double-digests with BstEII and EcoRI, BglIII and HindIII were done. A BstEII-BglIII double-digest produced fragments of 5.40 and 0.60 Md whereas a BstEII-HindIII double-digest produced fragments of 3.10 and 3.00 Md. This suggests that the BglIII site lies very near to the EcoRI site nearest to the BglIII site. To confirm its proximity to an EcoRI site, a BstEII-EcoRI double-digest was done. This produced fragments of 4.20 and 2.10 as expected. The third small band expected from the BstEII-EcoRI double-digest was not seen on agarose gels. From this data, the restriction endonuclease cleavage map shown in Figure 4.5 was constructed.

Plasmids pTTC2, pTTC3 and pTTC4 also, were examined by restriction endonuclease mapping exactly as described above for pTTC1. It was found that all 4 pTTC plasmids had a size of 6.30 Md and all had identical restriction endonuclease cleavage maps. This finding was unexpected because the pTTC plasmids are smaller than the expected hybrid consisting of pTT1 linked to the BamHI-BglIII CAT-containing fragment from pBD64. Therefore, in each case, a site-specific deletion must have occurred to produce the final hybrid. The nature of the deleted sequences is unknown but they are most likely to have been from the pBD64 region of the hybrid since the restriction endonuclease cleavage sites of pTT1 are all present within pTTC1-4. Instability of pCI94 sequences present in hybrid plasmids has been reported by several workers (e.g. Gryczan *et*

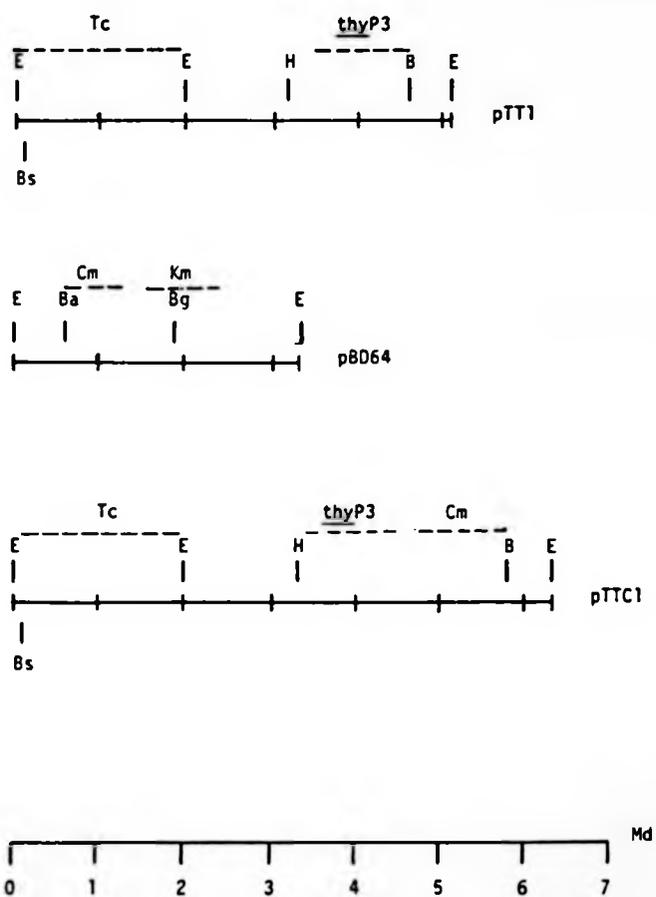


Figure 4.5: Restriction endonuclease cleavage maps of pTTCl and the parental plasmids used in its construction

al., 1980b). The plasmid pBD64 was itself produced by deletion of sequences from the pC194-containing hybrid, pBD12 (Gryczan et al., 1980b).

Another interesting feature of the pTTC hybrids is that the same BglIII fragment has been regenerated in each case. This suggests that in each case the BamHI-BglIII fragment, from pBD64, was inserted into the pTT1 BglIII site in the same orientation. If the BamHI-BglIII pBD64 fragment was inserted in the opposite orientation, the BglIII site of pTTC1-4 would be converted to a Sau3A site, i.e. GATC, and a BglIII site would be present nearer the HindIII site of pTTC1-4.

The reasons for the selective orientation of the BamHI-BglIII pBD64 fragment and the selective deletion, possibly of the same fragment, are unknown but may possibly have been essential to produce a stable plasmid carrying the Tc^r, Cm^r and Thy⁺ markers.

4.2.6 Transformation of Competent Cells with pTTC1

Since the hybrid CAT-containing plasmids pTTC1, pTTC2, pTTC3 and pTTC4 appeared to be identical, further investigations were done using only pTTC1. The properties of this plasmid were examined by transforming competent B. subtilis BD393 cells with unfractionated pTTC1, ccc monomeric pTTC1 and EcoRI-digested pTTC1. B. subtilis strain BD393 was used because this strain was much easier to grow than strain QB943. Table 4.8 shows the results of such an experiment.

Whereas unfractionated pTTC1 gave rise to two types of transformant, the first type being Tc^r, Cm^r, Thy⁺, and the second type Tc^s, Cm^s, Thy⁺, monomeric pTTC1 and EcoRI-digested pTTC1 gave rise to only the latter type of transformant. A sample of each type of transformant was screened for the presence of extrachromosomal plasmid DNA and it was found that antibiotic-resistant transformants, i.e. Tc^r, Cm^r, Thy⁺, contained extrachromosomal plasmid DNA whereas antibiotic-sensitive transformants, i.e. Tc^s, Cm^s, Thy⁺,

Transforming DNA	Initial selection	Transformants per μg of DNA	Phenotype of transformants (%)
pTTC1	Thy ⁺	2.0×10^4	69 Tc ^r , Cm ^r , Thy ⁺ 31 Tc ^s , Cm ^s , Thy ⁻
	Tc ^r	1.9×10^3	100 Tc ^r , Cm ^r , Thy ⁺
	Cm ^r	1.5×10^3	100 Tc ^r , Cm ^r , Thy ⁺
Monomeric pTTC1	Thy ⁺	2.2×10^4	100 Tc ^s , Cm ^s , Thy ⁺
	Tc ^r	0.0	-
	Cm ^r	0.0	-
<u>EcoRI</u> -digested pTTC1	Thy ⁺	6.0×10^3	100 Tc ^s , Cm ^s , Thy ⁺
	Tc ^r	0.0	-
	Cm ^r	0.0	-

TABLE 4.8: Transformation of competent *B. subtilis* BD393 cells

Transformants were spread onto TSBA containing 12 μg per ml of either Tc or Cm and SMS agar lacking thymine and incubated for up to 2 days at 37°C.

did not. Therefore, in this respect pTTCI behaved in a similar manner to pTTI.

The fact that neither Tc^r , Cm^s , Thy^+ nor Tc^s , Cm^r , Thy^+ transformants were produced suggested that plasmid fragmentation did not occur. Also, since no Tc^s , Cm^r , Thy^+ transformants were detected, this suggested that the entire pTTCI plasmid did not integrate into the host chromosome. If integration had occurred the production of Tc^s , Cm^r , Thy^+ transformants would have been expected assuming that the CAT gene was functionally expressed. As stated earlier, functional expression of the pC194 CAT gene upon integration into the *B. subtilis* chromosome has been reported (Rapoport *et al.*, 1979; Haldenwang *et al.*, 1980).

To determine whether the Tc^s , Cm^s , Thy^+ transformants expressed the CAT gene even at low levels, CAT assays were done on ten Tc^s , Cm^s , Thy^+ pTTCI transformants. As controls, assays were done on non-transformed *B. subtilis* BD393 cells and also on Tc^r , Cm^r , Thy^+ pTTCI transformants. Whereas the Tc^r , Cm^r , Thy^+ transformant produced 0.4 IU of CAT activity per ml of culture; the Tc^s , Cm^s , Thy^+ transformants in addition to the non-transformed strain produced less than 0.01 IU of CAT activity per ml of culture. This finding suggested that the CAT gene of pTTCI probably did not integrate into the chromosome along with the *thyP3* gene. If it did integrate, it was not expressed even at low levels.

Although the results obtained with the CAT gene-containing plasmids can be explained by non-integration of the CAT gene along with the *thyP3* gene, the results do not give any conclusive evidence for non-integration. Therefore integration into the *B. subtilis* chromosome of the Tc-resistance genes, CAT gene, and *thyP3* gene, was examined by hybridisation analysis.

4.2.7 Hybridisation Analysis of Antibiotic Sensitive, Thy^+ Transformants

The aim of these experiments was to determine more precisely, which regions of pTTI and pTTCI became integrated into the *B. subtilis* chromosome when the

transformants produced were antibiotic-sensitive but Thy⁺. Such transformants were examined by Southern analysis as detailed below.

Strain QBT5, a Tc^S, Thy⁺, pTT1 transformant of *B. subtilis* QB943, and strain BDTC5, a Tc^S, Cm^S, Thy⁺, pTTCl transformant of *B. subtilis* BD393 were used as a source of chromosomal DNA. As a probe for the *thyP3* gene, the 1.5 Md *Bgl*III fragment of pCD1 was used. This *Bgl*III fragment was used in order to eliminate the 0.47 Md *Bgl*III-*Eco*RI fragment of pCD1 which has been shown to hybridise to a specific *Eco*RI fragment of *B. subtilis* chromosomal DNA (E.M. Rubin, cited in Duncan *et al.*, 1978), thus making interpretation of the hybridisation results simpler. Five µg of pCD1 was digested with *Bgl*III and after agarose gel electrophoresis, the 1.5 Md, *thyP3*-containing fragment was isolated by electroelution. The use of pCD1 as a source of the *thyP3* gene also prevented contamination from *B. subtilis* chromosomal DNA sequences since pCD1 was isolated from *E. coli*

As a source of the CAT gene the plasmid pHV14 (Ehrlich, 1978a) was used. This is a hybrid plasmid which was constructed by inserting *Hind*III-digested pC194 into the *Hind*III site of pBR322 (Ehrlich, 1978a). pC194 was excised from pHV14 by digestion of 5 µg of pHV14 with *Hind*III and after agarose gel electrophoresis, the 1.8 Md pC194-containing *Hind*III fragment was isolated by electroelution. Contamination from *B. subtilis* chromosomal DNA was again prevented since pHV14 was isolated from *E. coli*. pAB224 DNA, isolated from *B. subtilis* BD170, was used as a source of Tc-resistance genes.

The *thyP3* gene, CAT gene and Tc-resistance genes DNA fragments each were radioactively labelled by nick-translation. For each hybridisation experiment, using one of the above mentioned probes, the following was done: 10 µg aliquots of chromosomal DNA from strains QBT5 and BDTC5, were digested separately with *Bgl*III and *Eco*RI. After agarose gel electrophoresis the separated DNA fragments were transferred to a nitrocellulose membrane. The membrane was then probed using radioactively labelled DNA fragments and

finally subjected to autoradiography. As controls, both EcoRI and BglII-digested chromosomal DNA from a non-transformed B. subtilis strain, BD224, was used in each experiment. Also, end-labelled HindIII-digested λ DNA was included as a size standard to permit estimation of the sizes of the hybridisation bands from the autoradiographs.

In the first experiment, the thyP3 gene from pCD1 was used as a probe. As can be seen from Figure 4.6, the thyP3 probe hybridised to two regions of both BglII-digested (Lane A), and EcoRI digested (Lane B) B. subtilis BD224 chromosomal DNA. However, the same probe hybridised to three regions of B. subtilis strain QBT5 and strain BDTC5 chromosomal DNAs, which were isolated from a Tc^S, Thy⁺ pTT1 transformant and a Tc^S, Cm^S, Thy⁺ pTTC1 transformant, respectively. Lanes C and D show hybridisation to strain QBT5 chromosomal DNA digested with BglII and EcoRI respectively and Lanes E and F show hybridisation to strain BDTC5 chromosomal DNA digested with BglII and EcoRI respectively. The additional band present in each lane presumably results from hybridisation of the thyP3 probe to a chromosomally integrated thyP3 gene. The additional band present after BglII digestion of the chromosomal DNAs, which has a size of approximately 8.0 Md, is the same size in Lanes C and E. Similarly, the additional band present after EcoRI digestion of chromosomal DNAs, which has a size of approximately 4.2 Md, is the same size in Lanes D and F.

These results suggest that the integrated thyP3 gene is present in the chromosomes of both the pTT1 and pTTC1, antibiotic-sensitive, Thy⁺ transformants, in a similar position, i.e. after integration the thyP3 gene lies between BglII sites separated by 8.0 Md and EcoRI sites separated by 4.2 Md. Also, because the corresponding QBT5 and BDTC5 chromosomal DNA, thyP3-containing, digestion fragments are the same size, the size of the integrated thyP3-containing DNA fragment must be the same size in each case. Therefore these results suggest that a specific integration event occurred upon



Figure 4.6: Photograph of an autoradiograph showing hybridisation of a 1.5Md BglII fragment of pCD1 to restriction endonuclease-digested chromosomal DNAs

Lane A BglII-digested BD224 DNA (12.0, 6.2Md)
 Lane B EcoRI-digested BD224 DNA (12.0, 1.5Md)
 Lane C BglII-digested QBT5 DNA (12.0, 8.0, 6.2Md)
 Lane D EcoRI-digested QBT5 DNA (12.0, 4.2, 1.5Md)
 Lane E BglII-digested BDTC5 DNA (12.0, 8.0, 6.2Md)
 Lane F EcoRI-digested BDTC5 DNA (12.0, 4.2, 1.5Md)
 Lane G end-labelled HindIII-digested λ DNA

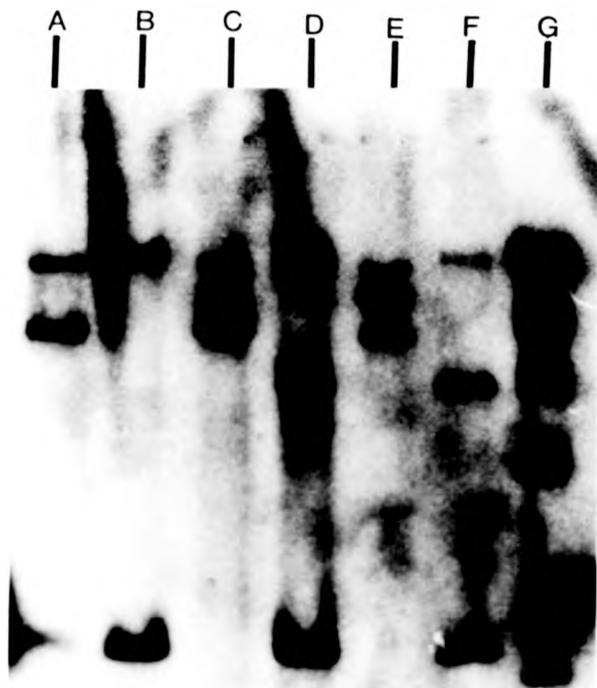


Figure 4.6: Photograph of an autoradiograph showing hybridisation of a 1.5Md BqIII fragment of pCD1 to restriction endonuclease-digested chromosomal DNAs

Lane A BqIII-digested BD224 DNA (12.0, 6.2Md)
 Lane B EcoRI-digested BD224 DNA (12.0, 1.5Md)
 Lane C BqIII-digested QBT5 DNA (12.0, 8.0, 6.2Md)
 Lane D EcoRI-digested QBT5 DNA (12.0, 4.2, 1.5Md)
 Lane E BqIII-digested BDTCS DNA (12.0, 8.0, 6.2Md)
 Lane F EcoRI-digested BDTCS DNA (12.0, 4.2, 1.5Md)
 Lane G end-labelled HindIII-digested λ DNA

transformation with pTT1 and pTTC1 resulting in the same size fragment being integrated into the host chromosome, at a similar site, in each case.

The above results clearly demonstrated integration of the thyP3 gene but gave no information of the nature of the sequences also present in the chromosome of a non-transformed strain, which hybridised to the thyP3 probe. The chromosomal DNAs of all three strains tested produced bands of approximately 12.0 Md and 1.5 Md after digestion with EcoRI and of approximately 12.0 Md and 6.2 Md after digestion with BglII.

As stated earlier, the 0.47 Md BglII-EcoRI fragment of pCD1 hybridises to a specific EcoRI fragment of B. subtilis chromosomal DNA (cited in Duncan *et al.*, 1978). However, for this reason this fragment was not included as part of the thyP3 probe used in this investigation. Therefore the hybridisation bands seen also in the control lanes cannot be the result of hybridisation of chromosomal DNA sequences to this fragment. A possible explanation for the two hybridisation bands seen also in the control lanes (A and B), is hybridisation of the thyP3 probe to the B. subtilis chromosomal thyA gene. As stated earlier, E.M. Rubin (cited in Duncan *et al.*, 1978) found that the thyP3 gene was homologous to the B. subtilis chromosomal thyA gene. If the thyA gene has an internal BglII and EcoRI site then this could explain the presence of two hybridisation bands in Lanes A and B.

Subsequent to this work Galizzi *et al.* (1981) have demonstrated hybridisation of the thyP3 gene to a unique EcoRI fragment and a unique BglII fragment of B. subtilis chromosomal DNA. Also Stroynowski (1981a) has demonstrated hybridisation of the thyP3 gene to a specific EcoRI fragment of B. subtilis chromosomal DNA. Since the thyA gene appears not to contain an internal BglII or EcoRI site, only one of these hybridisation bands observed in Lanes A and B of Figure 4.6 can result from hybridisation of the thyP3 probe to the chromosomal thyA gene. The nature of the other sequences, present in the B. subtilis chromosome, which have homology with the thyP3 probe, are unknown.

Figure 4.7 is a photograph of an autoradiograph showing the result of hybridising the pC194 probe to chromosomal DNA digested separately with BglIII and EcoRI. Only a weak hybridisation band was seen in each lane. The lanes containing chromosomal DNA from a pTTC1 Tc^S, Cm^S, Thy⁺ transformant (Lanes E and F) did not contain an additional band representing hybridisation of the pC194 probe to a chromosomally integrated CAT gene. Therefore, this clearly demonstrated that the CAT gene of pTTC1 did not integrate into the chromosome of pTTC1 Tc^S, Cm^S, Thy⁺ transformants. This again suggests that a specific integration event occurred resulting in only part of the plasmid pTTC1 being integrated into the host chromosome, to produce Tc^S, Cm^S, Thy⁺ pTTC1 transformants.

The weak hybridisation band, which has a size of approximately 8.0 Md after BglIII cleavage and 4.2 Md after EcoRI cleavage, of the chromosomal DNAs, must be due to hybridisation of the pC194 probe to a specific chromosomal DNA sequence. The nature of this chromosomal sequence is unknown, has not been reported elsewhere and was not investigated further.

Figure 4.8 is a photograph of an autoradiograph showing the result of hybridising the pAB224 probe to chromosomal DNAs digested separately with BglIII and EcoRI. No hybridisation to chromosomal DNA was seen in any lane suggesting that pAB224 sequences do not become integrated into the chromosome of Tc^S, Thy⁺ pTT1 and Tc^S, Cm^S, Thy⁺ pTTC1 transformants.

In summary, the results of the Southern analysis suggest that upon transformation of competent B. subtilis thymine auxotrophs with either pTT1 or pTTC1, antibiotic-sensitive, Thy⁺ transformants are produced by an integration event resulting in a specific segment of the plasmid, containing the thyP3 gene, being inserted into a specific site in the host chromosome. Non-integration of the Tc^r genes and CAT gene explains the antibiotic sensitivity of such Thy⁺ transformants.



Figure 4.7: Photograph of an autoradiograph showing hybridisation of a pC194 probe to restriction endonuclease-digested chromosomal DNAs

Lane A BglIII-digested BD224 DNA (8.0Md)
 Lane B EcoRI-digested BD224 DNA (4.2Md)
 Lane C BglIII-digested QBT5 DNA (8.0Md)
 Lane D EcoRI-digested QBT5 DNA (4.2Md)
 Lane E BglIII-digested BDTC5 DNA (8.0Md)
 Lane F EcoRI-digested BDTC5 DNA (4.2Md)
 Lane G end-labelled HindIII-digested λ DNA

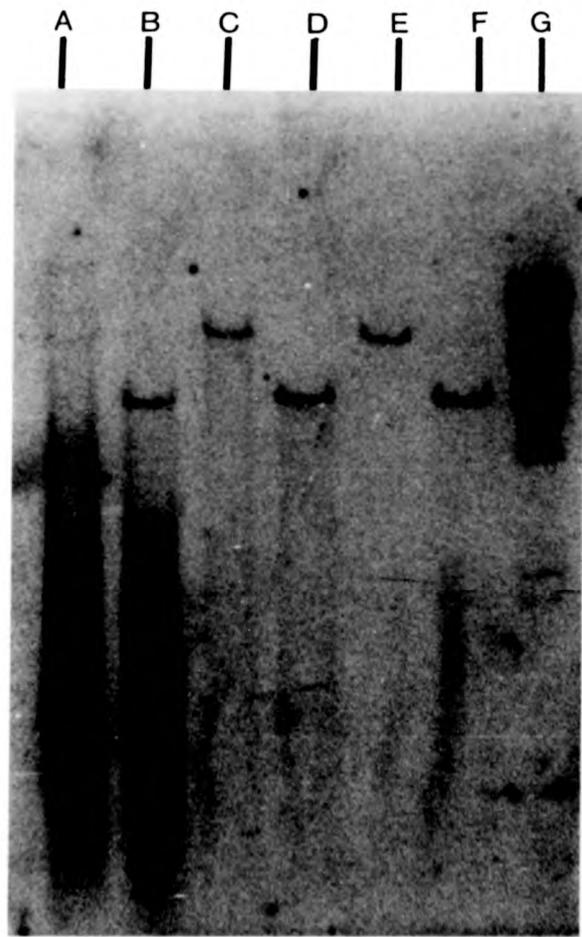


Figure 4.7: Photograph of an autoradiograph showing hybridisation of a pC194 probe to restriction endonuclease-digested chromosomal DNAs

- Lane A BglII-digested BD224 DNA (8.0Md)
- Lane B EcoRI-digested BD224 DNA (4.2Md)
- Lane C BglII-digested QBT5 DNA (8.0Md)
- Lane D EcoRI-digested QBT5 DNA (4.2Md)
- Lane E BglII-digested BDTCS DNA (8.0Md)
- Lane F EcoRI-digested BDTCS DNA (4.2Md)
- Lane G end-labelled HindIII-digested λ DNA



Figure 4.8: Photograph of an autoradiograph showing hybridisation of a pAB224 probe to restriction endonuclease-digested chromosomal DNAs

- Lane A end labelled HindIII-digested λ DNA
- Lane B BglII-digested BD224 DNA
- Lane C EcoRI-digested BD224 DNA
- Lane D BglII-digested QBT5 DNA
- Lane E EcoRI-digested QBT5 DNA
- Lane F BglII-digested BDTC5 DNA
- Lane G EcoRI-digested BDTC5 DNA
- Lane H undigested pAB224 DNA

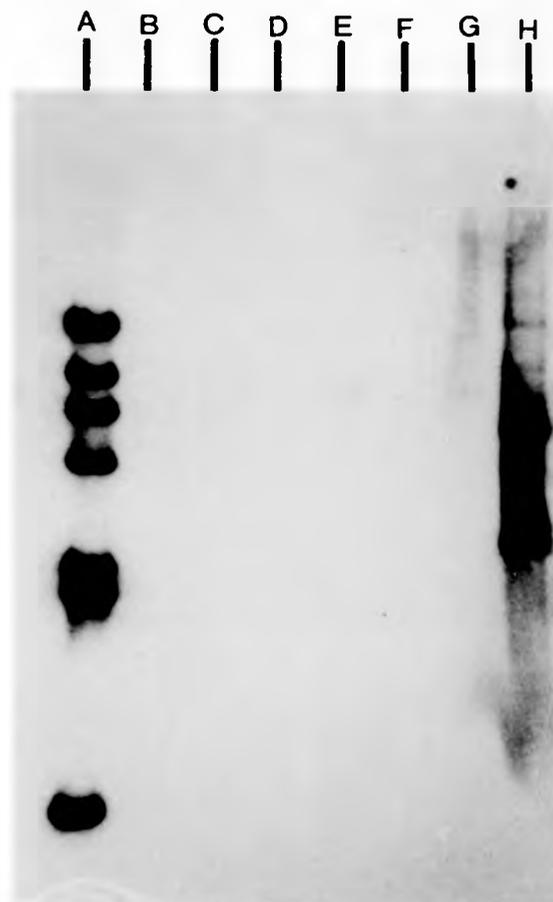


Figure 4.8: Photograph of an autoradiograph showing hybridisation of a pAB224 probe to restriction endonuclease-digested chromosomal DNAs

Lane A end labelled HindIII-digested λ DNA
 Lane B BglIII-digested BD224 DNA
 Lane C EcoRI-digested BD224 DNA
 Lane D BglIII-digested QBT5 DNA
 Lane E EcoRI-digested QBT5 DNA
 Lane F BglIII-digested BDTCS DNA
 Lane G EcoRI-digested BDTCS DNA
 Lane H undigested pAB224 DNA

4.2.8 Stability of Hybrid Plasmids

The stability of the hybrid thyP3-containing plasmids pTT1, pTT2, pTT3 and pTTC1 was investigated by growing batch-cultures of plasmid-bearing strains under different selective conditions and determining the phenotype of the cells after overnight growth in complex medium. The stability of these plasmids was of particular interest considering their unusual properties; it was demonstrated that the thyP3 gene has extensive DNA sequence homology with the B. subtilis chromosome and that under certain conditions the thyP3 region of the hybrids could integrate into the B. subtilis chromosome. However, extrachromosomal maintenance of the plasmids occurred even though the host was recombination-proficient (recE₄⁺). The results of the stability analysis experiments are shown in Table 4.9.

In the absence of any selection pressure, plasmid-borne markers were lost from all the strains tested though at markedly different rates. Strains harbouring pTT1 and pTT2 lost both the Tc^r and Thy⁺ phenotypes simultaneously suggesting that segregational instability occurred. This also suggested that cells harbouring either pTT1 or pTT2 may not contain a chromosomally integrated thyP3 gene in addition to the plasmid-borne thyP3 gene. The reason why pTT1-free strains were detected at a higher frequency than pTT2-free strains is unknown but could be related to the larger size of pTT2.

Strains harbouring pTT3 lost their Tc^r but not the Thy⁺ phenotype in the absence of any selection. Such Tc^s, Thy⁺ clones were found not to contain any extrachromosomal plasmid DNA and therefore probably contained an integrated copy of the thyP3 gene. Integration of the thyP3 gene into the chromosome could have occurred either upon initial transformation with the plasmid or during subsequent vegetative growth of the transformed cells.

The Tc^r, Cm^r and Thy⁺ plasmid-borne genes of pTTC1 were simultaneously lost from plasmid-bearing cells suggesting segregational instability. As in the case of pTT1 and pTT2, this result suggested that pTTC1-bearing cells may not

Plasmid	Selection pressure	Phenotype (%)	
pTT1	Thy ⁺	100	Tc ^r , Thy ⁺
	Tc ^r	100	Tc ^r , Thy ⁺
	None	96	Tc ^r , Thy ⁺
		4	Tc ^s , Thy ⁻
pTT2	Thy ⁺	100	Tc ^r , Thy ⁺
	Tc ^r	100	Tc ^r , Thy ⁺
	None	77	Tc ^r , Thy ⁺
		23	Tc ^s , Thy ⁻
pTT3	Thy ⁺	80	Tc ^r , Thy ⁺
		20	Tc ^s , Thy ⁺
	Tc ^r	100	Tc ^r , Thy ⁺
	None	56	Tc ^r , Thy ⁺
		44	Tc ^s , Thy ⁺
	pTTC1	Thy ⁺	100
Tc ^r		100	Tc ^r , Cm ^r , Thy ⁺
Cm ^r		100	Tc ^r , Cm ^r , Thy ⁺
None		59	Tc ^r , Cm ^r , Thy ⁺
		41	Tc ^s , Cm ^s , Thy ⁻

Table 4.9: Stability analysis of pTT1, pTT2, pTT3 and pTTC1

contain a chromosomally integrated thyP3 gene in addition to a plasmid-borne thyP3 gene. The larger size of pTTC1 in comparison to pTT1 and pTT2 may account for the greater number of plasmid-free cells detected.

If plasmid-bearing strains were grown under selective pressure for retention of plasmid-borne markers, a different pattern of plasmid stability was observed. If selection for antibiotic-resistance was applied, no plasmid-borne markers were lost in any cases. This demonstrated that under these conditions, plasmid fragmentation may not occur. If selection was made for retention of the thyP3 gene, by growing cells in TYS broth not containing added thymine, pTT1, pTT2 and pTTC1 also retained their antibiotic-resistance markers. However, pTT3-bearing cells, in some cases, lost the Tc-resistance marker. This suggested that cells harbouring pTT3 contained, in addition to the plasmid-borne thyP3 gene, a chromosomally integrated thyP3 gene.

4.3 DISCUSSION

The work in this chapter details the construction and characterisation of hybrid plasmids containing the thyP3 gene of the *B. subtilis* bacteriophage Φ 3T. The successful cloning of the thyP3 gene demonstrated that the unique EcoRI site of pAB224 can be used to clone foreign DNA fragments without inactivating essential plasmid replication functions and also without affecting expression of the pAB224 Tc-resistance genes. Several interesting observations were made from analysis of the properties of the hybrid plasmids constructed.

Firstly, previous work by Canosi *et al.* (1978), Mottes *et al.* (1979) and Contente and Dubnau (1979a), established that ccc monomeric plasmid DNA was inactive in the transformation of *B. subtilis* competent cells and that most of the transforming activity of a plasmid preparation was due to the presence of oligomeric forms of plasmid DNA. However, Contente and Dubnau (1979b) demonstrated that ccc monomeric, as well as linear plasmid DNA was active in the transformation of competent cells providing that they contained a

homologous resident plasmid. The results presented in this chapter demonstrated that monomeric forms of pTT1, pTT2, pTT3 and pTTC1 were active in the transformation of competent cells devoid of a homologous resident plasmid. However, at least in the case of pTT1 and pTTC1, transformation probably resulted in the integration of a particular region of the plasmids, containing the thyP3 gene, into a specific site in the host chromosome. Although monomeric forms of these plasmids could not give rise to antibiotic-resistant transformants containing extrachromosomal plasmid DNA, multimeric forms of the plasmids could. Therefore monomeric and multimeric forms of the plasmids must be processed by different mechanisms upon transformation of competent cells. The reason why transformation with multimeric species of plasmid did not lead to integration of the thyP3 gene into the host chromosome is unclear. However, a possible explanation comes from an examination of a model of our current understanding of plasmid transformation (Figure 4.9). Normally, transformation with a monomeric plasmid molecule does not produce a transformed cell because only a single strand of the plasmid enters a competent cell and as such is unable to establish itself as an autonomously replicating, double-stranded molecule. However, in the case of the thyP3-containing plasmids, this single-stranded molecule could pair with a homologous region of the host chromosome and undergo a recombination event, leading to integration of the thyP3 region of the plasmid, into the host chromosome. Therefore, in some respects, transformation with monomeric plasmid DNA is analogous to transformation with chromosomal DNA.

Transformation with multimeric forms of the thyP3-containing plasmids probably occurs in a similar fashion to transformation with plasmids such as pAB224 (Figure 4.9, b). Hence any single-stranded plasmid DNA which enters a competent cell is probably made unavailable for recombination with the host chromosome by pairing with a complementary strand of plasmid DNA. Thus multimeric forms of the thyP3-containing plasmids can establish themselves as

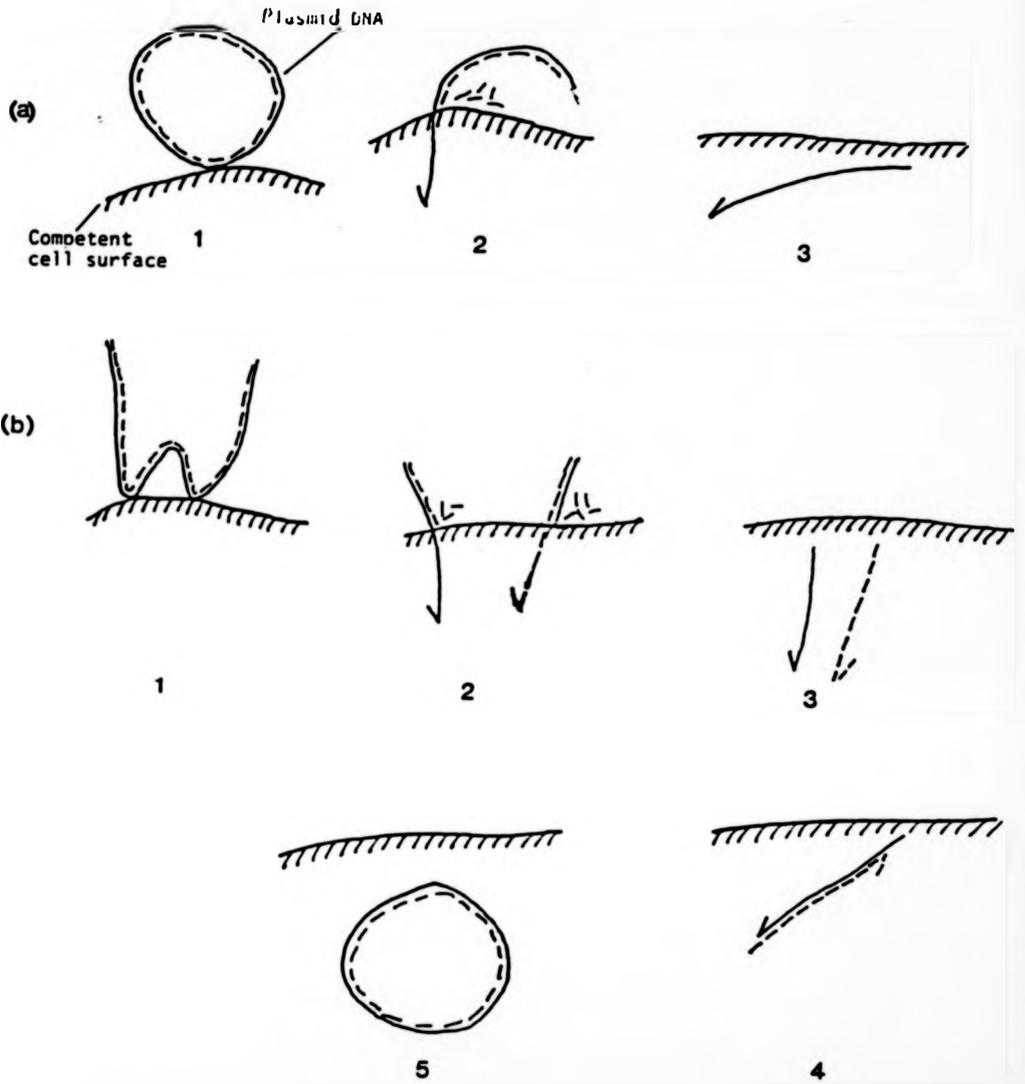


Figure 4.9: Schematic diagram showing the processing of (a) monomeric plasmid DNA and (b) multimeric plasmid DNA by competent *B. subtilis* cells (adapted from Canosi *et al.*, 1981)

- 1 Plasmid DNA binds to the competent cell surface.
- 2, 3 Uptake of single-stranded plasmid DNA.
- 4, 5 Base-pairing of complementary single strands and repair to produce fully double-stranded plasmid DNA.

autonomously replicating plasmids. The hybrid plasmid pTT3 may be processed by a different mechanism since cells harbouring an extrachromosomal plasmid may also contain an integrated copy of the thyP3 gene. However, this was not demonstrated conclusively.

Subsequent to the observations made with the thyP3-containing plasmids, there have been several reports of transformation of competent *B. subtilis* cells with monomeric plasmid DNA (Canosi *et al.*, 1981; Bensi *et al.*, 1981; Iglesias *et al.*, 1981). These workers demonstrated that monomeric plasmid DNA could be activated for transformation by insertion into a plasmid molecule of a DNA segment homologous to a region of the *B. subtilis* chromosome. Also, transformation by such monomeric plasmid DNA was found to be recE₄-dependent and resulted, not in the integration of the homologous segment into the host chromosome as seen with the thyP3-containing plasmids, but the production of an autonomously replicating extrachromosomal plasmid.

The reason why transformation with monomeric plasmids containing the thyP3 gene, constructed in this investigation, did not give rise to antibiotic-resistant transformants, containing extrachromosomal plasmid DNA, whereas the plasmids described by the above workers did, is unclear. However, it must be noted that in the case of the thyP3-containing plasmids, perfect plasmid-chromosome homology did not exist. To determine whether perfect plasmid-chromosome homology would enable monomeric thyP3-containing plasmids to transform competent cells to antibiotic-resistance, monomeric pTT1 and pTTC1 were used to transform competent cells already harbouring a thyP3 gene (a Tc^S, Thy⁺ pTT1 transformant was used as host). However, even in this case, with perfect plasmid-chromosome homology, transformation to antibiotic-resistance was not observed. Therefore, perfect plasmid-chromosome homology alone is not sufficient to activate monomeric plasmids for transformation to antibiotic-resistance.

The second interesting observation made from analysis of the thyP3-containing plasmids was that, in certain instances, transformation with plasmid DNA resulted in integration of plasmid-borne sequences, in particular the thyP3 gene, into the host chromosome. However, once established as autonomously replicating plasmids, chromosomal integration probably did not occur, at least in the case of pTT1, pTT2 and pTTC1.

Total or partial integration, into the B. subtilis chromosome, of plasmids carrying B. subtilis chromosomal DNA inserts has been reported by several workers (Rapoport *et al.*, 1979; Tanaka and Sakaguchi, 1978; Jayaraman *et al.*, 1981; Haldenwang *et al.*, 1980; Duncan *et al.*, 1977, 1978; Ehrlich, 1976; Phillips *et al.*, 1980; Galizzi *et al.*, 1981; Stroynowski, 1981b). The following conclusion can be drawn from the results of these workers. Extrachromosomal maintenance of hybrid plasmids which contain fragments of DNA which are homologous to the host chromosome, can occur. However, such plasmids are often unstable and part of, or the entire, hybrid plasmid may integrate into the host chromosome. Integration can nonetheless be prevented by the use of a recombination-deficient (recE₄) host strain. Transformation with plasmids which have sequence homology with the B. subtilis chromosome, but which are incapable of autonomous replication in B. subtilis, leads to the immediate integration into the host chromosome of part of, or the entire, plasmid. Conversely, plasmids which are capable of autonomous replication in B. subtilis may integrate partially, or completely, either upon transformation or during subsequent vegetative growth.

The thyP3-containing plasmids constructed in this investigation are unusual in that once extrachromosomal replication has been established, the plasmids appear to be relatively stable in that they do not integrate into the host chromosome during vegetative growth; or if they do integrate they in addition simultaneously exist extrachromosomally. pTT3 may be an exception to this rule

since the thyP3 gene may integrate into the host chromosome during vegetative growth.

The third interesting finding that came from analysis of the thyP3-containing plasmids was that the thyP3 gene, or sequences flanking this gene, have homology with two separate regions of the B. subtilis chromosome. The 1.5 Md BglIII fragment of pCD1 hybridised to 12.0 Md and 1.5 Md EcoRI fragments, and 12.0 Md and 6.2 Md BglIII fragments of chromosomal DNA from a non-transformed B. subtilis strain. Only one of these hybridisation bands, in each case, represented hybridisation to the chromosomal thyA gene, which E.M. Rubin (cited in Duncan *et al.*, 1978) had shown to be homologous to the thyP3 gene.

Subsequent to this finding, Galizzi *et al.* (1981) and Stroynowski (1981a, b) have investigated ϕ 3T sequences homologous to the B. subtilis chromosome. Galizzi *et al.*, (1981) observed hybridisation of the thyP3-containing plasmid pPV21 to a 9.0 Md EcoRI fragment and a 7.0 Md BglIII fragment of chromosomal DNA from a non-transformed B. subtilis strain. However, Stroynowski (1981a) observed hybridisation of the thyP3-containing plasmid pFT thyP3 to an EcoRI fragment of greater than 10 Md.

The differences in sizes of the thyA-containing DNA fragment reported by these workers may be due to different size fragments produced by the different strains used. However, it is also possible that the different sizes reported were due to different size standards used to calculate the size of the thyA-containing fragments.

From the results of Galizzi *et al.* (1981) and Stroynowski (1981a), it is likely that the 12.0 Md EcoRI hybridisation band observed in this investigation corresponds to the B. subtilis chromosomal thyA gene. However, from the results of Galizzi *et al.* (1981) it is not clear whether the 12.0 Md or the 6.2 Md BglIII hybridisation band contains the thyA gene; Galizzi *et al.* (1981) reported the thyA-containing BglIII fragment to be 7.0 Md.

The results of Stroynowski (1981a, b), also suggested a possible explanation for the presence of two hybridisation bands observed after hybridisation of the thyP3 probe to chromosomal DNA from a non-transformed B. subtilis strain in this investigation. Stroynowski (1981a) observed that the thyP3-containing plasmid pFT502 was homologous to two regions of chromosomal DNA from a non-transformed B. subtilis strain. In addition to an EcoRI hybridisation band of greater than 10 Md, one of 1.25 Md was also observed. In contrast, a pFT thyP3 probe hybridised to only one EcoRI fragment of greater than 10 Md. pFT502 had a Φ 3T DNA fragment of about 2.0 Md whereas that of pFT thyP3 was only 0.37 Md. Therefore pFT502 contained Φ 3T sequences other than the thyP3 gene and it must be these additional sequences which hybridised to a specific region of the B. subtilis chromosome. The 1.25 Md hybridisation band, observed after hybridisation to the pFT502 probe, was shown to be due to the presence of SP8 prophage sequences in the B. subtilis chromosome. Strains cured of SP8 prophage did not give a hybridisation band of 1.25 Md after hybridisation to the pFT502 probe.

Hybridisation, using total Φ 3T sequences as a probe, produced about 20 bands after EcoRI-digestion of B. subtilis chromosomal DNA (Stroynowski, 1981a). However, hybridisation to a strain cured of SP8 produced only four bands. One of these, of greater than 10 Md, represented hybridisation of the thyP3 gene to the chromosomal thyA gene. The other bands of 5.6, 1.45 and 1.3 Md, which displayed only weak homology, were the result of hybridisation of Φ 3T sequences to chromosomal sequences other than the thyA gene and SP8 prophage.

Since the hybridisation probe used in this present investigation was a 1.5 Md BglII fragment, approximately 1.1 Md of this DNA must be Φ 3T sequences other than the thyP3 gene. Therefore, the 1.5 Md EcoRI hybridisation band, and also one of the BglII bands produced after hybridisation of the 1.5 Md thyP3-containing probe to B. subtilis chromosomal DNA probably represented

hybridisation of sequences other than the thyP3 gene to SP β prophage sequences. However, hybridisation to chromosomal DNA sequences other than the thyA gene and SP β sequences cannot be ruled out.

The fourth point of interest concerns the site and mechanism of integration of the thyP3 gene into the *B. subtilis* chromosome. Total plasmid integration of thyP3-containing plasmids has been reported in several cases. Duncan *et al.* (1977, 1978) observed total integration of the plasmid pCD1 into a site near the chromosomal thyA gene. Duncan *et al.* (1978) also observed total integration of pCD4 and pCD6. These hybrid plasmids consisted of a 0.5 Md region of pCD1, which is ϕ 3T sequences other than the thyP3 gene, in addition to pMB9 and the ϕ 22 thy gene. Phillips *et al.* (1980) subsequently showed that pCD4 integrated near the SP β chromosomal attachment site and may even have integrated into SP β sequences. Galizzi *et al.* (1981) also observed total integration of the thyP3-containing plasmid pPV21; the major site of integration was the thyA locus but integration at the ϕ 3T att site (near the SP β att site) was also observed.

In contrast to total plasmid integration Ehrlich *et al.* (1976) and Stroynowski (1981b) observed integration of only the thyP3 gene into the *B. subtilis* chromosome. Although Ehrlich *et al.* (1976) did not determine the site of integration of their thyP3-containing plasmids, Stroynowski (1981b) found that integration into the thyA gene and also SP β sequences occurred. Integration into SP β sequences was found to occur preferentially with large DNA fragments such as those produced by BamHI-digestion of phage ϕ 3T, or intact ϕ 3T. Small fragments of DNA, containing the thyP3 gene, such as those produced by EcoRI or BglII digestion of ϕ 3T, or cloned thyP3 genes, integrated into the chromosomal thyA gene.

Although the site of integration of the thyP3-containing plasmids constructed in this investigation was not determined, results from the Southern analysis of pTT1 and pTTCl transformants suggest that it was not at the thyA locus. If it was at the thyA locus, an additional band would not have been seen

on the autoradiographs. Therefore, at least in the cases of pTT1 and pTTC1, integration was probably into SP β prophage sequences. Since pTT1 and pTTC1 contain the 0.47 Md fragment of pCD1, which is also present in pCD4, pTT1 and pTTC1 may in fact integrate at the same location as pCD4, i.e. near the Φ T att site.

Therefore, in conclusion, the plasmid vector pAB224 has been shown to be useful for the cloning of a foreign gene into its unique EcoRI site. The thyP3-containing hybrids constructed had several unusual properties in that they had extensive DNA sequence homology with the B. subtilis chromosome and in certain circumstances part of the hybrids could integrate into a specific site in the host chromosome.

Analysis of the properties of the thyP3-containing hybrid suggested that a recE4 strain may not be necessary to prevent integration of plasmid sequences which are homologous to the host chromosome. Also, that by selecting for one plasmid-borne marker, it may be possible to maintain the structural integrity of other plasmid-borne genes.

CHAPTER V

PENICILLINASE- β -GALACTOSIDASE FUSIONS IN *E. coli* AND *B. subtilis*

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5.1 INTRODUCTION

5.1.1 Protein Secretion

Many proteins, from both eukaryotes and prokaryotes, which are specifically transported across membranes, have been found to possess a common feature. In general, proteins destined for secretion are synthesised as precursors, containing at their N-terminus, an extension of 15 to 30 non-polar, hydrophobic amino acids, a signal-sequence or -peptide (see Figure 5.1), which are not found in the mature protein. This signal-peptide, which apparently facilitates translocation of the protein across the cell membrane, is subsequently removed by proteolytic cleavage. Although a vast amount of work has concerned the mechanism of protein secretion by eukaryotes and Gram-negative bacteria, comparatively little is known about the mechanism of protein secretion by Gram-positive organisms. However, present indications are that the general mechanism of protein secretion is the same in all organisms (Talmadge *et al.*, 1980b).

It is hoped that using genetic manipulation, regulatory signals from a secreted protein can be attached to a protein of interest to promote the secretion of that protein into either the periplasmic space for *E. coli* or the culture medium for *B. subtilis* (or other species of *Bacillus*). For example, rat proinsulin already has been shown to be secreted by *E. coli* cells when fused to a bacterial signal-peptide (Villa-Komaroff *et al.*, 1978; Talmadge *et al.*, 1980a).

Although species of *Bacillus* are known to secrete a large variety of proteins into the culture medium, very little is known about the N-terminal amino acids of the precursor forms of these proteins. Recently, the *B. coagulans* α -amylase gene has been cloned in *E. coli* (Cornelis *et al.*, 1982) and the *B. amyloliquefaciens* α -amylase gene has been cloned in *B. subtilis* (Yoneda *et al.*, 1979; Palva, 1982). Also, the nucleotide sequence of the promoter and 5' end of the *B. amyloliquefaciens* α -amylase gene has been determined (Palva *et al.*, 1981).

The *B. amyloliquefaciens* α -amylase gene has been shown to be translated *in vitro*, into a protein of 60,000 daltons (Palva, 1982) which was slightly larger than the secreted form of the enzyme which had a size of 58,000 daltons. Therefore the 60,000 dalton protein probably contained the intact α -amylase signal-peptide in addition to the mature α -amylase polypeptide. From the DNA sequence of the cloned α -amylase gene (Palva *et al.*, 1981), a signal peptide of 31 amino acid residues was predicted. Although this is fairly large, it has a structure similar to that of typical signal-peptides of exported proteins found in Gram-negative organisms (see Figure 5.1).

Probably the most widely studied secreted protein of a *Bacillus* species is the penicillinase produced by the constitutive mutant *B. licheniformis* strain 749/C (Dubnau and Pollock, 1965). This strain produces several different forms of the penicillinase protein which appear to be synthesised as a common larger precursor and are produced by successive proteolytic cleavage steps (Yamamoto and Lampen, 1976; Sarvas *et al.*, 1978; Davis and Tai, 1980). Under normal growth conditions, greater than 50% of the penicillinase protein is present as a secreted form (exoenzyme) whereas the rest is in a membrane-bound form (Sargent *et al.*, 1968).

The penicillinase protein initially is synthesised as a precursor of 34,500 daltons and has been detected *in vitro* (Sarvas *et al.*, 1978). The N-terminus of this precursor, which is probably analogous to the signal-peptides found in most secreted proteins, is subsequently removed to produce a 31,000 dalton membrane-bound form of the enzyme (Simons *et al.*, 1978). A second cleavage can then occur to remove the N-terminal hydrophobic peptide anchoring the enzyme to the cytoplasmic membrane and the water-soluble exoenzyme is released from the cell. The exopenicillinase is known to be heterogeneous in size (Ambler and Meadway, 1969; Crane and Lampen, 1974) and two different forms of the enzyme have been detected (Simons *et al.*, 1978). The smaller secreted form, or "exo-small" is 265 amino acid residues long; the "exo-large" form has an

MKATKLVLLGAVILGSTLLAG CSS	<i>E. coli</i> prolipoprotein Inouya <i>et al.</i> , 1977
MKKSLLVKASAVATLVPMLSFA AEG	<i>E. coli</i> f1 major coat protein Chang <i>et al.</i> , 1978
MKIKTGARILALSALTMMFSASALA KIE	<i>E. coli</i> maltose binding protein Bedouelle <i>et al.</i> , 1980
MMITLAKLPLAVAVAAGVMSAQAMA VAF	<i>E. coli</i> λ receptor protein Emr <i>et al.</i> , 1980
MKKLALSLSLVLAFSSATAAFA AIP	<i>S. typhimurium</i> J protein Higgins and Ames, 1981
MKKTVLALSLLIGLGATAASYA AIP	<i>S. typhimurium</i> LAO protein Higgins and Ames, 1981
ANEGNAMGFIMRPVSASSHAT AOL	<i>S. dysenteriae</i> outer membrane protein Braun and Cole, 1982
MKKLLFAIPLVVPFYSHS A	<i>E. coli</i> f1 minor coat protein Schaller <i>et al.</i> , cited in Emr <i>et al.</i> , 1980
MSIQHRVALIPFFALFCLPVFA HPE	<i>E. coli</i> β -lactamase Sutcliffe, cited in Emr <i>et al.</i> , 1980
MYTSGYHRSSSFSSAASKIA AVS	<i>E. coli</i> outer membrane protein Beck and Bremner, 1980
MKOSTIALALLPLFTPVTKA RTP	<i>E. coli</i> alkaline phosphatase Inouya <i>et al.</i> , 1982
MIQKRKRTVSFRLYLMCTLLFVSLPITKTA VNG	<i>B. amyloliquefaciens</i> α -amylase Palva <i>et al.</i> , 1981
MKLVFSTLKLKKAVALLLFSCVALAG CANNOTNA S [↑] PAEKNE KTE	<i>B. licheniformis</i> penicillinase Neugebauer <i>et al.</i> , 1981
	

Figure 5.1: Amino acid sequence of some prokaryotic signal-peptides

M = Met	K = Lys	A = Ala	T = Thr	L = Leu	V = Val	G = Gly
I = Ile	S = Ser	C = Cys	P = Pro	F = Phe	E = Glu	R = Arg
Q = Gln	Y = Tyr	H = His	W = Trp			

additional 8 amino acid residues (Ambler and Meadway, 1969; Simons et al., 1978; Izui et al., 1980).

Smith et al. (1981) demonstrated that the penicillinase protein was secreted co-translationally by B. licheniformis cells and in extracts was found to be synthesised by membrane-associated polysomes. Proteins of 36,000, 33,000 and 29,000 daltons were synthesised in vitro which corresponded to the precursor, membrane-bound, and exopenicillinases respectively. In both E. coli and B. subtilis the penicillinase protein contained a cysteinyl residue at or near the N-terminus in addition to glycerol and fatty acid residues (Nielsen et al., 1981). Lai et al. (1981) also observed the glycerol and fatty acid residues covalently attached to the penicillinase protein synthesised in E. coli. These modifications, which occur in growing B. licheniformis cells, are thought to be responsible for the retention on the outer side of the plasma membrane of B. licheniformis cells of 50% of the total culture penicillinase.

Recently the B. licheniformis 749/C penicillinase structural gene penP, has been cloned in E. coli using both bacteriophage (Brammar et al., 1980; Neugebauer et al., 1981) and plasmid (Gray and Chang, 1981; Imanaka et al., 1981b) vectors and the nucleotide sequence of the promoter-proximal region (Kroyer and Chang, 1981) (Figure 5.2) and the entire penP gene (Neugebauer et al., 1981) have been determined.

The nucleotide sequence analysis showed that sequences preceding the penP structural gene have features typical of regulatory signals for gene expression in prokaryotes. Also the amino acid sequence predicted from the nucleotide sequence of the cloned gene (Figure 5.2), suggested that the penicillinase protein is synthesised as a precursor with an extension of 34 amino acid residues present at the N-terminus of the exo-large penicillinase. This signal-peptide is similar to those of secreted proteins from other prokaryotes (Figure 5.1) except that the N-terminus of the penicillinase protein is more basic due to the presence of 4 lysine residues.

-35

GAA ACG AGG TCA TCA TTT CCT TCC GAA AAA ACG GTT GCA TTT AAA TCT TAC ATA TGT

-10

AAT ACT TTC AAA GAC TAC ATT TGT AAG ATT TGA TGT TTG AGT CGG CTG AAA GAT CGT

ACG TAC CAA TTA TTG TTT CGT GAT TGT TCA AGC CAT AAC ACT GTA GGG ATA GTG GAA

SD

AGA GTG CTT CAT CTG GTT ACG ATC AAT CAA ATA TTC AAA CGG AGG GAG ACG ATT TTT

PstI

ATG AAA TTA TGG TTC AGT ACT TTA AAA CTG AAA AAG GCT GCA GCA GTG TTG CTT TTC
M K L W F S T L K L K K A A A V L L F

TCT TGC GTC GCG CTT GCA GGA TGC GCT AAC AAT CAA ACG AAT GCC TCG CAA CCT GCC
S C V A L A G C A N N Q T N A S Q P A

CAG GAA CAA TTT GAT GCA AAA CTC GGG ATC TTT
E K N E K T E L G I F

Figure 5.2: DNA sequence of the promoter-proximal region of the *B. licheniformis* 749/C *penP* gene (adapted from Kroyer and Chang, 1981 and Neugebauer *et al.*, 1981).

M = Met	K = Lys	A = Ala	T = Thr	L = Leu
V = Val	G = Gly	S = Ser	C = Cys	P = Pro
F = Phe	E = Glu	Q = Gln	W = Trp	N = Asn

The -35 and -10 regions are RNA polymerase recognition sites.
The SD (Shine-Dalgarno) sequence is the ribosome binding site.

5.1.2 LacZ Fusions

In order to study gene regulation it is often useful to fuse the genetic regulatory elements of a particular gene of interest to the structural part of the well characterised E. coli lacZ gene (Miller and Reznikoff, 1980). A simple, sensitive assay for the lacZ gene-product, β -galactosidase, has been developed in addition to an in situ assay enabling bacterial colonies producing the enzyme to easily be detected (Miller, 1972; Miller and Reznikoff, 1980). The enzyme has been well characterised and found to consist of a tetramer of four identical subunits each of approximately 116,000 daltons (Miller and Reznikoff, 1980) which gives a size of 464,000 daltons for the tetramer. It is the tetrameric form of the enzyme which is probably the only active form and at low ionic strengths and/or protein concentrations, the enzyme dissociates into inactive monomers (Miller and Reznikoff, 1980).

Various techniques for fusing the lacZ gene to different DNA sequences have been developed for use in E. coli (Casadaban, 1976; Casadaban and Cohen, 1980; Casadaban et al., 1980) and when fused to a foreign gene, the activity of β -galactosidase can be used as a convenient indicator of the level of expression of the fused gene. This technique has proved particularly useful for analysing transcriptional and translational regulatory signals of genes whose products are difficult to assay, e.g. the E. coli tRNA gene, tyrT (Berman and Beckwith, 1979) and the E. coli polypeptide elongation factor Tu gene, tufB (Takebe and Kaziro, 1982).

Since the N-terminus of the β -galactosidase protein is not essential for enzymic activity, fusions can be constructed that replace the 5'-coding segment of the lacZ gene with other DNA sequences (e.g. Müller-Hill and Kania, 1974; Heidecker and Müller-Hill, 1977; Brickman et al., 1979). Such so called protein fusions still retain functional β -galactosidase activity and have been used widely for investigations into protein secretion. By linking β -galactosidase to the N-terminus of the E. coli alkaline phosphatase enzyme, Inouya and Beckwith (1977)

were able to isolate and sequence part of the alkaline phosphatase signal-peptide. Also, using similar techniques, it has been possible to fuse the lacZ gene to the 5' end of several genes which code for E. coli secreted proteins such as the lamB, malE, malF and phoA genes (Silhavy et al., 1976; 1977; Bassford and Beckwith, 1979; Bassford et al., 1979; Sarthy et al., 1979; Hall et al., 1982; Herrero et al., 1982; Hall et al., 1982). The distribution of such fusion proteins within the cell has been investigated and it has been shown that in the case of the maltose binding protein (the lamB gene-product) lamB-lacZ fusions have in some cases resulted in secretion of the fusion protein into the outer membrane of the cell (Hall et al., 1982). However, in the case of periplasmic proteins such as the malE gene-product (Herrero et al., 1982), synthesis of β -galactosidase-containing fusion proteins has resulted in the fusion proteins becoming embedded in the inner membrane of the cell. This often resulted in cell lysis upon prolonged synthesis of the fusion protein. Therefore, although it has been firmly established that protein signal-peptides play a major role in protein secretion, the presence of a signal-peptide alone may not be sufficient to promote the secretion of a particular protein of interest. The nature of the particular protein may also be of prime importance.

The work in this section details the construction and characterisation of β -galactosidase-containing fusion proteins in E. coli and B. subtilis.

5.2 RESULTS

5.2.1 Construction and Characterisation of penP-lacZ Fusions in E. coli

As detailed in the introduction to this chapter, the E. coli lacZ gene has widely been used to study both the regulation of gene expression and the structure and function of protein signal-peptides. In this present investigation it was decided to use the E. coli β -galactosidase enzyme as part of a model system consisting of a penP-lacZ fusion, to investigate the expression and secretion of fusion proteins

in B. subtilis. However, as a preliminary investigation to determine whether such a fusion would yield a protein with β -galactosidase activity, the fusion was initially constructed in E. coli. Genetic manipulation in this organism is much simpler than in B. subtilis and also the penP gene transcriptional and translational regulatory signals have been shown to be functional in E. coli (Brammar et al., 1980).

From analysis of the nucleotide sequence of the penP gene (Neugebauer et al., 1981; Kroyer and Chang, 1981) and a 5' terminal region of the lacZ gene (Casadaban et al., 1980), it appeared possible to fuse the lacZ gene to a 5'-terminal segment of the penP gene so as to position the lacZ gene in the same translational reading frame as the penP gene and at a site upstream from the coding sequences of the penP signal-peptide (Figure 5.3). The BamHI site preceding the lacZ structural gene of pMC1396 (Figure 5.4) can be linked to the BglIII site which lies within the penP structural gene (Figures 5.2 and 5.3). Thus a fusion protein consisting of the penicillinase signal-peptide and mature protein N-terminus linked to β -galactosidase, should be produced.

The plasmid pUB1660 (Figure 5.5) was used as a source of the penP gene. This vector consists of the B. licheniformis 749/C penP gene from the recombinant λ bacteriophage λ pen (Brammar et al., 1980) linked to the E. coli vector pACYC184 (Chang and Cohen, 1978). Plasmids pUB1660 and pMC1871, isolated from strains of E. coli, were characterised by restriction endonuclease analysis as shown in Table 5.1. The DNA fragment sizes obtained were in good agreement with the restriction endonuclease cleavage maps shown in Figures 5.5 and 5.6.

Prior to cloning, the BamHI lacZ-containing fragment of pMC1871 was purified by electroelution. A BamHI digest of this plasmid produced fragments of 2.0, 2.0 and 0.7 Md. The presence of two fragments of similar size (2.0 Md) meant that the lacZ-containing fragment could not be purified after BamHI digestion of pMC1871 and therefore the plasmid was first digested with PstI; this

penP

ATG AAA TTA TGG TTC AGT ACT TTA AAA CTG AAA AAG GCT GCA GCA GTG TTG CTT TTC

TCT TGC GTC GCG CTT GCA GGA TGC GCT AAC AAT CAA ACG AAT GCC TCG CAA CCT GCC

CAG GAA CAA TTT GAT GCA AAA CTC GGG ATC TTT GCA TTG GAT ACA GGT ACA AAC CGG

ACG GTA GCG TAT CGG CCG GAT GAG CGT TTT GCT AAC TTT GCT TCG ACC ATT AGG GCT

BclII

TTA ACT GTA GGC GTG CTT TTG CAA CAG AAA TCA ATA GAA GAT CT

BamHI lacZ gene

G GAT CCG GGC GTC GTT TTA

CAA CGT CGT GAC TGG GAA AAC CCT GGC GTT ACC CAA CTT AAT CGC CTT GCA GCA TAT

CCC CCC TTC CCC AGC TGG CGT AAT

Figure 5.3: Predicted DNA sequence of a penP-lacZ fusion (adapted from Kroyer and Chang, 1981 and M.C. Casadaban, personal communication)

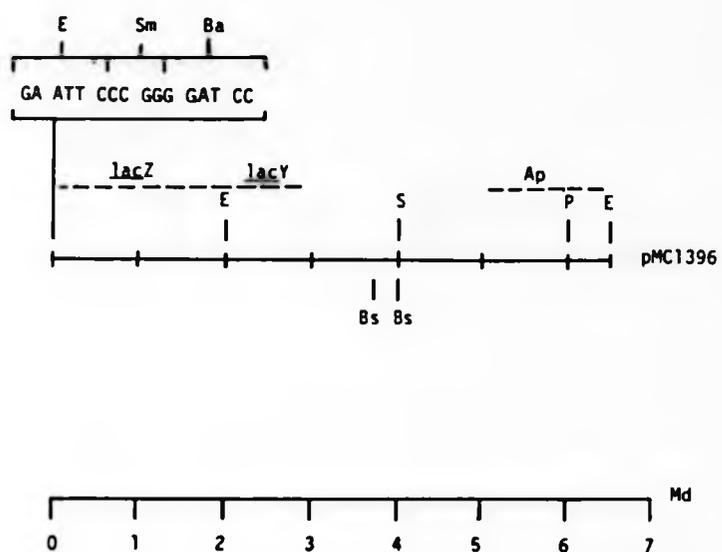


Figure 5.4: Restriction endonuclease cleavage map of pMC1396 and the DNA sequence at the 5' end of the *lacZ* gene (adapted from Casadaban et al., 1980)

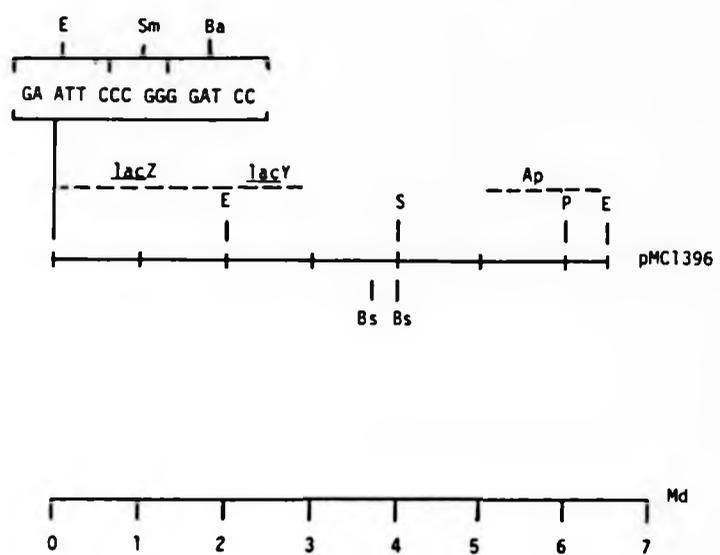


Figure 5.4: Restriction endonuclease cleavage map of pMC1396 and the DNA sequence at the 5' end of the *lacZ* gene (adapted from Casadaban *et al.*, 1980)

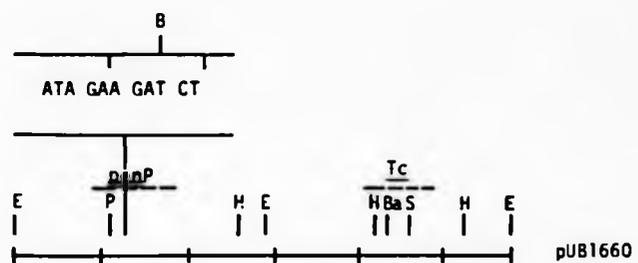


Figure 5.5: Restriction endonuclease cleavage map of pUB1660 and the DNA sequence of the BclI region (adapted from Docherty, 1981)

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)	
	pMC1871	pUB1660
<u>EcoRI</u>	2.20, 1.80, 0.70	2.70*
<u>BamHI</u>	2.00*, 0.70	5.60
<u>PstI</u>	2.90, 2.00	5.50
<u>SalI</u>	2.10, 1.95, 0.80	5.60
<u>SmaI</u>	4.60	-
<u>HindIII</u>	-	3.10, 1.50, 1.05
<u>BglII</u>	-	5.60

TABLE 3.1: DNA fragment sizes of pMC1871 and pUB1660 obtained after restriction endonuclease digestions

* represents 2 fragments of the same size

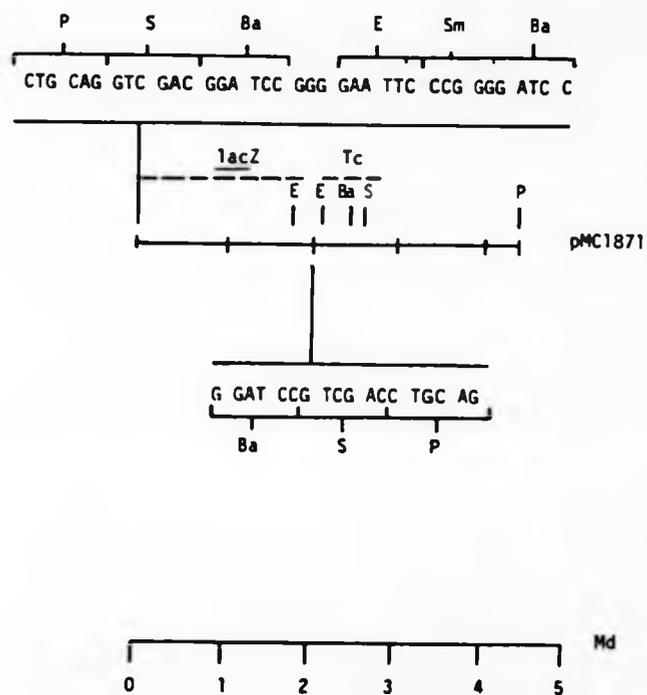


Figure 5.6: Restriction endonuclease cleavage map of pMC1871 and the DNA sequences flanking the *lacZ* gene (M.C. Casadaban, personal communication)

produced fragments of 2.9 and 2.0 Md. 40 µg of pMC1871 was digested with 40 units of PstI for 2 h at 37°C and after agarose gel electrophoresis, the 2.0 Md lacZ-containing fragment was isolated by electroelution. This fragment was digested further with 20 units of BamHI for 2 h at 37°C and after agarose gel electrophoresis the 2.0 Md BamHI fragment was isolated by electroelution. One µg of BglII-digested pUB1660 was ligated to about 1 µg of the 2.0 Md BamHI, lacZ-containing fragment of pMC1871 in a final reaction volume of 20 µl. 10 µl of this ligation-mix was then used to transform competent E. coli MC1061 cells. As controls, competent cells were transformed with 1 µg of pUB1660 DNA and 1 µg of pMC1871 DNA. Transformants were spread onto L-agar containing BCIG solution in addition to either 50 µg per ml of Ap or 20 µg per ml of Tc.

pUB1660 produced 4.1×10^5 Ap^r and 1.7×10^5 Tc^r transformants whilst pMC1871 produced 0 Ap^r and 2.1×10^4 Tc^r transformants. No detectable β-galactosidase activity was produced since all the transformants were white indicating a lacZ⁻ genotype. The pUB1660-pMC1871 fragment ligation-mix produced 1.7×10^3 Ap^r and 2.1×10^3 Tc^r transformants. Whereas none of these Ap^r transformants produced β-galactosidase activity (white colonies), 23.5% of the Tc^r transformants did produce β-galactosidase activity (blue colonies). The plasmids pEClac1, pEClac2 and pEClac3 were isolated from three of the latter type of transformant and further characterised as detailed below.

Firstly, the plasmids were transformed into competent E. coli MC1061 cells (Table 5.2). As expected, pUB1660 produced Tc^r and Ap^r transformants all of which were lacZ⁻ and pMC1871 produced Tc^r transformants which were all lacZ⁻ also. Whereas the hybrid pEClac2 produced only Tc^r, Ap^s, lacZ⁺ transformants, both pEClac1 and pEClac3 produced Tc^r, Ap^s, lacZ⁺ and Tc^r, Ap^r, lacZ⁻ transformants. Analysis of these plasmids by agarose gel electrophoresis revealed that pEClac2 contained a plasmid of approximately

Transforming DNA	Transformants per μg DNA	Phenotype of transformants (%)		
		Tc ^r , Ap ^s , <u>lacZ</u> ⁺	Tc ^r , Ap ^r , <u>lacZ</u> ⁻	Tc ^r , Ap ^s , <u>lacZ</u> ⁻
pUB1660	6.0 x 10 ⁵ Tc ^r	100	0	0
	8.0 x 10 ⁵ Ap ^r	100	0	0
pMC1871	5.0 x 10 ⁵ Tc ^r	0	0	100
	0.0 Ap ^r	-	-	-
pEClac1	1.2 x 10 ⁶ Tc ^r	85	15	0
	4.5 x 10 ⁵ Ap ^r	0	100	0
pEClac2	2.2 x 10 ⁵ Tc ^r	100	0	0
	0.0 Ap ^r	-	-	-
pEClac3	5.6 x 10 ⁵ Tc ^r	95	5	0
	8.3 x 10 ⁴ Ap ^r	0	100	0
No DNA	0.0 Tc ^r	-	-	-
	0.0 Ap ^r	-	-	-

TABLE 3.2: Transformation of *E. coli* MC1061 cells

Plates were incubated overnight at 37°C and a further 1 day at ambient temperature. Media contained BCIG solution and either Tc or Ap at a concentration of 25 μg per ml.

8.0 Md whereas pEClac1 and pEClac3 each contained a plasmid of approximately 8.0 Md in addition to one of approximately 6.5 Md.

A photograph of restriction endonuclease digestions of pEClac1, pEClac2 and pEClac3 is shown in Figure 5.7. The smaller plasmid species present in pEClac1 and pEClac3 each have a single recognition site for PstI, BglII and BamHI and may be equivalent to pUB1660 which also has single recognition sites for these enzymes and which also transforms *E. coli* to Tc^r, Ap^r, lacZ⁻. The larger plasmid species present in pEClac1, pEClac3 and also pEClac2, each have single recognition sites for BamHI and PstI but no recognition site for BglII. Therefore these plasmid species may be the expected pUB1660-pMC1871 fragment, hybrid.

Since pEClac2 contained only one plasmid species, further investigations employed only this plasmid. Restriction endonuclease digestions of pEClac2 are shown in Table 5.3 and restriction endonuclease cleavage maps are shown in Figure 5.8. pEClac2 has the structure of the predicted pUB1660-pMC1871 fragment hybrid except that pEClac2 is slightly larger and also has a deletion of the HindIII site which lies within the pACYC184 region of the hybrid.

β -galactosidase assays were done on *E. coli* cultures to obtain a quantitative measure of enzyme activity (Table 5.4). An uninduced, lacZ⁺, *E. coli* culture (strain W3110) produced a low level of β -galactosidase activity but upon induction this level increased approximately two thousand fold. As expected *E. coli* strain MC1061 (lacZ⁻) harbouring pMC1871 did not produce β -galactosidase activity even upon induction. In contrast this strain harbouring pEClac2 produced approximately 17 units per bacterium per minute of β -galactosidase activity. This level did not increase upon induction suggesting that the enzyme was synthesised constitutively. This would be expected if synthesis was regulated by the penP genetic regulatory signals; the penicillinase protein also is synthesised constitutively in *E. coli* cells (Brammar et al., 1980). The level of β -galactosidase activity in strain MC1061 harbouring pEClac2 was five to

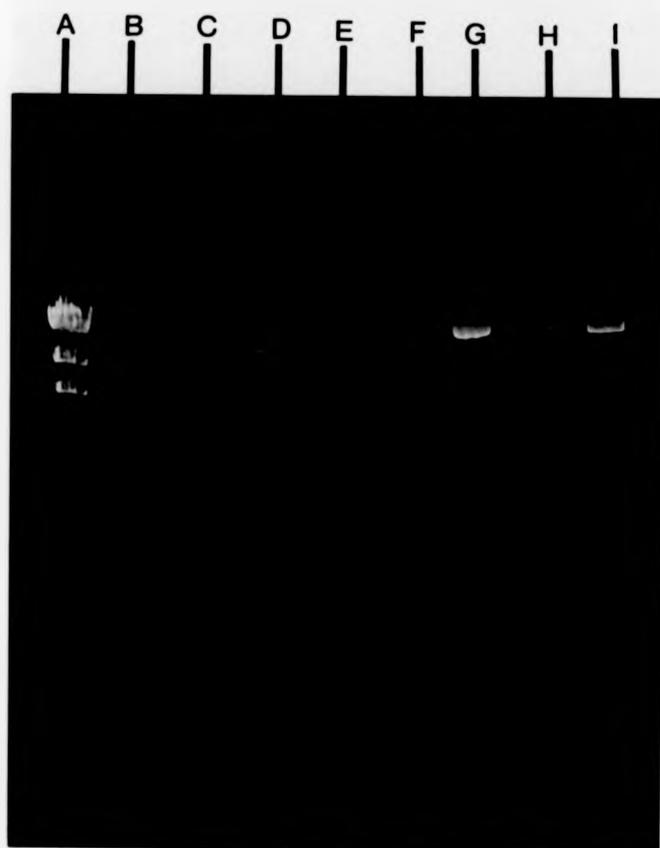


Figure 5.7: Photograph showing nEC1ac1, pEC1ac2 and pEC1ac3 after agarose gel electrophoresis

- Lane A HindIII-digested λ DNA
- Lane B PstI-digested pEC1ac2 DNA (8.40Md)
- Lane C HindIII-digested pEC1ac2 DNA (8.30, 1.60Md)
- Lane D BamHI-digested pEC1ac2 DNA (8.30Md)
- Lane E SalI-digested pEC1ac2 DNA (8.20Md)
- Lane F EcoRI-digested pEC1ac2 DNA (3.15, 2.95, 1.75Md)
- Lane G undigested nEC1ac3 DNA
- Lane H undigested nEC1ac2 DNA
- Lane I undigested nEC1ac1 DNA

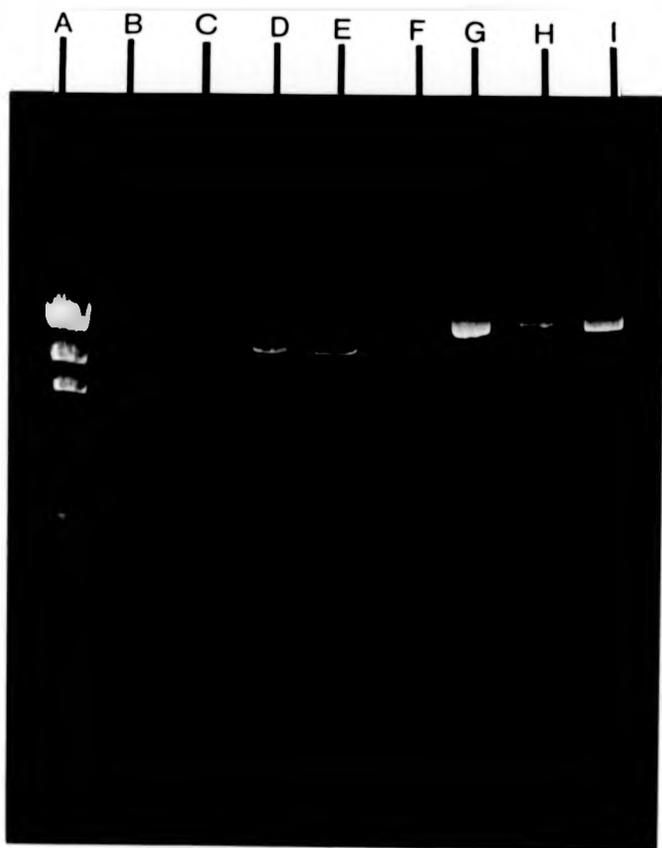


Figure 5.7: Photograph showing nEClac1, pEClac2 and pEClac3 after agarose gel electrophoresis

- Lane A HindIII-digested λ DNA
- Lane B PstI-digested pEClac2 DNA (8.40Md)
- Lane C HindIII-digested pEClac2 DNA (6.30, 1.60Md)
- Lane D BamHI-digested pEClac2 DNA (8.30Md)
- Lane E SalI-digested pEClac2 DNA (8.20Md)
- Lane F EcoRI-digested pEClac2 DNA (3.15, 2.95, 1.75Md)
- Lane G undigested nEClac3 DNA
- Lane H undigested nEClac2 DNA
- Lane I undigested nEClac1 DNA

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>Bam</u> HI	8.30
<u>Pst</u> I	8.40
<u>Sal</u> I	8.20
<u>Hind</u> III	6.30, 1.60
<u>Eco</u> RI	3.15, 2.95, 1.75
<u>Bam</u> HI- <u>Pst</u> I	5.00, 2.70
<u>Bam</u> HI- <u>Sal</u> I	8.20, 0.20
<u>Bam</u> HI- <u>Hind</u> III	6.10, 1.60, 0.20
<u>Bam</u> HI- <u>Eco</u> RI	3.15, 1.65, 1.60, 1.35
<u>Pst</u> I- <u>Sal</u> I	5.50, 2.60
<u>Pst</u> I- <u>Hind</u> III	3.30, 3.05, 1.73
<u>Pst</u> I- <u>Eco</u> RI	2.90, 2.15, 1.55, 1.20
<u>Sal</u> I- <u>Hind</u> III	6.25, 1.65, 0.30
<u>Sal</u> I- <u>Eco</u> RI	3.20, 1.70, 1.55, 1.50
<u>Hind</u> III- <u>Eco</u> RI	3.00, 1.75, 1.30, 1.20, 0.30

TABLE 5.3: DNA fragment sizes of pEClac2 obtained after restriction endonuclease digestions

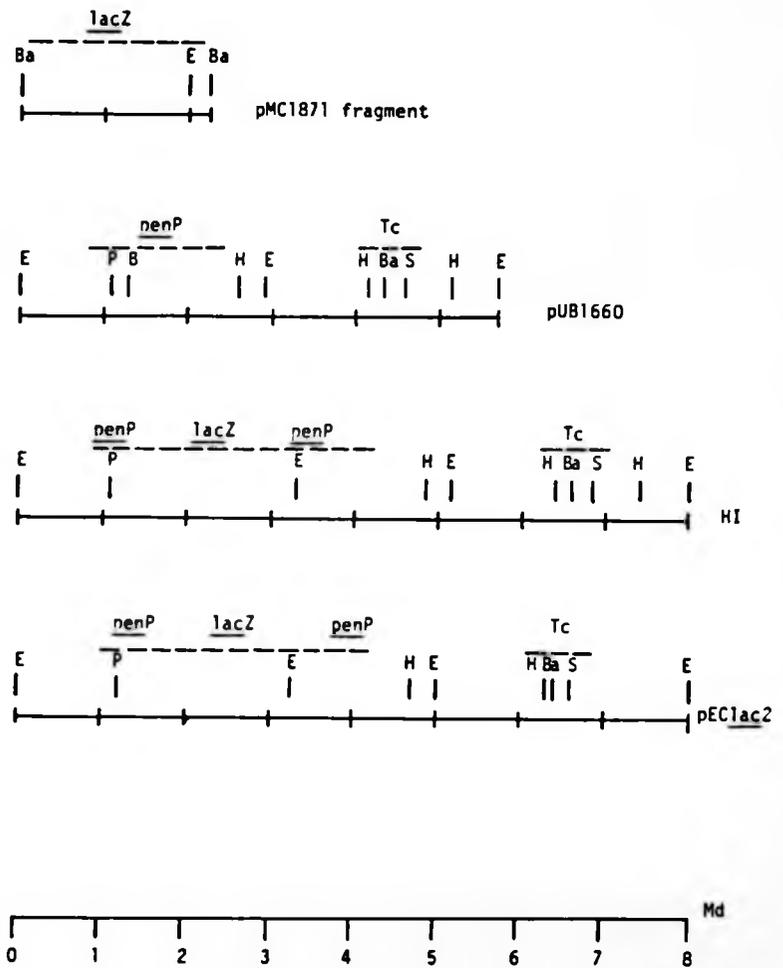


Figure 5.8: Restriction endonuclease cleavage maps of pEC1lac2 and the parental plasmids used in its construction. HI is the predicted pUB1660-pMC1871 hybrid.

Strain	β -galactosidase activity	
	units per bacterium per minute	units per ml of culture per minute
W3110	3.3	4.9
W3110 + I	6105.0	9035.4
MCI061-pEClac2	17.3	18.9
MCI061-pEClac2 + I	16.7	18.0
MCI061-pMCI871	0.0	0.0
MCI061-pMCI871 + I	0.0	0.0

TABLE 5.4: β -galactosidase activity of *E. coli* cultures

β -galactosidase assays were done as detailed in section 2.2.19. Cells were grown in 5ml of M9 broth in a 1oz bottle and incubated overnight at 37°C with vigorous aeration. IPTG (I) was added to a final concentration of 200 μ g/ml where necessary.

six-fold higher than that of an uninduced lacZ⁺ E. coli (strain W3110), but about 300-fold lower than the latter strain induced with IPTG. The low level of β -galactosidase produced by MC1061 harbouring pEClac2 compared to an induced, lacZ⁺ E. coli strain could be due to several factors. Firstly, the penicillinase- β -galactosidase fusion protein may be synthesised at only a low level. Brammar *et al.* (1980) found that E. coli produced a 600-fold lower level of penicillinase than the parental B. licheniformis 749/C strain from which the penP gene was isolated. It was suggested by these same workers that poor translation of the penP mRNA could be responsible for low level synthesis of the penicillinase protein since the penP gene is efficiently transcribed *in vitro* by the E. coli RNA polymerase (M. Sarvas, cited in Brammar *et al.*, 1980). Secondly, the pEClac2 penicillinase- β -galactosidase fusion protein may have a low specific activity compared to the wild type β -galactosidase protein. This phenomenon has been reported by other workers (e.g. Hall *et al.*, 1982). Thirdly, the above fusion protein may be more unstable than the wild type β -galactosidase protein and hence may be rapidly degraded. Instability of such proteins has previously been described (Muller-Hill and Kania, 1974; Bassford *et al.*, 1979).

A general phenomenon was observed with all the E. coli clones harbouring a penP-lacZ fusion. Incubation at ambient temperature for up to 7 days resulted in the appearance of white outgrowths from the blue colonies. These outgrowths were not further investigated but their occurrence suggested that production of a penicillinase- β -galactosidase fusion protein may be toxic to cells producing the protein. Hence there may be a strong selection pressure for non-producing cells. The white outgrowths are probably variants producing little or no fusion protein. Similar findings have previously been reported by other workers (e.g. Bassford *et al.*, 1979).

No further investigations of these β -galactosidase-producing strains was done since the desired information had been obtained, i.e. a penP-lacZ fusion of the type described above produced a protein with β -galactosidase activity.

5.2.2 Construction and Characterisation of pTAH1

Since the penP-lacZ fusion of pEClac2 was shown to produce a protein with β -galactosidase activity in E. coli, a similar fusion was constructed in B. subtilis. Therefore a vector for insertion of the lacZ gene into the BglII site within the penP gene for use in B. subtilis, was made. The EcoRI site of pAB224 (Bingham et al., 1980) was shown to be capable of accommodating foreign DNA without inactivating essential plasmid functions (see Chapter IV). Therefore this vector was chosen to support replication, in B. subtilis, of the penP gene. Plasmid pUB1660 (Figure 5.5) was used as a source of the penP gene since it can conveniently be removed on a 2.8 Md EcoRI fragment.

One μg of EcoRI-digested pAB224 was ligated to 1 μg of EcoRI-digested pUB1660 in a final reaction volume of 20 μl . This ligation-mix then was used to transform competent B. subtilis BD393 cells. Transformants were spread onto TSBA containing either 25 μg per ml of Tc or 10 μg per ml of Ap and incubated for up to 2 days at 37°C.

pAB224 produced 8.5×10^4 Tc^r and 0 Ap^r transformants whereas pUB1660 did not produce any transformants; the ligation-mix gave 2.0×10^4 Tc^r and 0 Ap^r transformants. After toothpicking onto TSBA containing 50 μg per ml of Ap, 5% of the Tc^r ligation-mix transformants were found to be Ap^r. One of these Tc^r, Ap^r clones was chosen for further investigation and after growth in TYS broth containing 25 μg per ml of Tc and 50 μg per ml of Ap, the plasmid pTAH1 was subsequently isolated.

Transformation of competent B. subtilis BD393 cells with pTAH1 produced 1.9×10^5 Tc^r but 0 Ap^r transformants per μg of DNA. However, all the Tc^r transformants were found to be also Ap^r after replica-plating onto TSBA containing 50 μg per ml of Ap. Therefore pTAH1 must contain the penP gene from pUB1660. The reason why direct selection for Ap^r transformants was unsuccessful is unknown but similar findings with the penP gene have been reported (Gray and Chang, 1981). Induction of the penP gene is not required

since the penicillinase protein is synthesised constitutively (Dubnau and Pollock, 1965; Brammar *et al.*, 1980).

A restriction endonuclease cleavage map of pTAH1 was constructed to confirm the presence of the penP gene. Restriction endonuclease digestions are shown in Table 5.5 and Figure 5.9 and restriction endonuclease cleavage maps are shown in Figure 5.10. The hybrid pTAH1 has the expected structure from inserting the 2.8 Md penP-containing fragment of pUB1660 into the single EcoRI site of pAB224 except that pTAH1 contains an additional 0.3 Md EcoRI fragment of unknown origin. This suggests that pTAH1 may have arisen either by deletions from a larger hybrid or insertion of a 0.3 Md fragment into the expected hybrid. The restriction endonuclease cleavage map does show that the required penP gene is present in pTAH1 and that the BglII site within this gene is present. Since these were the requirements of the hybrid, no further characterisation was done.

5.2.3 Construction and Characterisation of penP-lacZ Fusions in B. subtilis

The plasmid pTAH1 can be used to construct a penP-lacZ fusion in B. subtilis in a similar manner to those constructed in E. coli (section 5.2.1). To accomplish this, about 1 µg of the BamHI, lacZ-containing fragment of pMC1871, isolated as previously described (section 5.2.1) was ligated to 1 µg of BglII-digested pTAH1 in a final reaction volume of 20 µl. This ligation-mix then was used to transform competent B. subtilis IG20 cells. Transformants were spread onto TSBA containing BCIG solution and 25 µg per ml of Tc, incubated overnight at 37°C and a further 1 day at ambient temperature.

Whereas pAB224 and pTAH1 produced 7.2×10^2 and 3.5×10^3 Tc^r, lacZ⁻ (white colonies) transformants per µg of DNA respectively, 2 µg of the ligation-mix DNA produced 1.5×10^4 Tc^r transformants, 4% of which were lacZ⁺ (blue colonies). Ten of these Tc^r, lacZ⁺ ligation-mix transformants were streaked onto TSBA containing either 25 µg per ml of Tc and BCIG solution or 10 µg per

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>Pst</u> I	5.40
<u>Bgl</u> II	5.30
<u>Hind</u> III	5.40
<u>Bst</u> EII	5.00
<u>Eco</u> RI	2.85, 2.00, 0.30
<u>Pst</u> I- <u>Bgl</u> II	5.25, 0.15
<u>Pst</u> I- <u>Hind</u> III	4.20, 1.50
<u>Pst</u> I- <u>Bst</u> EII	3.45, 1.55
<u>Pst</u> I- <u>Eco</u> RI	2.15, 1.80, 1.20, 0.30
<u>Bgl</u> II- <u>Hind</u> III	4.40, 1.30
<u>Bgl</u> II- <u>Bst</u> EII	3.35, 1.80
<u>Bgl</u> II- <u>Eco</u> RI	2.05, 1.60, 1.35, 0.30
<u>Hind</u> III- <u>Bst</u> EII	2.90, 2.20
<u>Hind</u> III- <u>Eco</u> RI	2.65, 2.10, 0.25, 0.20*
<u>Bst</u> EII- <u>Eco</u> RI	2.95, 1.90, 0.15, 0.05*

TABLE 3.3: DNA fragment sizes of pTAH1 obtained after restriction endonuclease digestions

* These fragments were not seen on agarose gels and are estimated sizes.



Figure 5.9: Photograph showing restriction endonuclease-digested pTAH1 plasmid DNA

Lanes A and F HindIII-digested λ DNA
 Lane B HindIII-digested pTAH1 DNA (5.40Md)
 Lane C EcoRI-digested pTAH1 DNA (2.85, 2.00, 0.30Md)
 Lane D BstEII-digested pTAH1 DNA (5.30Md)
 Lane E PstI-digested pTAH1 DNA (5.0Md)
 The 0.3Md EcoRI fragment (Lane C) cannot be seen on this gel



Figure 5.9: Photograph showing restriction endonuclease-digested pTAH1 plasmid DNA

Lanes A and F HindIII-digested λ DNA
 Lane B HindIII-digested nTAH1 DNA (5.40nd)
 Lane C EcoRI-digested nTAH1 DNA (2.85, 2.00, 0.30nd)
 Lane D BstEII-digested nTAH1 DNA (5.30nd)
 Lane E PstI-digested pTAH1 DNA (5.01nd)
 The 0.3nd EcoRI fragment (Lane C) cannot be seen on this gel

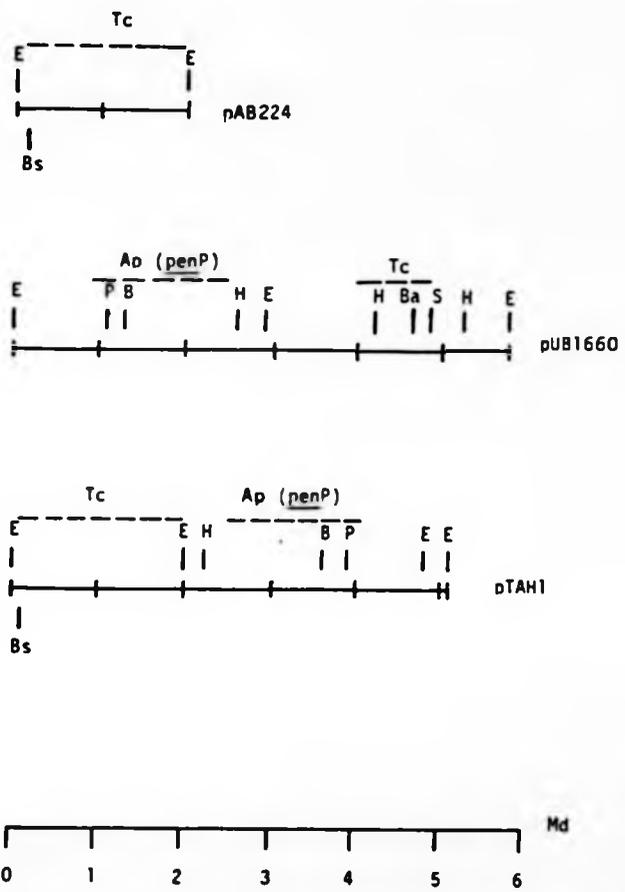


Figure 5.10: Restriction endonuclease cleavage maps of pTAH1 and the parental plasmids used in its construction

ml of Ap. After overnight incubation at 37°C all the clones were found to be Tc^r, Ap^s, lacZ⁺. This suggested that the lacZ gene had in fact been inserted into, and inactivated the penP gene of pTAH1.

A general phenomenon observed with the transformants was that many rapidly growing white outgrowths appeared after incubation at ambient temperature for up to 7 days. Blue areas of each clone were restreaked but white outgrowth always appeared. These outgrowths presumably produced little or no β-galactosidase activity and were probably spontaneous variants of the β-galactosidase-producing clones. Another phenomenon observed was that upon restreaking, the intensity of the blue colour of the colonies decreased. Thus it appeared that the clones were gradually producing lower levels of β-galactosidase activity; the reasons for this are unknown. However, colonies which produced β-galactosidase activity grew much more slowly than did non-producing colonies. If the penicillinase-β-galactosidase fusion protein was toxic to the cells, then producing cells may be at a growth disadvantage. Thus there may be a selection pressure to decrease or halt synthesis of the fusion protein.

After restreaking the B. subtilis IG20 Tc^r, lacZ⁺ clones, several times, four of the clones stopped producing β-galactosidase activity (white colonies) whilst the remaining six clones produced only weak activity (pale blue colonies). Plasmid DNA was isolated from 2 of these Tc^r, lacZ⁺ clones (pTAH lacZ5 and pTAH lacZ10) after growth in TYS broth containing 25 μg per ml of Tc. Figure 5.11 shows the predicted structure of the hybrids.

Both plasmid preparations contained several species of plasmid DNA, some of which were smaller than the 7.3 Md expected hybrid. B. subtilis IG20 cells were transformed with 0.1 μg of each plasmid preparation in an attempt to separate the different plasmid species. Transformants were spread onto TSBA containing BCIG solution and 25 μg per ml of Tc, incubated overnight at 37°C and a further 1 day at ambient temperature. pTAHlacZ5 produced 2.9 x 10⁴ Tc^r transformants 4% of which were lacZ⁺, whilst pTAHlacZ10 produced 8.8 x 10⁴

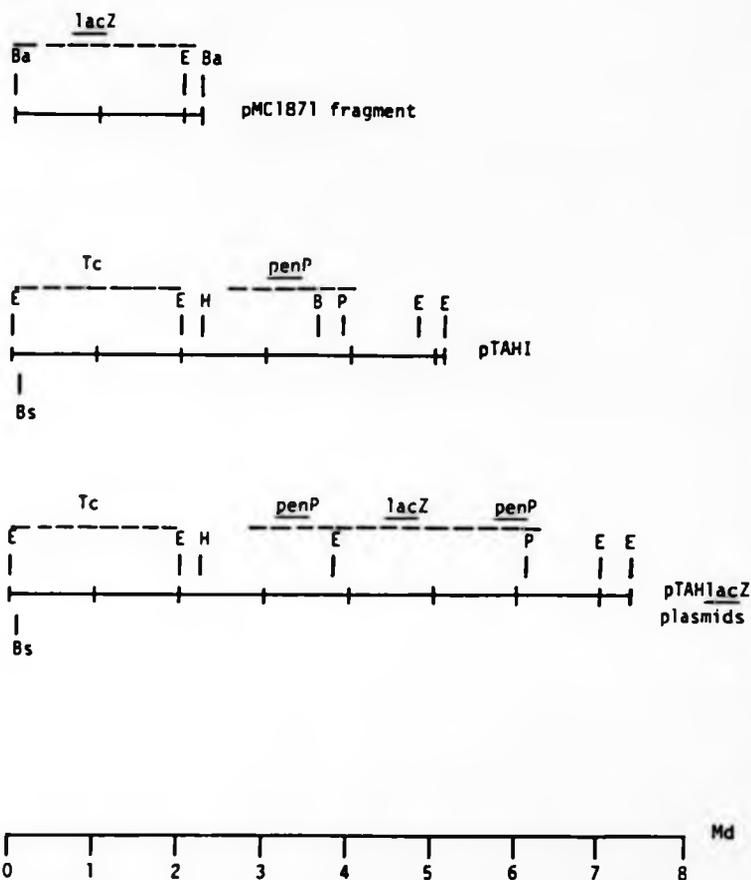


Figure 5.11: Restriction endonuclease cleavage maps of the predicted structure of the pTAHlacZ plasmids and the parental plasmids used in their construction

Tc^r transformants, 35% of which were lacZ⁺. The lacZ⁺ colonies were an intense blue colour, much more intense than the original colonies from which the plasmids were isolated. This suggested that the reduction in β -galactosidase activity upon restreaking of the parental clones was not the result of a plasmid mutation giving a lower level of synthesis of the penicillinase- β -galactosidase fusion protein. The Tc^r, lacZ⁻ pTAHlacZ5 and pTAHlacZ10 transformants probably resulted from transformation with a plasmid species lacking a functional penP-lacZ fusion.

Two each of pTAHlacZ5 and pTAHlacZ10 Tc^r, lacZ⁺ transformants were streaked onto TSBA containing 25 μ g per ml of Tc and BCIG solution, incubated overnight at 37°C and a further 1 day at ambient temperature. White outgrowths appeared in 3 out of 4 cases. The IG20-pTAHlacZ10 clone which did not produce white outgrowths was used as a source of plasmid DNA. This clone may contain only a single species of plasmid DNA.

The plasmid pTAHlacZ101 was isolated and used to transform competent *B. subtilis* IG20 cells. Whereas transformation with 1 μ g of pTAH1 gave 2.4×10^4 Tc^r transformants, transformation with 0.5 μ g of pTAHlacZ101 produced 1.8×10^6 Tc^r transformants of which greater than 90% produced β -galactosidase activity. However, the presence of some Tc^r, lacZ⁻ pTAHlacZ101 transformants again suggested that more than one plasmid species was present in this preparation. Analysis of pTAHlacZ101 on agarose gels confirmed that a homogeneous plasmid preparation had not been obtained; several species of plasmid DNA were observed (Figure 5.12).

Some of the lacZ⁺ pTAHlacZ101 transformants produced white outgrowths which again suggested instability of β -galactosidase activity production. No further attempts were made to produce a homogeneous plasmid preparation since it was thought likely that deletion derivatives of the lacZ-containing plasmids were produced spontaneously during vegetative growth of the cells.



Figure 5.12: Photograph showing the different plasmid species present in a pTAH1acZ101 preparation

Lane A undigested nHC1396 (6.6Md)
Lane B undigested nAB224 DNA (2.0Md)
Lane C undigested pUB1660 DNA (5.8Md)
Lane D undigested pTAH1acZ101 DNA



Figure 5.12: Photograph showing the different plasmid species present in a pTAHlacZ101 preparation

Lane A undigested nMC1396 (6.6Md)
Lane B undigested nAB224 DNA (2.0Md)
Lane C undigested pUB1660 DNA (5.8Md)
Lane D undigested pTAHlacZ101 DNA

β -galactosidase assays were done on B. subtilis cells harbouring pTAHlacZ101 to determine whether β -galactosidase activity was secreted into the culture medium (Table 5.6). The assays revealed that 50% of the total β -galactosidase activity detected was in the supernatant fluid. The remaining 50% may have been cell-bound since the same level of activity was detected irrespective of whether cells were lysed prior to being assayed.

The total level of β -galactosidase activity detected was about 3-fold lower than the level detected in E. coli cultures. However, a direct comparison of activity cannot be made since cells were grown under different culture conditions. The higher level detected in E. coli may have been due to a gene dosage effect. The copy number of the E. coli plasmid pEClac3 may be higher than that of the B. subtilis plasmid pTAHlacZ101, the plasmid replicons pACYC184 and pAB124 respectively have copy numbers of 20 (Chang and Cohen, 1978) and 5 to 7 (Bingham, 1980) respectively. Also, the relative rates of transcription and translation of the penP-lacZ hybrids may have been different in E. coli and B. subtilis and in addition the stability of the hybrid protein may be different in both these hosts.

To determine at which part of the growth cycle maximum β -galactosidase activity was produced, attempts were made to follow β -galactosidase activity production throughout the growth cycle. B. subtilis IG20 harbouring pTAHlacZ101 was inoculated into 100 ml of TYS broth containing 25 μ g per ml of Tc in a 1500 ml ribbed Erlenmeyer flask. Incubation was carried out at 37°C with vigorous aeration. The inoculum consisted of either a single colony from a fresh transformation or a 1 ml inoculum from a fresh 10 ml culture in a 1 oz bottle. Many attempts were made to follow β -galactosidase activity production but very little or no (less than 0.1 unit per minute per bacterium) β -galactosidase activity was detected. The reasons for this failure are unknown and the results were unexpected. No further attempts were made to follow production of β -galactosidase activity with strains containing pTAHlacZ101.

Strain	Sample	β -galactosidase activity	
		units per bacterium per minute	units per ml per minute
IG20	Intact cells	0.0	0.0
	Lysed cells	0.0	0.0
	Supernatant fluid	0.0	0.0
IG20-pTAHlacZ101	Intact cells	2.9	2.9
	Lysed cells	3.0	3.0
	Supernatant fluid	2.6	2.6

TABLE 5.6: β -galactosidase activity of *B. subtilis* cultures

β -galactosidase assays were done as detailed in section 2.2.19. Cells were grown in 5ml of TYS broth in a loz bottle and incubated overnight at 37°C with vigorous aeration.

Cultures of *B. subtilis* IG20 harbouring pTAHlacZ101 were grown under different conditions to determine the effect on β -galactosidase activity production. Also, superoxide dismutase (SOD) assays were done to measure the degree of cell lysis. SOD is a particularly suitable enzyme since it is extremely stable, unaffected by oxygen, does not require cofactors, reducing agents nor metal ions, and is relatively resistant to proteolysis, extremes of pH and detergents and organic solvents. The enzyme has a high specific activity; at 30,000 units per mg of protein it is possible to detect as little as 2 ng of enzyme in a 250 μ l sample (A. Atkinson, personal communication). Table 5.7 shows the results of the β -galactosidase and SOD assays.

The levels of β -galactosidase activity of the different cultures cannot be directly compared since each culture was probably at a different point in the growth cycle and the production of β -galactosidase activity may be growth cycle dependent. However, the results do show that the level of β -galactosidase activity produced was extremely variable as was the relative amounts of the enzyme found in the cell fraction and the supernatant fluid fraction. This latter level varied between less than 0.1% to 100% of the total activity. Although no significant conclusions can be drawn concerning the effect of growth temperature and culture conditions upon β -galactosidase activity production, it appeared that growth in TYS broth yielded more β -galactosidase activity than growth in SMS broth. The results of the SOD assays showed that β -galactosidase activity detected in the supernatant fluid did not result from the release of enzyme due to cell lysis. This suggested that β -galactosidase activity was actually secreted out of the cells into the culture medium. No further enzyme assays were done with *B. subtilis* IG20 harbouring pTAHlacZ101.

Since the pTAHlacZ5, pTAHlacZ10, and pTAHlacZ101 plasmid preparations each contained greater than one species of plasmid DNA, the stability of pTAHlacZ101 and pTAH1 was investigated to determine whether plasmid

Culture conditions	OD _{600nm}	β-galactosidase activity (units per bacterium per minute)		β-galactosidase activity (units per ml of culture per minute)		SOD activity		
		CF	SF	CF	SF	CF	SF	
<u>SMS broth</u>								
37°C 1	1.18	0.8	0.5	0.9	0.6	2.1	<0.4	
2	0.78	0.8	0.5	0.6	0.4	1.4	<0.4	
3	2.81	0.3	0.3	0.8	0.7	5.8	0.4	
37°C 1	1.45	2.6	0.0	3.8	0.0	2.9	<0.4	
2	0.11	2.7	1.4	0.3	0.2	ND	ND	
3	0.39	2.8	0.5	1.1	0.2	0.6	<0.4	
<u>TYS broth</u>								
37°C 1	0.82	3.3	3.8	2.7	3.1	1.7	<0.4	
2	0.62	3.1	4.4	1.9	2.7	1.3	0.4	
3	5.55	0.0	0.8	0.0	4.4	10.6	1.0	
30°C 1	1.49	7.4	4.0	11.0	5.0	3.2	<0.4	
2	0.43	5.0	0.0	2.2	0.0	0.9	<0.4	
3	5.57	4.7	1.3	26.2	7.2	12.1	1.2	

TABLE 5.7: Enzyme activities of *B. subtilis* IG20-pTAHlacZ101 cultures

1. 5ml of broth in a loz bottle, with aeration
2. 10ml of broth in a 100ml Erlenmeyer flask, no aeration
3. 40ml of broth in a 250ml Erlenmeyer flask, with aeration

CF = Cell fraction

SF = Supernatant fraction

ND = Not detectable

< 0.4 represents the limit of sensitivity of the assay

fragmentation and/or plasmid loss occurred during vegetative growth of the cells. Table 5.8 shows the results of the analyses.

If selection pressure was made for either Tc- or Ap-resistance then cells harbouring pTAH1 did not lose the non-selected plasmid-borne marker. However, if no selection pressure was applied, 2% of the cells were found to have lost both plasmid-borne markers suggesting complete plasmid loss.

Cells harbouring pTAHlacZ101 lost plasmid-borne markers irrespective of the selection pressure applied. If selection pressure for Tc-resistance was applied then 67% of the cells became lacZ⁻ whilst the remaining 33% remained lacZ⁺. Conversely, if no selection pressure was applied, 97% of the cells became Tc^s, lacZ⁻ whilst the remaining 3% retained both plasmid-borne markers, i.e. were Tc^r, lacZ⁺.

These results suggested that in the case of pTAHlacZ101 and to a much lesser degree with pTAH1, in the absence of Tc selection, complete plasmid loss may have occurred. Also, if Tc selection was applied fragmentation of pTAHlacZ101 may have occurred. Thus in contrast to pTAH1, pTAHlacZ101 is extremely unstable.

The reasons for this instability of pTAHlacZ101 are unknown. However, since cells producing β -galactosidase activity grew more slowly than those not producing the enzyme, there may be a strong selection pressure for those cells not producing β -galactosidase activity. As stated previously, the toxic effects of secreted fusion proteins containing the lacZ gene in *E. coli* have been reported (e.g. Silhavy *et al.*, 1978; Schwartz *et al.*, 1982). Because of the extreme instability of the penP-lacZ containing plasmids described in this chapter, no further characterisation of the plasmids was attempted.

Plasmid	Selection pressure	Phenotype (%)				
		Tc ^r , Ap ^r	Tc ^s , Ap ^s	Tc ^r , lacZ ⁺	Tc ^r , lacZ ⁻	Tc ^s , lacZ ⁻
pTAH1	Tc	100	0	-	-	-
	Ap	100	0	-	-	-
	None	98	2	-	-	-
pTAH-lacZ101	Tc	-	-	33	67	0
	None	-	-	3	0	97

TABLE 5.8: Stability analysis of *B. subtilis* IG20 pTAH1 and pTAHlacZ101

5.2.4 Construction and Characterisation of an *E. coli* - *B. subtilis* Bifunctional Plasmid Containing a penP-lacZ Fusion

Analysis of penP-lacZ-containing plasmids constructed in *B. subtilis* proved difficult due to the presence of multiple species of plasmid DNA. Therefore another similar fusion was constructed in *E. coli* but using a bifunctional plasmid vector. Plasmid DNA isolated from *E. coli* could then be characterised prior to its introduction into *B. subtilis*. This was accomplished by inserting the plasmid pMC1396 into pTAH1 as detailed below. However, initially a restriction endonuclease cleavage map of pMC1396 was constructed (Table 5.9 and Figure 5.4).

The penP-lacZ fusion was constructed as follows. One μg of pMC1396 was digested with BamHI and ligated to 1 μg of BglII-digested pTAH1 in a final reaction volume of 20 μl ; 10 μl of this ligation-mix was used to transform competent *E. coli* MC1061 cells. Transformants were spread onto L-agar containing BCIG solution and 25 μg per ml of Ap, incubated overnight at 37°C and a further 1 day at ambient temperature. Whereas transformation with 1 μg of pMC1396 produced 1.3×10^6 Ap^r transformants which were all lacZ⁻ (white colonies), transformation with 1 μg of the ligation-mix produced 1.1×10^5 Ap^r transformants 4% of which were lacZ⁺ (blue colonies). Three Ap^r, lacZ⁺ ligation-mix transformants were checked for resistance to 25 μg per ml of Tc. Clones harbouring an intact pMC1396 hybrid should be Tc^r since the Tc-resistance genes of pAB224 are phenotypically expressed in *E. coli* (Eccles *et al.*, 1981). Two of the clones tested were Tc^r whereas the third was Tc^s. The plasmids pEBlac1, pEBlac2 and pEBlac3 were isolated from these clones and used to transform competent *E. coli* MC1061 and *B. subtilis* IG20 cells (Table 5.10).

Firstly, pEBlac2, isolated from the Ap^r, Tc^s, lacZ⁺ clone, produced Ap^r, Tc^s, lacZ⁺ *E. coli* transformants but no *B. subtilis* transformants. Therefore this plasmid may have arisen by the deletion or inactivation of pAB224 sequences

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>EcoRI</u>	4.40, 2.05
<u>BstEII</u>	6.50, 0.38
<u>PstI</u>	6.60
<u>Sall</u>	6.60
<u>BamHI</u>	6.60
<u>HindIII</u>	-
<u>EcoRI-BstEII</u>	2.50, 2.05, 1.80, 0.30
<u>EcoRI-PstI</u>	3.85, 2.05, 0.50
<u>EcoRI-Sall</u>	2.60, 2.10, 2.00
<u>EcoRI-BamHI</u>	4.45, 2.05, <0.05*
<u>BstEII-PstI</u>	4.20, 2.00, 0.30
<u>BstEII-Sall</u>	6.50, 0.30, <0.05*
<u>BstEII-BamHI</u>	3.75, 2.50, 0.30
<u>PstI-Sall</u>	4.45, 2.05
<u>PstI-BamHI</u>	5.90, 0.45
<u>Sall-BamHI</u>	4.10, 2.60

TABLE 3.9: DNA fragment sizes of pMC1396 obtained after restriction endonuclease digestions

* These fragments were not seen on agarose gels and are estimated sizes

Transforming DNA	Selection	<u>E. coli</u>		<u>B. subtilis</u>	
		Transformants	% <u>lacZ</u> ⁺	Transformants	% <u>lacZ</u> ⁺
pEB <u>lac</u> 1	Tc	5.2 x 10 ²	100	8.0 x 10 ⁴	0
	Ap	4.6 x 10 ⁴	100	0.0	0
pEB <u>lac</u> 2	Tc	0.0	-	0.0	-
	Ap	7.8 x 10 ⁴	100	0.0	-
pEB <u>lac</u> 3	Tc	2.6 x 10 ³	100	6.4 x 10 ⁴	100
	Ap	1.6 x 10 ⁵	100	0.0	-

TABLE 3.10: Transformation of E. coli MC1061 and B. subtilis IG20 competent cells with pEBlac1, pEBlac2 and pEBlac3

E. coli and B. subtilis transformants were spread onto L-agar and TSBA respectively. Media contained BCIG solution and either 25µg per ml of Ac or Ap. Plates were incubated overnight at 37°C and then for a further 1 day at ambient temperature.

from a larger hybrid. No further work with this plasmid was done since it could not transform *B. subtilis* to Tc-resistance.

Secondly, pEBlac1, isolated from an Ap^r, Tc^r, lacZ⁺ clone, produced Tc^r, Ap^r, lacZ⁺ *E. coli* transformants but only Tc^r, Ap^s, lacZ⁻ *B. subtilis* transformants. A possible explanation for this was obtained from analysis of a restriction endonuclease cleavage map of pEBlac1 (Table 5.11 and Figures 5.13 and 5.14). It appears that pEBlac1 resulted from the insertion of pMC1396 into the BglIII site of pTAH1 but in the opposite orientation required to place the lacZ gene under control of the penP regulatory signals. Since insertion of pMC1396 in this orientation produced a plasmid which was lacZ⁺ in *E. coli*, the lacZ gene must be transcribed and translated utilising regulatory signals other than those of the penP gene. This is also supported by the fact that this plasmid is lacZ⁻ in *B. subtilis*. The regulatory signals utilised by *E. coli* for β-galactosidase synthesis cannot have been recognised or utilised by *B. subtilis*. Non-utilisation of *E. coli* genetic regulatory signals by *B. subtilis* is a well documented phenomenon (e.g. Ehrlich, 1978a). Since pEBlac1 was lacZ⁻ in *B. subtilis* no further characterisation of this plasmid was done.

Thirdly, pEBlac3, also isolated from an Ap^r, Tc^r, lacZ⁺ clone, produced Tc^r, Ap^r, lacZ⁺ *E. coli* transformants and Tc^r, Ap^s, lacZ⁺ *B. subtilis* transformants. This plasmid has the expected properties of a pMC1396-pTAH1 hybrid. The plasmid was further characterised by restriction endonuclease cleavage analysis (Table 5.12 and Figures 5.15 and 5.16) the results of which confirmed that pEBlac3 has the predicted structure of the pTAH1-pMC1396 hybrid.

β-galactosidase assays were done on cultures of *B. subtilis* IG20 harbouring pEBlac3 using similar conditions to those detailed earlier for *B. subtilis* IG20 cells harbouring pTAHlacZ101 (Section 5.2.3). The results obtained (Table 5.13) were similar to those reported previously (Section 5.2.3) for *B. subtilis* IG20 harbouring pTAH lacZ101 and similar conclusions can be drawn. Although no

from a larger hybrid. No further work with this plasmid was done since it could not transform *B. subtilis* to Tc-resistance.

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Thirdly, pEBlac3, also isolated from an Ap^r, Tc^r, lacZ⁺ clone, produced Tc^r, Ap^r, lacZ⁺ *E. coli* transformants and Tc^r, Ap^s, lacZ⁺ *B. subtilis* transformants. This plasmid has the expected properties of a pMC1396-pTAH1 hybrid. The plasmid was further characterised by restriction endonuclease cleavage analysis (Table 5.12 and Figures 5.15 and 5.16) the results of which confirmed that pEBlac3 has the predicted structure of the pTAH1-pMC1396 hybrid.

β -galactosidase assays were done on cultures of *B. subtilis* IG20 harbouring pEBlac3 using similar conditions to those detailed earlier for *B. subtilis* IG20 cells harbouring pTAHlacZ101 (Section 5.2.3). The results obtained (Table 5.13) were similar to those reported previously (Section 5.2.3) for *B. subtilis* IG20 harbouring pTAH lacZ101 and similar conclusions can be drawn. Although no

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>Pst</u> I	10.00, 0.60
<u>Hind</u> III	12.00
<u>Eco</u> RI	4.30, 3.40, 2.05, 1.35, 0.30
<u>Sal</u> I	12.00
<u>Bst</u> EII	6.70, 4.20, 0.30
<u>Pst</u> I- <u>Hind</u> III	7.35, 3.75, 0.65
<u>Pst</u> I- <u>Eco</u> RI	3.85, 3.35, 2.05, 1.10, 0.40, 0.30, 0.20
<u>Pst</u> I- <u>Sal</u> I	9.30, 2.05, 0.60
<u>Pst</u> I- <u>Bst</u> EII	7.50, 1.95, 1.65, 0.60, 0.30
<u>Hind</u> III- <u>Eco</u> RI	4.40, 3.20, 2.10, 1.35, 0.35, 0.30
<u>Hind</u> III- <u>Sal</u> I	7.00, 5.15
<u>Hind</u> III- <u>Bst</u> EII	5.20, 4.20, 2.30, 0.35
<u>Eco</u> RI- <u>Sal</u> I	3.50, 2.50, 2.10, 2.10, 1.37, 0.30
<u>Eco</u> RI- <u>Bst</u> EII	3.45, 2.40, 1.90, 1.75, 1.30*, 0.30*
<u>Sal</u> I- <u>Bst</u> EII	7.40, 3.80, 0.30, <0.10**

TABLE 5.11: DNA fragment sizes of pEBlacI obtained after restriction endonuclease digestions

* Represents two fragments of the same size

** This fragment was not seen on agarose gels and is an estimated size

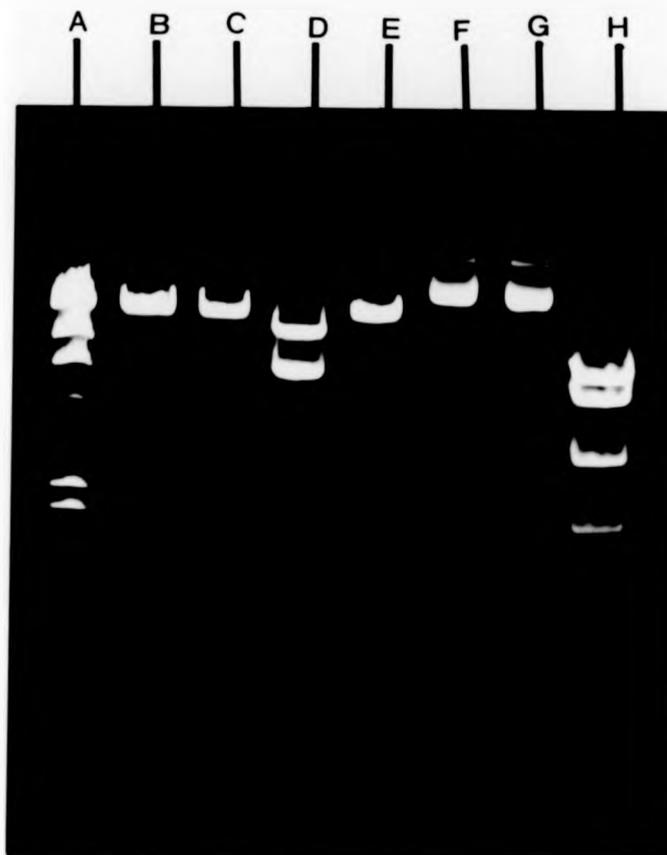


Figure 5.13: Photograph showing restriction endonuclease-digested nEBlacI DNA after agarose gel electrophoresis

- Lane A HindIII-digested λ DNA
- Lane B PstI-digested nEBlacI (10.00, 0.60Md)
- Lane C SalI-digested nEBlacI (12.00Md)
- Lane D BstEII-digested nEBlacI (6.70, 4.20, 0.30Md)
- Lane E HindIII-digested nEBlacI (12.00Md)
- Lane F BamHI-digested pEBlacI (undigested)
- Lane G BglII-digested nEBlacI (undigested)
- Lane H EcoRI-digested nEBlacI (4.30, 3.40, 2.35, 1.35, 0.30Md)

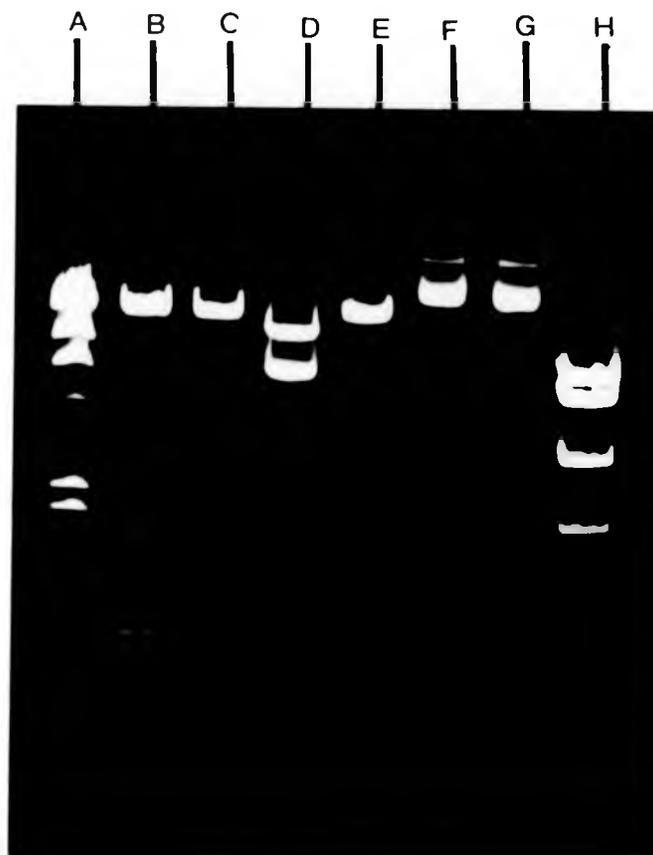


Figure 5.13: Photograph showing restriction endonuclease-digested pEB1ac1 DNA after agarose gel electrophoresis

- Lane A HindIII-digested pEB1ac1 DNA
- Lane B PstI-digested pEB1ac1 (10.00, 0.60Md)
- Lane C SalI-digested pEB1ac1 (12.00Md)
- Lane D BstEII-digested pEB1ac1 (6.70, 4.20, 0.30Md)
- Lane E HindIII-digested pEB1ac1 (12.00Md)
- Lane F BamHI-digested pEB1ac1 (undigested)
- Lane G BnlII-digested pEB1ac1 (undigested)
- Lane H EcoRI-digested pEB1ac1 (4.30, 3.40, 2.05, 1.35, 0.30Md)

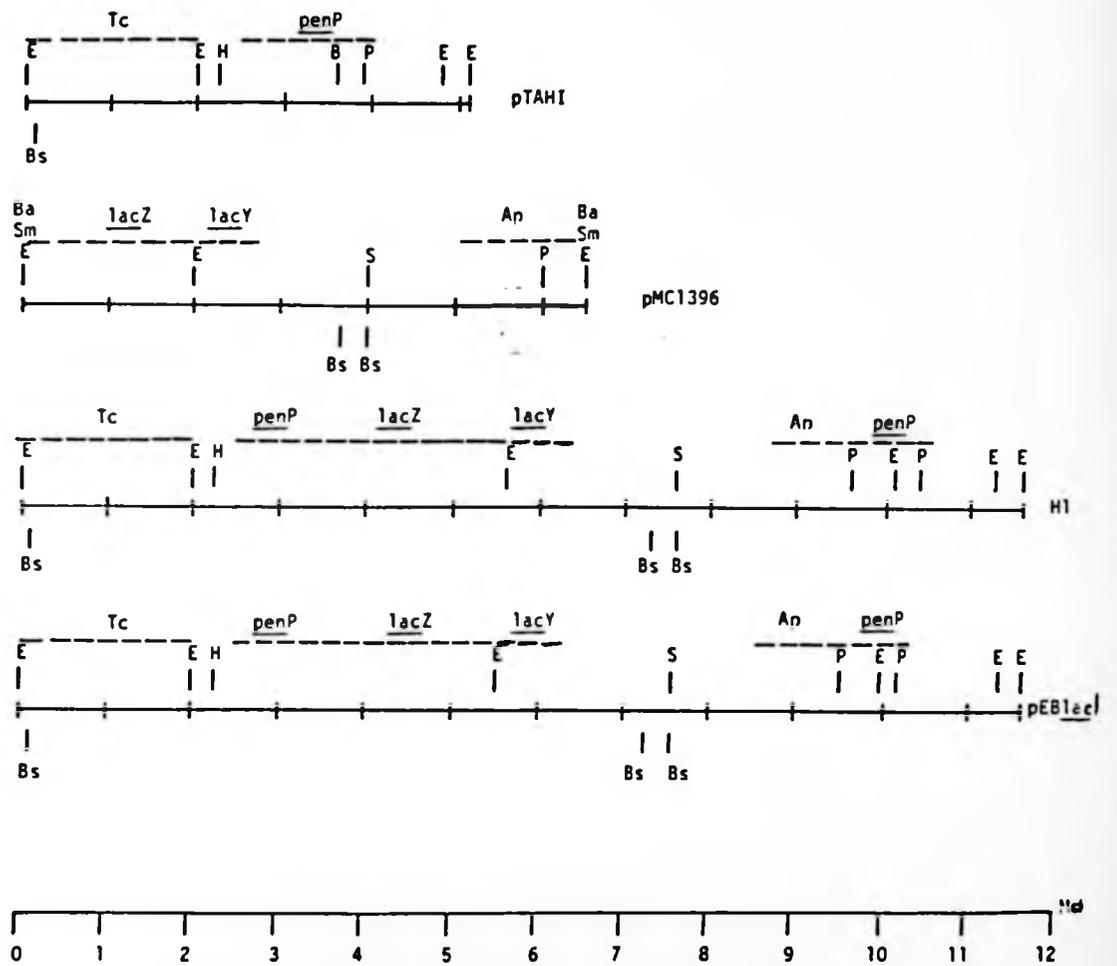


Figure 5.14: Restriction endonuclease cleavage maps of pEBlacI and the parental plasmids used in its construction. H1 is the structure of the predicted hybrid

Restriction endonuclease digestions	Plasmid DNA fragment sizes (Md)
<u>Pst</u> I	6.50, 570
<u>Hind</u> III	11.80
<u>Eco</u> RI	4.70, 3.20, 2.00, 1.60, 0.30
<u>Sal</u> I	12.00
<u>Bst</u> EII	5.85, 5.50, 0.30
<u>Pst</u> I- <u>Hind</u> III	6.00, 3.60, 2.00
<u>Pst</u> I- <u>Eco</u> RI	4.05, 2.17, 2.00, 1.60, 1.03, 0.45, 0.25
<u>Pst</u> I- <u>Sal</u> I	5.60, 3.55, 1.95
<u>Pst</u> I- <u>Bst</u> EII	3.70*, 1.85, 1.40, 0.25
<u>Hind</u> III- <u>Eco</u> RI	4.70, 3.30, 2.03, 1.37, 0.35, 0.29
<u>Hind</u> III- <u>Sal</u> I	8.20, 3.80
<u>Hind</u> III- <u>Bst</u> EII	5.50, 3.70, 2.22, 0.23
<u>Eco</u> RI- <u>Sal</u> I	3.35, 2.55, 2.15, 2.05, 1.67, 0.31
<u>Eco</u> RI- <u>Bst</u> EII	3.17, 2.55, 2.07, 1.90, 1.70, 0.29, 0.20, <0.10**
<u>Sal</u> I- <u>Bst</u> EII	5.90, 5.20, 0.25, <0.10**

TABLE 3.12: DNA fragment sizes of pEBlac3 obtained after restriction endonuclease digestions

* Represents two fragments of the same size

** These fragments were not seen on agarose gels and are estimated sizes

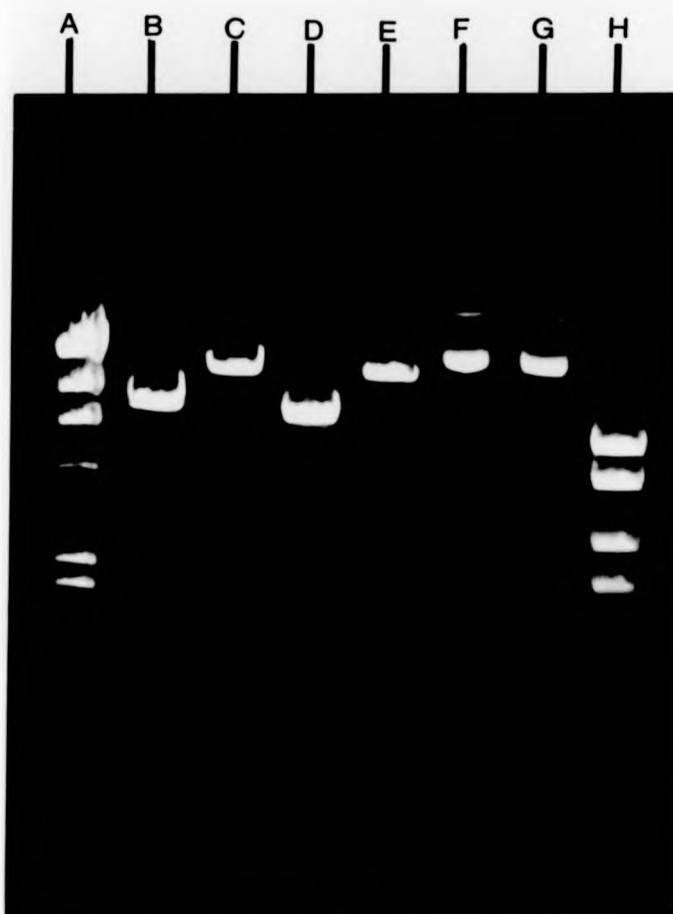


Figure 5.15: Photograph showing restriction endonuclease-digested pEBlac3 DNA after agarose gel electrophoresis

Lane A HindIII-digested λ DNA
 Lane B PstI-digested nEBlac3 (6.50, 5.70Md)
 Lane C SalI-digested pEBlac3 (12.00Md)
 Lane D BstEII-digested pEBlac3 (5.85, 5.50, 0.30Md)
 Lane E HindIII-digested pEBlac3 (11.80Md)
 Lane F BamHI-digested pEBlac3 (undigested)
 Lane G BglII-digested nEBlac3 (undigested)
 Lane H EcoRI-digested nEBlac3 (4.70, 3.20, 2.00, 1.60, 0.30Md)
 The 6.50 and 5.70 PstI fragments (Lane B) and the 5.55 and 5.50 BstEII fragments (Lane D) are not clearly resolved on this gel.



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 Lane F BamHI-digested pEBlac3 (undigested)
 Lane G BnlII-digested pEBlac3 (undigested)
 Lane H EcoRI-digested pEBlac3 (4.70, 3.20, 2.00, 1.60, 0.30Md)
 The 6.50 and 5.70 PstI fragments (Lane B) and the 5.85 and 5.50 BstEII fragments (Lane D) are not clearly resolved on this gel.

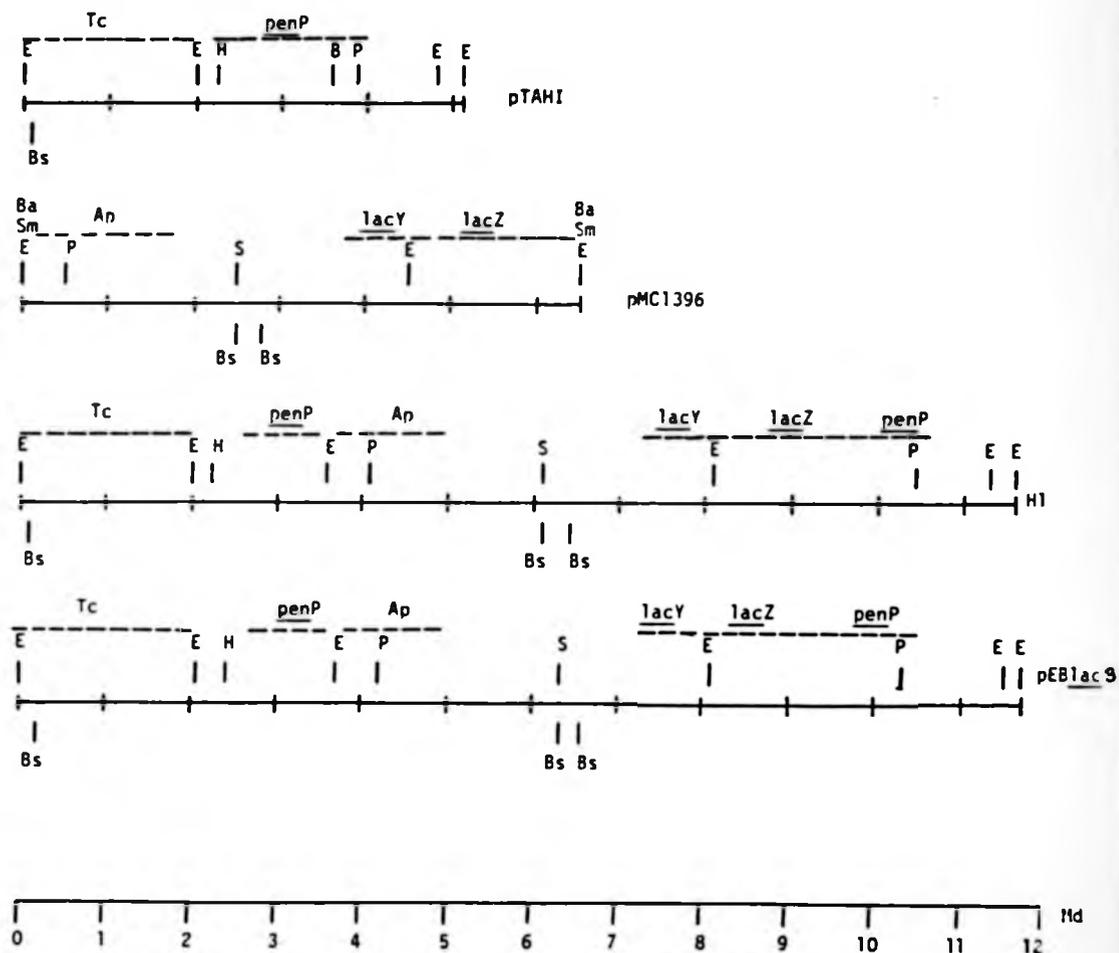


Figure 5.16: Restriction endonuclease cleavage maps of pEB1ac3 and the parental plasmids used in its construction. H1 is the structure of the predicted hybrid

Culture conditions	OD _{600nm}	β-galactosidase activity (units per bacterium per minute)		β-galactosidase activity (units per ml of culture per minute)		SOD activity (units/ml)		
		CF	SF	CF	SF	CF	SF	
<u>SMS broth</u>								
37°C 1	1.46	0.0	0.0	0.0	0.0	2.6	<0.4	
2	1.16	0.0	0.7	0.0	0.8	2.2	<0.4	
3	4.60	0.0	0.0	0.0	0.0	8.9	<0.4	
37°C 1	1.22	0.1	0.6	0.2	0.7	1.9	<0.4	
2	0.13	0.0	5.2	0.0	0.7	ND	ND	
3	1.54	0.0	0.3	0.0	0.4	3.1	<0.4	
<u>TYS broth</u>								
37°C 1	0.74	2.4	4.5	1.8	3.3	1.6	<0.4	
2	0.94	1.0	1.2	0.9	1.1	1.8	<0.4	
3	4.36	0.3	1.5	2.2	6.5	8.2	0.6	
30°C 1	1.34	0.4	1.3	0.5	1.7	2.8	<0.4	
2	0.52	2.5	0.8	1.3	0.4	1.1	<0.4*	
3	7.80	1.0	0.3	7.8	2.3	15.5	0.9	

TABLE 5.13: Enzyme activities of *B. subtilis* IG20-pEB_{lac}3 cultures

- 5ml of broth in a loz bottle, with aeration
- 10ml of broth in a 100ml Erlenmeyer flask, no aeration
- 40ml of broth in a 250ml Erlenmeyer flask, with aeration

CF = Cell fraction

SF = Supernatant fraction

ND = Not detectable

< 0.4 represents the limit of sensitivity of the assay

direct comparison of individual enzyme levels can be made, it does appear that in general growth in TYS broth leads to higher levels of β -galactosidase activity production. Also, when β -galactosidase activity production was detected in the supernatant fluid, it was probably not the result of cell lysis. This was concluded from the SOD assay data which showed that cell lysis did not contribute significantly to β -galactosidase activity detected in the supernatant fluid.

As was observed previously (sections 5.2.1 and 5.2.3) with penP-lacZ fusions, incubation at ambient temperature for several days resulted in the appearance of white outgrowths; these appeared at a much higher frequency in B. subtilis than in E. coli. Also, upon restreaking of the B. subtilis fusion-containing clones on BCIG-containing media the intensity of the blue colour of the clones decreased. Thus although the use of pEBlac3 enabled the plasmid to be characterised prior to its introduction into B. subtilis, the plasmid was unstable when introduced into B. subtilis.

Stability analysis of pEBlac3 revealed that as with pTAHlacZ101, pEBlac3 also was extremely unstable in B. subtilis (Table 5.14). In the absence of Tc selection only 4% of the cells retained the Tc-resistance marker whereas less than 1% retained the lacZ marker. If Tc selection was applied then 21% of the cells retained the lacZ marker.

The instability of pEBlac3 in B. subtilis was investigated further to try and determine whether the gradual decrease in β -galactosidase activity production, upon restreaking, and the complete loss of β -galactosidase activity was due to a host mutation affecting either the plasmid copy number or expression of the lacZ gene or whether it was due to a plasmid mutation.

Firstly, four white outgrowths (B. subtilis strains IG20-pEBlac3w1-4) from a B. subtilis IG20-pEBlac3 (Tc^r , $lacZ^+$) transformant were restreaked onto TSBA containing Tc (12 μ g per ml) and BCIG solution to confirm that no β -galactosidase activity was produced. Then the stability of each Tc^r , $lacZ^-$ plasmid was determined (Table 5.14). This showed that these plasmids were

Plasmid	Selection pressure	Phenotype (%)		
		Tc ^r , <i>lacZ</i> ⁺	Tc ^r , <i>lacZ</i> ⁻	Tc ^s , <i>lacZ</i> ⁻
pEB <i>lac</i> 3	Tc	21	79	0
	None	0	4	96
pEB <i>lac</i> 3w1	None	0	98	2
pEB <i>lac</i> 3w2	None	0	94	6
pEB <i>lac</i> 3w3	None	0	100	0
pEB <i>lac</i> 3w4	None	0	100	0

TABLE 5.14: Stability analysis of *B. subtilis* IG20 harbouring pEB*lac*3 and the *lacZ*⁻ derivatives pEB*lac*3w1-4

extremely stable compared to the parental plasmid, pEB*lac*3. Next, plasmid DNA was isolated from two Tc^r, *lacZ*⁻ *B. subtilis* IG20 clones (pEB*lac*3w1 and IG20 pEB*lac*3w4) and used to transform competent *B. subtilis* IG20 cells. pEB*lac*3w1 and pEB*lac*3w4 gave 7.8×10^2 and 1.3×10^4 Tc^r, *lacZ*⁻ transformants per μg of DNA respectively whereas pEB*lac*3 gave 1.6×10^3 Tc^r, *lacZ*⁺ transformants per μg of DNA. Therefore the plasmids pEB*lac*3w1 and pEB*lac*3w4, isolated from a *lacZ*⁻ derivative of *B. subtilis* IG20-pEB*lac*3, have either an inactivated or deleted *lacZ* gene. Since in contrast to pEB*lac*3 these plasmids are stably maintained in *B. subtilis*, it may be the production of β -galactosidase activity by *B. subtilis* clones harbouring pEB*lac*3 which results in plasmid instability.

To confirm that it was not a host mutation which resulted in the loss of β -galactosidase activity of *B. subtilis* IG20-pEB*lac*3 derivatives, a Tc^s clone was isolated from *B. subtilis* strain IG20-pEB*lac*3w1. This was done by growing a culture of *B. subtilis* IG20-pEB*lac*3w1 in antibiotic-free medium (TYS broth) and screening clones for a Tc^s phenotype. A Tc^s, *lacZ*⁻ strain, *B. subtilis* IG20101, was isolated and transformed with pEB*lac*3. If a host mutation had occurred in this strain then the pEB*lac*3 transformants should not produce β -galactosidase activity. Out of 1.1×10^3 Tc^r transformants obtained, 96% were found to be *lacZ*⁺. Therefore IG20 101 cannot have a chromosomal mutation affecting β -galactosidase activity production. No further analysis of these plasmids was done.

5.3 DISCUSSION

Although there have been many reports of the expression of foreign genes in *B. subtilis* (e.g. Hardy et al., 1981), to date there have been no reports of the secretion by *B. subtilis* cells of a foreign protein fused to a bacterial signal-peptide. The aim of the work detailed in this chapter was to determine whether a penicillinase- β -galactosidase fusion protein of the type described would lead to

the secretion into the culture medium, by B. subtilis cells, of a protein with β -galactosidase activity.

The penicillinase protein of B. licheniformis 749/C is a well characterised enzyme, the DNA sequence of which is known (Neugebauer et al., 1981). Therefore the signal peptide of this protein was an obvious choice to use to promote the secretion of a fusion protein. Secretion of β -galactosidase was attempted for several reasons. The enzyme has been well characterised (Miller and Reznikoff, 1980), a partial DNA sequence is known (Casadaban et al., 1980), and simple assays for the enzyme are available (Miller, 1972). However the enzyme has several disadvantages when used as part of a model system to study protein secretion. Firstly, the enzyme, with a subunit molecular weight of approximately 116,000 daltons, is extremely large in comparison to most proteins secreted by B. subtilis (Priest, 1977). Secondly, a tetramer of the enzyme is required for β -galactosidase activity (Miller and Reznikoff, 1980). Therefore if a β -galactosidase-containing fusion protein was secreted by B. subtilis cells, association of monomer subunits would be required for detection of β -galactosidase activity. This would have to occur in either the cell membrane or culture medium. Thirdly, β -galactosidase possesses several essential sulphhydryl groups (Miller and Reznikoff, 1980) which could become oxidised under the aerobic growth conditions employed. Thus the enzyme could be inactivated. Therefore, if secretion of β -galactosidase could be attained, it should be possible to obtain secretion of almost any protein of interest.

Penicillinase- β -galactosidase fusions were introduced into E. coli and B. subtilis and low levels of β -galactosidase activity were detected in both hosts. The cellular location of the fusion protein in E. coli was not determined but it may have been secreted to the outer membrane since this is the location of the unfused penicillinase protein in E. coli cells (cited in Lai et al., 1981). Both cell-bound and extracellular forms of the enzyme were detected in B. subtilis cultures. The enzymic activity detected in the supernatant fluid was unlikely to

be the result of cell lysis since assays for the cytoplasmic enzyme SOD revealed that there was no appreciable cell lysis. Therefore it appeared that β -galactosidase activity was secreted into the culture medium by B. subtilis cells. The amount of β -galactosidase activity produced was extremely variable as was the location of this activity. Therefore it was not possible to purify enzyme from the culture fluid. Purification would have enabled characterisation of the protein and in particular sequence analysis of its N-terminus to determine the point of cleavage.

Plasmids containing penicillinase- β -galactosidase fusion proteins were unstable in both E. coli and B. subtilis and white colony variants producing no or very little β -galactosidase activity readily appeared. The fusion-containing plasmids were much more unstable in B. subtilis than in E. coli and for this reason a fusion-containing plasmid which could replicate in both E. coli and B. subtilis was constructed. Plasmid DNA isolated from E. coli was then characterised prior to introduction into B. subtilis. The bifunctional penP-lacZ-containing plasmid also contained the lacY gene. This did not appear to affect β -galactosidase production in B. subtilis, the effect on E. coli cells was not investigated.

The DNA sequence around the fusion sites of the penP-lacZ-containing plasmids was not determined. However, restriction endonuclease analysis of such plasmids isolated from E. coli suggested that the correct fusion had been constructed.

Further developments of vector plasmids to promote the secretion of proteins of commercial importance would be necessary for industrial use. An inducible promoter would be useful for regulated gene expression. In addition, for industrial purposes it would be necessary to obtain secretion of an unfused protein. This would require correct positioning of the gene of interest to ensure correct processing to remove the signal-peptide. Clearly then much more work needs to be done to develop a useful "secretion vector" for use in Bacilli,

however the demonstration of secretion of a penP-lacZ fusion protein by B. subtilis shows the promise of the system.

CHAPTER VI

B. licheniformis STRAIN LO2 AS A HOST FOR

GENETIC MANIPULATION

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6.1 INTRODUCTION

Other than E. coli, B. subtilis is the most widely studied prokaryotic organism and thus was a natural choice as an alternative to E. coli as a host for genetic manipulation. Initially, much impetus for developing B. subtilis genetic manipulation systems stemmed from the assumption that it is a safer host than E. coli. The major reason for this is that in contrast to E. coli, B. subtilis does not cause human infections (see section 1.4). However, today, probably the major reason for wanting to use B. subtilis is because of its potential industrial use, particularly its ability to secrete proteins into the culture medium (Priest, 1977).

For some industrial uses other species of Bacillus may prove to be more useful. Hence B. megaterium (Brown and Carlton, 1980; Vorobjeva et al., 1980), B. thuringiensis (Alikhanian et al., 1981; Martin et al., 1981; Miteva et al., 1981), B. licheniformis (Imanaka et al., 1981b) and B. stearothermophilus (Imanaka et al., 1982) are being investigated as potential hosts for genetic manipulation.

One of the reasons for wanting to use B. licheniformis strain LO2 (Bingham, 1979; 1980) as a host is that it is capable of growing at temperatures ranging from 28 to 62°C (Sharp, 1982) enabling investigations to be made into the expression of mesophilic and thermophilic genes at high and low temperatures. Also, the stability and action of various proteins, at high and low temperatures, can be investigated.

For industrial purposes, growth of a culture at 55 to 60°C may be advantageous since cooling of fermentation vessels can account for up to one third of the cost of a fermentation run (A. Atkinson, personal communication). Therefore it may be possible to make a substantial financial saving by using a host which is capable of growth at high temperatures. In addition, a large number of enzymes, such as glycerokinase and glycerol dehydrogenase, are obtained from thermophilic Bacillus strains (A. Atkinson, personal

communication). Such enzymes are of commercial importance since often they are extremely thermostable. Thus it may be possible using genetic manipulation, to improve the usefulness of such strains.

At the outset of this work, LO2 was classified as a B. stearothermophilus strain (Bingham, 1980). Subsequently the strain has been reclassified as a thermotolerant B. licheniformis strain (Sharp, 1982).

The successful development of B. licheniformis strain LO2 as a host for genetic manipulation is dependant upon two factors. First, suitable vector plasmids for the cloning and expression of foreign genes in this host need to be developed. Bingham *et al.* (1979; 1980; 1982) have isolated a small Tc-resistance plasmid from this strain and constructed deletion derivatives and hybrid plasmids which have been shown to replicate and express antibiotic-resistance in B. subtilis. Such vectors may be useful also with strain LO2.

Second, Bingham (1980) has developed a protoplast transformation system for strain LO2 enabling plasmid DNA to be introduced into this host. The plasmids pAB124 and pAB224 (Bingham *et al.*, 1980) transformed strain LO2 to Tc-resistance at high frequency (up to 10^6 Tc^r transformants per μ g of DNA) but the hybrid plasmid pAB324 (Bingham *et al.*, 1982) could not transform the same strain to either Tc- or Neo-resistance. It was suggested by Bingham (1980) that strain LO2 may not be capable of utilising the pAB324 plasmid replicon which is thought to be derived from pUB110.

B. licheniformis strain LO2 is far from ideal as a host for genetic manipulation. The expression of genes complementing auxotrophic mutations cannot easily be investigated in this host since it is prototrophic. Also, the strain cannot be made competent using procedures successfully developed for B. subtilis (Bingham, 1980); this may be attributed to the lack of suitable genetic markers necessary for the induction of competence. Furthermore, the strain produces extremely mucoid colonies making its handling difficult. Therefore it would be useful to isolate a series of mutants with defined genetic markers.

Several useful mutants would include mucoid-negative variants and amino acid-requiring mutants in addition to restriction and modification-deficient and recombination-deficient mutants.

The aim of the work detailed in this section was to develop a useful mutagenesis procedure for strain LO2, isolate a series of mutant strains and investigate the properties of such strains.

6.2 RESULTS

It was decided for reasons given below to try and develop a mutagenesis procedure for B. licheniformis strain LO2 using UV light as the mutagenic agent. Attempts to isolate auxotrophic mutants of this strain by nitrosoguanidine mutagenesis had not been very successful (A.H.A. Bingham, personal communication). Mutants isolated were unstable and reverted to the wild-type phenotype at high frequency. Mutagenesis with UV light is a relatively simple procedure and in addition to inducing transitions and transversions, also induces deletions (S.B. Primrose, personal communication). The latter type of mutants are particularly desirable because usually they are tightly blocked mutants which do not revert.

6.2.2 Conventional UV Mutagenesis of Strain LO2

Initial attempts at mutagenesis of strain LO2, using UV irradiation, were done using the method detailed in section 2.2.22. The efficiency of killing with UV irradiation varies greatly with different UV sources and experimental conditions, and therefore a survival curve was constructed to determine the optimum conditions for mutagenesis (Fig. 6.1).

Since a survival level of 0.1 to 10% usually is ideal for UV mutagenesis, an irradiation time of 8 s was chosen for mutagenesis of strain LO2. This gave a survival level of about 0.5%.

To test the usefulness of the mutagenic treatment, thymine-requiring (Thy⁻) mutants were first sought. These are readily selected since Thy⁻, but not Thy⁺

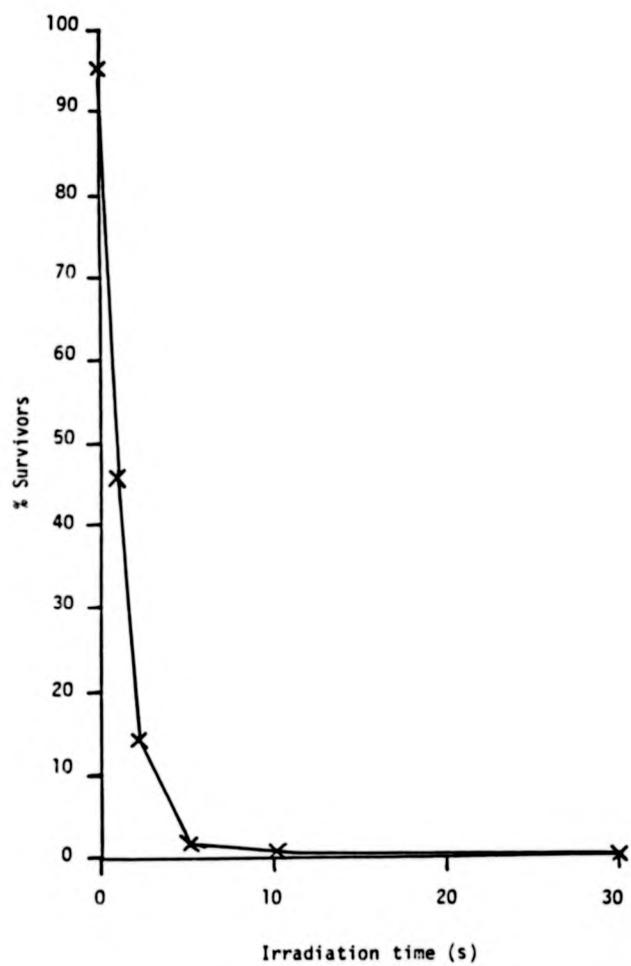


Figure 6.1: Survival of *B. lichenformis* strain L02 after UV irradiation

clones can grow on minimal medium containing thymine and trimethoprim (Tp). The minimum inhibitory concentration of B. licheniformis strain LO2 to Tp was determined. Cells were streaked onto SMS agar containing 50µg per ml of thymine and various concentrations of TP and incubated at 55°C for 4 days. Growth was inhibited completely by 5µg per ml of Tp whereas a Thy⁻ B. subtilis (strain BD393, trpC2, thyA, thyB) was capable of growth, at 37°C, on 25µg per ml of Tp. Therefore 15 and 25µg per ml of Tp were used for selection of Thy⁻ mutants of strain LO2.

After mutagenesis of a culture of strain LO2, plates were incubated for up to 7 days at 55°C to allow for growth of any mutants produced. Several attempts were made to isolate Thy⁻ mutants using this procedure but none were isolated. Consequently, this procedure was abandoned.

6.2.2 Plate UV Mutagenesis of Strain LO2⁻

Plate UV mutagenesis has been used successfully to isolate mutants of the Gram-positive organism Arthrobacter (S.B. Primrose, personal communication) and therefore this same procedure was tried with strain LO2.

Initially the irradiation time required to give between 100 and 1000 survivors per plate was determined as detailed in section 2.2.23. Survivors from the irradiated plates then were transferred, by replica-plating, to SMS agar containing 50µg per ml of thymine and either 15 or 25µg per ml of Tp. Plates were incubated at 55°C for up to 4 days. Table 6.1 shows the results obtained.

50 of the Tp^f mutants were tooth-picked onto SMS agar and SMS agar containing 50µg per ml of thymine and incubated for up to 4 days at 55°C to check for thymine auxotrophs; 6 (12%) of the Tp^f mutants were thymine-requiring. In separate but otherwise similar experiments, 20% and 24% of the Tp^f mutants produced were thymine-requiring.

Since, in the above experiments, a 30 s irradiation time gave the highest number of Tp^f mutants, this irradiation time was used subsequently for the

Irradiation time (sec)	Colony count	% Survivors	Tp ^r mutants		% Mutants per survivor	
			15µg/ml	25µg/ml	15µg/ml	25µg/ml
0	Confluent	-	0	0	-	-
5	Confluent	-	3	0	-	-
10	Confluent	9	0	-	-	-
20	Confluent	16	2	-	-	-
30	450	0.003	30	17	6.7	3.8
40	300	0.002	10	3	3.3	1.0

TABLE 6.1: Plate UV mutagenesis of B. licheniformis strain LO2

isolation of other mutants. Several mutagenesis experiments were done to produce multiply marked strains (see Table 6.2). After each mutagenesis experiment, survivors were tooth-picked out onto SMS agar to screen for presumptive mutants which were identified subsequently by tooth-picking onto SMS agar containing various nutritional supplements. The nutritional requirements of some presumptive mutants could not be identified. Such mutants may have had requirements different to those tested and may have been multiple auxotrophs. These mutants were not characterised further.

The frequency of auxotrophic mutants amongst the survivors of UV treatment ranged from 0.7 to 1.3% in different but otherwise similar experiments. The frequency of identifiable auxotrophs ranged from 0.14 to 0.2% of the survivors. Thus between 15 and 25% of the auxotrophic mutations were identified. The identified mutants were characterised further as follows. The spontaneous reversion frequency of each different auxotrophic marker was determined and the data obtained is presented in Table 6.3. All the mutations were relatively stable with reversion frequencies ranging from 8.0×10^{-7} to less than 1.5×10^{-10} . The mutants for which revertants were not detected may have been deletion mutants since these mutants cannot revert. Alternatively they may have been double or multiple mutants of the same allele or pathway.

6.2.3 Attempted Induction of Competence of Strain LO2 Mutants

One of the reasons for isolating a series of strain LO2 mutants was to demonstrate transformation of competent cells of such mutants with plasmid or chromosomal DNA. Such a system would be useful for several reasons including genetic analysis of the strain. The mutant strains chosen for investigation were L49 (His⁻, Met⁻) and L89 (His⁻, Met⁻, Poly⁻). These mutants were used for two reasons. Firstly, Dubnau and Pollock (1965) have shown that both His⁻ and Met⁻ mutants of B. licheniformis can be transformed by chromosomal DNA and, secondly, a rough colony variant of this strain could be transformed by

Mutant strain	Phenotype	Parental strain
L40	His ⁻ ,	LO2
L41	His ⁻ , Leu ⁻	L40
L42	His ⁻ , Thy ⁻	L40
L48	His ⁻ , Ileu ⁻	L40
L49	His ⁻ , Met ⁻	L40
L78	His ⁻ , Leu ⁻ , Thy ⁻	L41
L85	His ⁻ , Thy ⁻ , Poly ⁻	L110
L87	His ⁻ , Cys ⁻ , Poly ⁻	L110
L88	His ⁻ , Arg ⁻ , Poly ⁻	L110
L89	His ⁻ , Met ⁻ , Poly ⁻	L110
L90	His ⁻ , Lys ⁻ , Poly ⁻	L110
L91	His ⁻ , Gua ⁻ , Poly ⁻	L110
L92	His ⁻ , Ilv ⁻ , Lys ⁻ , Poly ⁻	L90
L110	His ⁻ , Poly ⁻	L40

TABLE 6.2: *B. licheniformis* strain LO2 mutants obtained after plate mutagenesis

Poly mutants are deficient in polyglutamate synthesis

Strain	Marker tested	Spontaneous reversion frequency
L110	His	$<1.5 \times 10^{-8}$
L41	Leu	$<1.5 \times 10^{-10}$
L85	Thy	1.9×10^{-9}
L48	Ileu	$<1.4 \times 10^{10}$
L49	Met	3.3×10^{-8}
L87	Cys	8.0×10^{-7}
L88	Arg	2.3×10^{-8}
L90	Lys	$<2.4 \times 10^{-9}$
L91	Gua	$<1.0 \times 10^{-9}$

TABLE 6.3: Spontaneous reversion frequency of *B. licheniformis* strain LO2 mutants

chromosomal DNA 1000 times more efficiently than a smooth colony variant of the same strain.

Chromosomal DNA, isolated from the prototrophic wild-type strain LO2, was used as a source of transforming DNA. This was used in preference to plasmid DNA because chromosomal DNA transformation of competent cells generally is more efficient than plasmid DNA transformation (see chapters III and IV). Attempts were made to transform B. licheniformis strain L49 and L89 to His⁺ or Met⁺. The methods of Bingham (1980) for transformation of B. subtilis competent cells and Dubnau and Pollock (1965) for transformation of B. licheniformis competent cells, were used.

Figure 6.2 shows a typical experiment using B. licheniformis strain L89 and the transformation procedure of Bingham (1980). In this and other transformation experiments attempted, neither His⁺ nor Met⁺ transformants were ever detected. No further attempts were made to transform competent cells of the B. licheniformis mutants.

6.2.4 Attempted Transformation of Strains LO2 Protoplasts

Bingham (1980) demonstrated that protoplasts of B. licheniformis strain LO2 could be transformed by plasmid DNA at a frequency of 10^6 transformants per μg of plasmid DNA. In addition, Bingham (1980) suggested that strain LO2 had a restriction system since plasmid DNA isolated from B. subtilis was 1 to 4 orders of magnitude less efficient in transformation as plasmid DNA isolated from strain LO2.

Since a series of strain LO2 mutants had been isolated (section 6.2.2) it was decided to examine the expression of various plasmid-borne genes in some such mutant strains. Of particular interest was the thyP3 gene of the B. subtilis bacteriophage $\phi 3T$, the Cm-resistance gene of the S. aureus plasmid pC194, the penP gene of B. licheniformis 749/C and the lacZ gene from E. coli. These genes previously have been cloned in pAB224 or derivatives of this plasmid (see

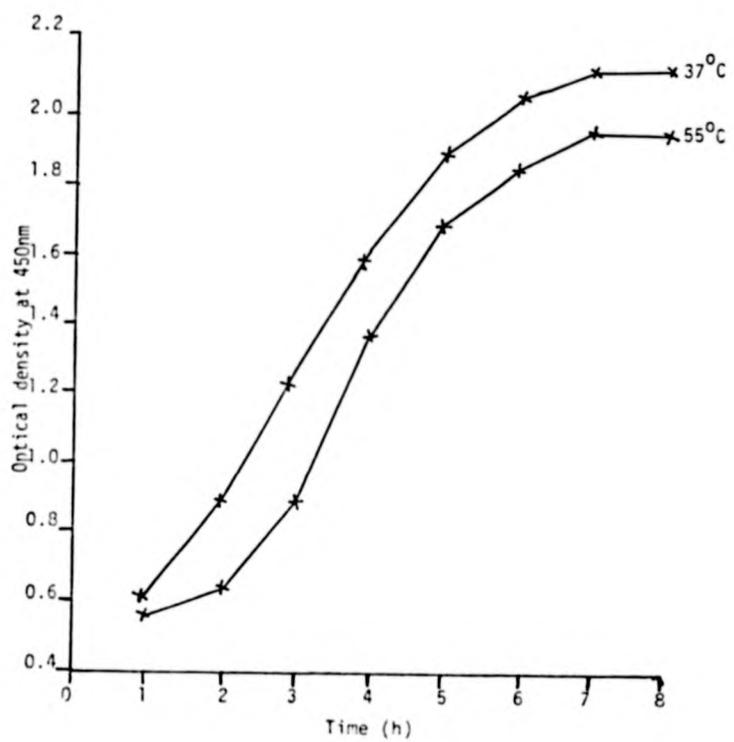


Figure 6.2: Transformation of *B. licheniformis* strain L89 cells were transformed with 2 μ g aliquots of *B. licheniformis* strain L02 DNA at 1h intervals using the method of Bingham (1980). Plates were incubated for 7 days at the growth temperature

Chapters III, IV and V) and hence should be able to transform and replicate in strain LO2. However, initial attempts were made to repeat the work of Bingham (1980).

The plasmid vector pAB224 was isolated from strain LO2 and used to transform protoplasts of strain LO2 using the method of Bingham (1980). Transformed protoplasts were spread onto TSBA, DM4 and DM4 containing 12 μ g per ml of Tc and incubated for up to 4 days at 55°C. Many attempts at transformation of strain LO2 met with failure for apparently unknown reasons.

Protoplasts of strain LO2 were successfully produced as evidenced by the production of spherical bodies seen using a light microscope. Also, these protoplasts were able to regenerate to the bacillary form when spread onto DM4 regeneration medium but not when spread onto TSBA. The percentage of osmotically-insensitive bacteria after lysozyme treatment, usually was less than 0.005% of the original population and the protoplast regeneration frequency ranged from 1 to 20% of the plated protoplasts. However, Tc^r transformants were never detected after spreading protoplasts onto DM4 containing Tc or replica-plating regenerated protoplasts from DM4 plates lacking Tc. The reason for the repeated failure to detect Tc^r transformants is unknown.

Table 6.4 shows a comparison of the results obtained in this present investigation with those obtained by Bingham (1980). Whereas Bingham (1980) usually obtained about 10⁴ osmotically insensitive rods, i.e. the colony count on TSBA, in this present investigation usually only about 10¹ osmotically-insensitive rods were obtained. This may reflect the extent of lysozyme treatment of the cells. However, similar conditions were used in both investigations and hence the lysozyme treatment should have been the same. The extent to which this affected transformation is unclear but it may have contributed to it.

Another possible explanation for the failure to obtain transformants in this investigation is that the PEG treatment was not successful. It has been reported

	Colony count		
	DM4	DM4-Tc (12µg per ml)	TSBA
Bingham (1980)	4.2×10^7	4.7×10^4	8.0×10^4
This report	2.0×10^7	0.0	1.6×10^1

TABLE 6.4: Transformation of *B. licheniformis* strain LO2 protoplasts by plasmid DNA

that different batches of PEG markedly affect the transformation frequency (Bingham, 1980): only one batch of PEG was used in this investigation.

No further attempts to transform strain LO2 protoplasts were made.

6.3 DISCUSSION

The work in this chapter details the construction and characterisation of mutants of B. licheniformis strain LO2. A procedure for the isolation of mutants was developed using UV light as the mutagenic agent. However, transformation of two of these mutants with chromosomal DNA could not be demonstrated. Also, attempts to transform protoplasts of B. licheniformis strain LO2 with plasmid DNA were unsuccessful.

The mutagenesis procedure used is a simple, rapid procedure with the advantage that different auxotrophs with the same phenotype, isolated in the same experiment, can be assumed to be independent mutants. Also, with this mutagenesis procedure, unlike others, there is no need to allow for segregation of mutant loci. The reasons for this are as yet unknown.

Subsequently, this mutagenesis procedure has been tried with B. subtilis (unpublished observation, and S.B. Primrose, personal communication). However, mutants could not be isolated using this procedure. Thus this mutagenic treatment may work with only certain species.

The competence transformation procedure of B. subtilis has been used extensively for both genetical and biochemical analysis of this species. A similar system could not be demonstrated with B. licheniformis strain LO2 (Bingham, 1980) and in addition with two mutants of strain LO2 as detailed in this chapter. In order to induce competence in strain LO2 it may be necessary to use different mutant strains to those tested or alternatively to use a different procedure for induction of competence.

The protoplast transformation procedure for strain LO2, developed by Bingham (1980), could not be repeated in this present investigation. The reasons

for this are unknown but the extent of lysozyme treatment and the batch of PEG used may have contributed to the failure.

At the outset of this investigation strain LO2 was classified as a B. stearothermophilus strain (Bingham, 1980). Transformation of neither competent cells nor protoplasts of this species had previously been reported. Subsequently Imanaka et al. (1982) have demonstrated high frequency transformation of B. stearothermophilus protoplasts by plasmid DNA. However, strain LO2 has been reclassified as a B. licheniformis strain (Sharp, 1982). Chromosomal DNA transformation of competent cells of B. licheniformis has been reported (e.g. Dubnau and Pollock, 1965) in addition to the transformation by plasmid DNA of competent cells (Docherty et al., 1981) and protoplasts (Imanaka et al., 1982) of B. licheniformis. Thus, because of the problems encountered with strain LO2 in this investigation, it may be useful to use one of the above mentioned B. stearothermophilus or B. licheniformis strains for future investigations.

CHAPTER VII

GENERAL DISCUSSION

At the outset of the work detailed in this thesis, several groups of workers were developing B. subtilis and other species of Bacillus as hosts for genetic manipulation (e.g. Gryczan et al., 1978, 1980a and b; Bingham, 1980; Docherty, 1981). Many advances had occurred with E. coli systems and thus genes from a wide variety of prokaryotic and eukaryotic organisms had been cloned and expressed in E. coli (e.g. Maniatis et al., 1978). In contrast, there had been only a limited number of reports of the cloning and expression of genes in B. subtilis (e.g. Gryczan et al., 1978; Keggins et al., 1978). It appeared that genes from only Gram-positive organisms were expressed when introduced into B. subtilis (e.g. Kreft et al., 1978) and thus B. subtilis was thought to be more stringent in its requirements for control signals for gene expression than E. coli.

Despite these problems, the Bacilli were thought to have great potential as hosts for genetic manipulation; they are safer hosts than E. coli (see Sections 1.2 and 1.3) and are capable of secreting large amounts of protein into the culture medium (see Section 1.10). The aim of the work detailed here was to investigate the usefulness of B. subtilis and B. licheniformis strain LO2 as hosts for genetic manipulation.

Since, at the outset of this work there had been only one report in the literature of the shotgun-cloning of chromosomal genes directly in B. subtilis (Keggins et al., 1978), initially, attempts were made to shotgun-clone the B. subtilis trpC gene and the B. licheniformis 749/C penP gene in B. subtilis (Chapter III). The plasmid vector pAB224 was used because previously, this plasmid had been shown to be capable of accommodating foreign DNA at its unique EcoRI site (Bingham, 1980). Many attempts at shotgun-cloning the trpC

and penP genes met with repeated failure. The possible reasons for this are detailed in Chapter III but a full explanation remains unclear. A low transformation frequency of ligation-mix DNA, in addition to a low insertion frequency of chromosomal DNA into the plasmid vector, may have contributed to the failure. Also, in the case of the trpC gene, genetic rearrangements may have been required for expression. In the case of the penP gene, the selection pressure applied during the attempted shotgun-cloning, i.e. Ap-resistance, probably contributed to the failure to shotgun-clone this gene; it was demonstrated subsequently (Chapter V) that direct selection for Ap-resistance cannot be applied with the penP gene.

Since difficulties with shotgun-cloning genes directly in B. subtilis, using plasmid vectors, have been encountered by many other workers (e.g. Gryczan et al., 1980a; A.J.P. Docherty and A.H.A. Bingham, personal communications), this approach for gene-cloning was not investigated further.

As alternatives to direct shotgun-cloning in B. subtilis, some workers (e.g. Dubnau et al., 1981) have used the plasmid-rescue shotgun-cloning system developed by Gryczan et al. (1980a). At present this system cannot be used to shotgun-clone B. subtilis chromosomal genes but the development of similar systems in other species of Bacillus would permit this. It may be possible to shotgun-clone the gene of interest using a bacteriophage vector. Several genes have been cloned using such vectors (e.g. Yoneda et al., 1979) but subsequent cloning into plasmid vectors is often necessary and problems with this have been encountered (Y. Yoneda and E. Kenny, personal communications).

As an alternative to shotgun-cloning in B. subtilis, several groups of workers (e.g. Rapoport et al., 1979) have made use of E. coli as an intermediate host. E. coli shotgun-cloning systems have been well tried and tested and in addition, the majority of foreign genes have been found to be expressed when introduced into E. coli. Hence expression of Bacillus genes in E. coli is probable and thus it is possible to screen for expression in E. coli.

In summary, if it is necessary to shotgun-clone a Bacillus gene, direct shotgun-cloning in B. subtilis using plasmid vectors may be attempted. However, problems are likely to be encountered. Thus it may be simpler to use an alternative approach such as the plasmid-rescue shotgun-cloning system, a bacteriophage vector, or E. coli as an intermediate host.

To investigate the usefulness of the plasmid pAB224 as a vector for use in B. subtilis, attempts were made to sub-clone the thyP3 gene of the B. subtilis bacteriophage $\Phi 3T$, from the plasmid pCD1 (Duncan *et al.*, 1977), into the unique EcoRI site of pAB224. Several hybrid plasmids containing the thyP3 gene were isolated and characterised. This confirmed the work of Bingham (1980) who found that the EcoRI site of pAB224 could be used to insert foreign DNA without inactivating essential plasmid functions.

The thyP3-containing plasmids were found to have unusual properties regarding plasmid transformation, integration and stability which are discussed in detail in Chapter IV. It was found that monomeric species of the thyP3-containing plasmids could not give rise to antibiotic-resistant transformants whereas multimeric forms of the plasmids could. Also, transformation with monomeric species of plasmid DNA resulted in integration of only the thyP3 region of the plasmids into a specific site in the B. subtilis chromosome.

The thyP3-containing plasmids were shown to have DNA sequence homology with specific regions of the B. subtilis chromosome. However, the plasmids could be maintained in an extrachromosomal state even though the host strain was recombination-proficient. Several workers (e.g. Rapoport *et al.*, 1979) have found that it is necessary to use a recE4 host to prevent recombination of homologous sequences present on a plasmid vector and hence the extrachromosomal maintainance of the thyP3-containing plasmids was unexpected.

In contrast to the parental vector pAB224, the hybrid thyP3-containing plasmids were segregationally unstable. The reasons for this are unclear but

such instability of hybrid plasmids has been widely reported in the literature (e.g. Goebel *et al.*, 1979; Mazza *et al.*, 1981). It was found that if clones harbouring the thyP3-containing plasmids were grown under selection pressure for retention of the thyP3 gene, then other plasmid-borne markers also could be retained. Thus the thyP3 gene may be a useful plasmid marker for use on an industrial scale. Clones containing this plasmid-borne gene could be grown in rich medium and selection for plasmid retention could be applied without the addition of antibiotics. The complex medium used, TYS broth, does not contain sufficient thymine for growth and hence the thyP3 gene is essential.

One of the major reasons for needing to use Bacilli for genetic manipulation is because of their ability to secrete proteins out of the cell into the culture medium (Priest, 1977). It can be envisaged therefore that Bacilli can be made to secrete a particular protein of interest. As a model system to investigate protein secretion, attempts were made to obtain secretion of the *E. coli* enzyme β -galactosidase by fusing the lacZ gene to the gene penP which codes for the penicillinase protein of *B. licheniformis* 749/C (Brammar *et al.*, 1979). The penicillinase protein is secreted in large amounts by *B. licheniformis* cells. However, approximately 50% of the penicillinase is in a membrane-bound form (Sargent *et al.*, 1968).

Plasmids were constructed (see Chapter V) which led to the fusion of the N-terminus of the penicillinase protein to all but 7 N-terminal amino acids of the β -galactosidase protein. Such plasmids were introduced into *E. coli* and *B. subtilis* and shown to direct low-level synthesis of a protein with β -galactosidase activity.

The location of the β -galactosidase activity in *B. subtilis* cultures was variable but in most cases enzymic activity was detected in the culture medium. Also, the results of superoxide dismutase assays suggested that β -galactosidase activity detected in the culture medium was not the result of cell lysis. Hence it appeared that β -galactosidase activity was actively secreted into the culture

medium. β -galactosidase normally is a cytoplasmic enzyme and as such is not "designed" to be secreted. Therefore, since secretion of a β -galactosidase-containing fusion protein was demonstrated, it should be possible to obtain secretion of almost any protein of interest.

The penicillinase- β -galactosidase fusions used in this investigation show the promise of the secretion system. However, it may not be possible, using this particular system, to develop a useful secretion system for use on an industrial scale. Ideally what is required is inducible synthesis leading to the high level production of a non-fused protein.

In addition to *B. subtilis* several other species of *Bacillus* may prove to be useful as hosts for genetic manipulation. The strain used in this investigation, *B. licheniformis* LO2, was thought to have potential as a host since it is capable of growing at temperatures ranging from 28°C to 62°C (Sharp, 1982). A protoplast transformation procedure for this strain has been developed (Bingham, 1980) and the plasmids pAB124, originally isolated from strain LO2 (Bingham et al., 1979) and the deletion derivative pAB224 (Bingham et al., 1980) have been introduced into it (Bingham, 1980).

To improve the usefulness of strain LO2, a mutagenesis procedure was developed (Chapter VI) and many mutant strains were constructed. Attempts to induce competence in 2 multiply marked mutants were unsuccessful and in addition, attempts to transform protoplasts of strain LO2 with plasmid DNA met with repeated failure. Therefore, at present, strain LO2 is far from ideal as a host for genetic manipulation.

In conclusion, using the plasmid vector pAB224, several hybrid plasmids were constructed and introduced into *B. subtilis*. This showed that the unique EcoRI site of this plasmid can be used to insert foreign DNA. By using the *B. licheniformis* 749/C penicillinase protein N-terminus, secretion of *E. coli* β -galactosidase by *B. subtilis* cells was demonstrated. However, neither chromosomal DNA transformation of competent *B. licheniformis* LO2 cells nor

plasmid DNA transformation of B. licheniformis LO2 protoplasts could be demonstrated. Hence further developments with this strain are required.

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The rapid isolation of mutants of some Gram-positive bacteria

L. Dijkhuizen, L. Keijer, V.J.C. Waddell *, S.B. Primrose *, D. Barstow ** and
A. Atkinson **

Department of Microbiology, University of Groningen, Kerklaan 30, Haren, The Netherlands; * Department of Biological Sciences, University of Warwick, Coventry CV4 7AL; and ** Centre for Applied Microbiological Research, Porton Down, Salisbury, England

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1. INTRODUCTION

Mutant analysis facilitates, in several ways, the study of cellular processes at the molecular level. With the proper series of mutants, for example, it is possible to define the enzymatic steps in a biochemical pathway. With a multiply-marked strain it is possible to map the structural gene for a protein of particular interest. Knowledge of the location of a marker simplifies the task of preparing strains containing different combinations of mutations which can be used to analyse the regulation of particular biochemical pathways.

A number of problems have been encountered when attempts have been made to isolate, for the first time, a stock of mutants of a hitherto neglected organism. First, well-tried methods for obtaining mutants of several bacterial species are lacking. Second, since two organisms rarely will respond in precisely the same way to a mutagenic treatment, or a mutant screening programme, it often happens that a published procedure has to be modified to yield optimal results. Finally, provision must be made for segregation of pure mutant cells before cloning so as not to isolate strains all bearing exactly the same mutation. Thus isolation of independent mutants requires independent mutagenic treatments. In this communication we described a method for isolating at high frequency independent mutants of a number of Gram-positive bacteria. The method was originally devel-

oped for use with an *Arthrobacter* sp. and appears to work best with this and other coryneform bacteria.

2. MATERIALS AND METHODS

All the bacteria used were from the culture collections maintained at the University of Warwick or the Centre for Applied Microbiological Research. *Arthrobacter* P1 and coryneform D7F have been described elsewhere [1,2].

The complex media used were TSBA (for *Bacillus* sp.) and Oxoid nutrient agar (all other strains). TSBA contained, per litre, 17 g Oxoid tryptone, 3 g Oxoid soya peptone, 5 g NaCl, 2.5 g K₂HPO₄, 2.5 g glucose and 15 g agar; the pH was adjusted to 7.3 with 2 N HCl prior to sterilization. The minimal medium used was that of Spizizen [3] except for *Arthrobacter*, when the medium of Levering et al. [1] was used. Minimal media were supplemented with amino acids as required. Nutritional pool plates were prepared as described by Clowes and Hayes [4].

For mutagenesis using UV light, cells were grown in complex media and used while still in the logarithmic phase of growth. Details of the irradiation procedure are given in the text.

3. RESULTS

Our original observations were made on a yellow pigmented, facultative methylotroph, *Arthrobacter* P1 (Levering et al., 1981). Portions (0.1 ml; approx. 10^7 cells) were spread on nutrient agar plates and irradiated with UV light for varying periods of time (20–90 s) until there were 10–1000 survivors per plate. Survivors from this irradiation were tested for their ability to grow on minimal medium, and all potential auxotrophs were retested both on minimal and complex medium. In six separate experiments the percentages of auxotrophs isolated in this way were 3.0%, 4.0%, 4.3%, 6.7%, 2.2% and 2.4%. For most mutagens, the number of viable mutants in the population will increase with irradiation dose up to a certain level, and then fall as killing overtakes the induction of new mutants. However, at the sampling times used with this method, there was no significant difference in the number of auxotrophs obtained. Attempts were made to identify the metabolic lesion in all the auxotrophs by picking them onto nutritional pool plates. Approx. 40% of the mutants failed to grow on any pool plate indicating multiple nutritional requirements. The remainder were identified as having lesions in one of the following biosynthetic pathways: leucine, lysine (2 isolates), tryptophan, arginine, glycine, serine, valine, proline, adenine (3 isolates), nicotinic acid, biotin, histidine, isoleucine and threonine. Two other mutants were also identified, one probably with a defect in methylamine transport and the other with a defect in pigment biosynthesis.

Because of the ease with which mutants of *Arthrobacter* P1 could be isolated, we mutagenized a variety of bacteria in a similar way and screened survivors for auxotrophs. No auxotrophs were found for *Escherichia coli* strain C (0/550), *Aeromonas hydrophila* strain HY (0/1150), *Pseudomonas aeruginosa* strain PAT (0/728), *Pseudomonas oxalaticus* (0/950) or *Bacillus subtilis* strains UW1 and BD224 (0/522 and 0/1080). However auxotrophs were found with coryneform D7F (16/141). Approx. 38% of the D7F auxotrophs had complex requirements and the remainder had single requirements which could be identified by use of nutritional pool plates.

Auxotrophs were also found with the thermophilic *Bacillus licheniformis* strain, LO2. In the first experiment with this strain, trimethoprin (Tp)-resistant mutants were isolated. On media containing 15 $\mu\text{g}/\text{ml}$ Tp they constituted 6.7% and 3.3% of survivors (0.003% and 0.002% survival, respectively) and on media containing 25 $\mu\text{g}/\text{ml}$ Tp they constituted 3.8% and 1.0% of survivors. In 3 separate experiments the proportion of Thy^- auxotrophs among the Tp^R mutants ranged from 12–24%. For the isolation of other auxotrophs, strain LO2 was irradiated on plates to 0.003% survival. The frequency of auxotrophs among the survivors ranged from 0.7–1.3% and the frequency of identifiable auxotrophs from 0.14% to 0.2% of survivors. Mutants have been isolated in this way which have defects in the biosynthesis of histidine, leucine, isoleucine/valine, methionine, cysteine, arginine, lysine, guanine, and polyglutamyl polypeptide, and double and triple auxotrophs have been constructed. All the auxotrophic markers tested were stable having reversion frequencies ranging from $8.0 \cdot 10^{-7}$ to less than $1.5 \cdot 10^{-10}$.

Under most cultural conditions, bacterial cells rarely contain a single genome. Thus after mutagenesis it is essential to grow the cells for a few generations to allow segregation of mutant loci. This may be unnecessary if mutagenesis is associated with appreciable killing since any viable but mutant genome is unlikely to find itself in the same bacterium as a viable but non-mutant genome. With the mutagenesis method described above there is no need to allow segregation of mutant loci. Furthermore, appreciable killing occurs and so inactivation of non-mutant genomes is highly probable. However, if killing of non-mutant genomes is the explanation for the success of the method then it should work with all bacteria. The failure to obtain mutants of a number of bacteria suggests that there is an alternative explanation. One possibility is that *Arthrobacter* P1, coryneform D7F and *B. licheniformis* normally have only one or two genomic copies whereas the other strains tested have three or four. If this were the case then lowering of the genomic copy number might facilitate mutagenesis. Consequently, *E. coli* and *A. hydrophila* were grown in nutrient broth and in chemostats at a dilution rate of 0.05/h and their

susceptibility to mutagenesis tested as described above. No auxotrophs were isolated regardless of the growth conditions.

4. DISCUSSION

The method of direct mutant isolation described in this communication has a number of advantages over conventional methods. First, it is extremely rapid and simple and requires no calibration of the UV source. Second, a range of different auxotrophs can be isolated from a single experiment. Third, because there are no detectable auxotrophs prior to irradiation, different isolates with the same phenotype obtained from the same plate can be assumed to be independent mutants. This is a particularly attractive feature if the aim of the mutants is to facilitate analysis of a biochemical pathway and is the major advantage over conventional methods of mutant isolation (see [5] for discussion). The biggest disadvantage with the method is that undoubtedly most of the auxotrophs isolated will carry multiple mutations. This is clear from the fact that a high proportion of them could not be characterized nutritionally. Even with the auxotrophs which could be identified there is still a high probability that they carry other "silent" mutations. Whether or not these prove troublesome would depend on the circumstances for which the mutants are to be used.

The reason why the method described here only works with certain organisms is not known. However, since the aim of the method was to obtain a stock of mutants and not to study mutagenesis itself, this hardly matters. Because of the simplicity of the method we recommend that it be tried first, before the conventional methods, when developing the genetics of a hitherto neglected organism.

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Properties of *thyP3*-Containing Plasmids in *Bacillus subtilis*

D. A. BARSTOW,* S. B. PRIMROSE† AND T. ATKINSON*

*Microbial Technology Laboratory, Centre for Applied Microbiology & Research, Porton Down, Salisbury, Wiltshire SP4 0JG, and †Department of Microbiology & Virus Research, Searle Research and Development, Lane End Road, High Wycombe, Bucks, England

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A series of hybrid plasmid molecules which contain both antibiotic resistance genes and the *thyP3* gene of the *Bacillus subtilis* bacteriophage $\phi 3T$ have been constructed. Monomeric or restriction enzyme-cleaved plasmid DNA is capable of transforming competent cells to thymine prototrophy only. However, multimeric plasmid DNA can transform competent cells to both thymine prototrophy and antibiotic resistance. Cells which have been transformed to thymine prototrophy only do not contain extrachromosomal plasmid DNA but instead contain the *thyP3* gene integrated into the host chromosome; the antibiotic resistance genes, however, do not become integrated into the chromosome. Although the *thyP3*-containing plasmids have extensive DNA sequence homology with the *B. subtilis* chromosome, they can be stably maintained, extrachromosomally, even in *recE*₄ hosts, in complex broth, and in the absence of antibiotics.

Bacillus subtilis has been used as a host for the cloning of homologous chromosomal genes (Segall & Losick, 1977; Mahler & Halvorson, 1977; Tanaka & Sakaguchi, 1978; Nagahari & Sakaguchi, 1978; Rapoport *et al.*, 1979; Canosi *et al.*, 1981; Jayaraman *et al.*, 1981). In addition to chromosomal genes considerable interest has been shown in the cloning of *B. subtilis* bacteriophage genes (Rutberg *et al.*, 1981), in particular the thymidylate synthetase gene (*thyP3*) of the bacteriophage $\phi 3T$ (Ehrlich *et al.*, 1976; Duncan *et al.*, 1978; Galizzi *et al.*, 1981) which complements thymine auxotrophs of both *B. subtilis* and *Escherichia coli*.

The $\phi 3T$ -*thyP3* gene has recently been shown to have DNA sequence homology with the *B. subtilis* chromosomal *thyA* gene (Duncan *et al.*, 1978; Galizzi *et al.*, 1981; Stroynowski, 1981) and the regions flanking the *thyP3* gene in $\phi 3T$ to have homology with a separate region of the *B. subtilis* chromosome (Stroynowski, 1981). When Ehrlich *et al.* (1976) cloned the *thyP3* gene into pSC101 they found that, upon transformation of *B. subtilis* with the hybrid plasmid, only the *thyP3* region of the plasmid was integrated into the host chromosome. Conversely, Dun-

can *et al.* (1978) showed that when the *thyP3* gene was cloned into pMB9, the entire recombinant plasmid, pCD1, became integrated into the *B. subtilis* chromosome. The bifunctional vector pHV15 (Cm^r, Ap^r in *E. coli*; Cm^r in *B. subtilis*) was used by Galizzi *et al.* (1981) to clone the *thyP3* gene. The resultant recombinant plasmid, pPV21, was found to be unstable in both *recE*₄ and *recE*₄ hosts with stable Cm^r, Thy^r clones being spontaneously produced only by integration of the entire plasmid into the host chromosome.

In order to examine the expression and integration of the *thyP3* gene more closely we initially cloned this gene into pAB224, a deletion derivative of pAB124 (Bingham *et al.*, 1979, 1980). This paper describes the properties of these *thyP3*-containing plasmids in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains used in this study and the plasmids they contain are described in Table 1.

Media. The minimal medium used for *B. subtilis* was that of Spizizen (1958). Glucose

TABLE I
STRAINS USED IN THIS STUDY

Strain	Genotype (plasmid)	Plasmid marker	Plasmid M_r (MDa)	Source
<i>E. coli</i> C600CD1	Thy (pCD1)	Tc ^r , thyP3	5.40	F. Young
<i>B. subtilis</i> IG224	<i>trpC2, r, m</i> , (pAB224)	Tc ^r	1.95	A. H. A. Bingham
QB943	<i>trpC2, pyrD, ilvA1, thyA, thyB</i>	—	—	R. J. Sharp
QBTT1	<i>trpC2, pyrD, ilvA1, thyA, thyB</i> , (pTT1)	Tc ^r , thyP3	5.70	This paper
QBTT2	<i>trpC2, pyrD, ilvA1, thyA, thyB</i> , (pTT2)	Tc ^r , thyP3	6.70	This paper
QBTT3	<i>trpC2, pyrD, ilvA1, thyA, thyB</i> , (pTT3)	Tc ^r , thyP3	5.70	This paper
BD393	<i>trpC2, lys, thyA, thyB</i>	—	—	A. J. P. Docherty
BDTTC1-4	<i>trpC2, lys, thyA, thyB</i> (pTTC1-4)	Tc ^r , Cm ^r , thyP3	6.30	This paper
BDBD64	<i>trpC2, metB10</i> , (pBD64)	Cm ^r , Neo ^r	3.20	A. J. P. Docherty
QB15	<i>trpC2, pyrD, ilvA1, thyA, thyB</i> (thyP3)	thyP3	—	This paper
BDTCS	<i>trpC2, lys, thyA, thyB</i> , (thyP3)	thyP3	—	This paper

was added to 1% (w/v), amino acid supplements to 100 µg/ml, and purine and pyrimidine base supplements to 50 µg/ml; 1% (w/v) agar was added for minimal plates. The complex medium used was TYS and TSBA plates as described by Bingham *et al.* (1979). *E. coli* was grown in L-broth. Tetracycline was used at final concentrations of 12 or 25 µg/ml and chloramphenicol at 10 µg/ml.

Preparation of plasmid DNA. Plasmid DNA from *E. coli* was isolated by CsCl-EtBr¹ gradient centrifugation of cleared lysates essentially as described by Clewell and Helinski (1969). Plasmid DNA from *B. subtilis* was prepared as described by Bingham *et al.* (1979). Plasmid DNA was stored in TE buffer

¹ Abbreviations used: Et, ethidium; TE buffer, 10 mM Tris-HCl, 0.1 mM Na₂EDTA, pH 8.0; TES, 30 mM Tris-HCl, 5 mM Na₂EDTA, 50 mM NaCl, pH 8.0, with 4 M HCl; TAE buffer, 40 mM Tris, 20 mM sodium acetate, 1 mM Na₂EDTA, pH 8.0, with acetic acid; ccc, covalently closed circular; CAT, chloramphenicol acetyltransferase; cDNA, chromosomal DNA; Tc, tetracycline; Cm, chloramphenicol.

(10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0) at -20°C.

In order to rapidly screen *B. subtilis* cells for extrachromosomal DNA miniplasmid preparations were performed as follows: 10-ml cultures were grown overnight in 1-oz bottles. Cells were spun down, washed twice in 5 ml TES (30 mM Tris-HCl, 5 mM Na₂EDTA, 50 mM NaCl, pH 8.0, 50 mM NaCl, pH 8.0, with 4 M HCl) and resuspended in 200 µl TES. EDTA (100 µl, 200 mM, pH 8.0) was added, followed by 100 µl lysozyme (10 mg/ml in TES). After 10 min on ice cells were incubated at 37°C for 6 min with occasional mixing. SDS (100 µl, 10% w/v) was added followed by 200 µl NaCl (5 M). After the mixture was left on ice for 1-2 h, cell debris was removed by centrifuging for 5 min in a microfuge. The supernatant fluid was extracted three or four times with chloroform-isoamyl alcohol (24:1 v/v) and 100-µl aliquots of the supernatant fluid were used for analysis on agarose gels.

Transformation of *B. subtilis*. Competent

cells were prepared as described by Bingham *et al.* (1979). Prior to plating on minimal agar, cells were centrifuged and resuspended in 1 ml of minimal medium. Phenotypes of transformants were checked by toothpicking 100 colonies onto appropriate selection plates.

Treatment of DNA with enzymes. T₄ DNA ligase and restriction enzymes were purchased from Bethesda Research Laboratories (Gaithersburg, Md.). DNA polymerase large fragment (Klenow) was purchased from Boehringer-Mannheim (Mannheim, Germany), and DNA polymerase was purchased from Amersham International Ltd. (Amersham, Buckinghamshire, England). Enzymes were used according to the instructions supplied by the manufacturers.

Agarose gel electrophoresis. DNA preparations were routinely electrophoresed on vertical slab gels using 1% (w/v) agarose (Bethesda Research Laboratories) in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 1 mM Na₂EDTA, pH 8.0, with acetic acid). Samples were electrophoresed at 125 V for 3–4 h or 20 V for 16–18 h.

Extraction of DNA from agarose gels. DNA was extracted from agarose gels by electroelution essentially as described by McDonnell *et al.* (1977).

End labelling λ DNA. λ DNA was end-labelled essentially as described by Downing *et al.* (1979).

Nick translation. Nick translation was carried out as described by Rigby *et al.* (1977).

Southern transfer and DNA-DNA hybridisation. Southern transfer and DNA-DNA hybridisations were carried out essentially as described by Southern (1975).

Autoradiography. Autoradiography was carried out at -70°C using Kodak X-ray film and Dupont Cronex Xtra Life intensifying screens.

Isolation of chromosomal DNA. Cells were grown overnight at 37°C in TYS broth, centrifuged, washed twice in 100 ml TES, and resuspended in 10 ml TES. EDTA (2 ml, 200 mM), pH 8.0, and 1 ml 10 mg/ml lysozyme in TES were added and the cells were placed on ice for 10 min. After 2 min at 37°C, 2 ml

10% Triton X-100 and 1 ml 10 mg/ml proteinase K (Boehringer-Mannheim, treated at 60°C for 10 min) were added. After being left on ice for 2–3 h the supernatant was extracted 3–6 times with redistilled phenol (equilibrated with TE buffer). The DNA was spooled 2–3 times after addition of 1/10th vol 3 M Na acetate, pH 5.0, and 2 vol 95% ethanol at -20°C. Finally the DNA was vacuum dried and resuspended in TE buffer.

Chloramphenicol-acetyltransferase assays. Cultures (20 ml) were grown in 1-oz bottles overnight at 37°C. Cells were centrifuged, washed once with 5 ml 0.1 M Tris-HCl, pH 7.8, and resuspended in 3 ml 0.1 M Tris-HCl, pH 7.8. Cells were disrupted by sonication on ice. The lysates were assessed for chloramphenicol acetyltransferase activity as described by Shaw (1975).

Plasmid stability. TYS broth (200 ml) was inoculated with a single colony from a fresh plate and incubated at 37°C with vigorous aeration overnight. Cell counts of each culture were made (on selective plates as necessary) and 100 colonies were toothpicked onto further selective plates to determine their phenotypes.

RESULTS

Construction and Characterisation of thyP3-containing plasmids

EcoRI-digested pAB224 and *EcoRI*-digested pCD1 were mixed, ligated, and used to transform competent *B. subtilis* QB943 cells. After initial selection for Thy^r transformants, three Tc^r clones were isolated by replica plating onto TSBA plates containing 25 µg/ml Tc. Miniplasmid preparations on these clones revealed the presence of the plasmids pTT1 (5.1 MDa), pTT2 (6.7 MDa), and pTT3 (5.7 MDa).

Restriction enzyme sites on pTT1, pTT2, pTT3, and pCD1 were identified by analysis of single and double restriction enzyme digests. Physical maps of the plasmids are shown in Fig. 1. All three pTT plasmids contain a 2-MDa *EcoRI* fragment corresponding to pAB224. The inserts, which vary in size—3.1,

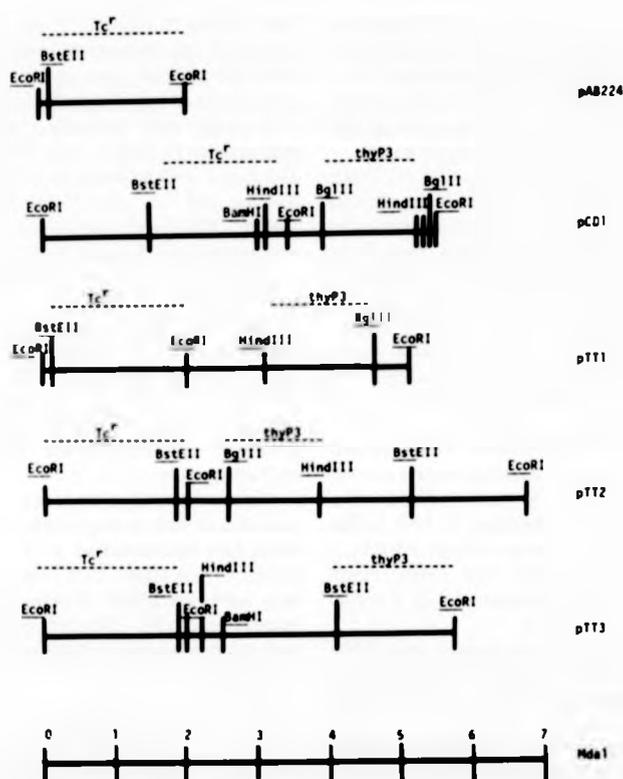


FIG. 1. Restriction enzyme maps of parental and hybrid plasmids.

3.7, and 4.7 MDa for pTT1, pTT3, and pTT2, respectively—contain the *thyP3* gene and additional sequences (Fig. 2). The presence of pAB224, *thyP3*, and pMB9 sequences in pTT1 was confirmed by hybridising nick-translated pTT1 to pAB124 and *EcoRI*-*BglII* double-digested pCD1 (data not presented). None of the constructed hybrids has the structure expected, consisting only of the *thyP3*-containing *EcoRI* fragment of pCD1 inserted into the single *EcoRI* site of pAB224. Genetic rearrangements have therefore occurred in each case, either upon transformation or during subsequent initial growth of the transformants.

Transformation of Competent Cells with Hybrid Plasmids

A comparison of the transforming activity of the three hybrid plasmids pTT1, pTT2, and pTT3 with the parental plasmids pCD1 and pAB224 was made by transforming competent *B. subtilis* QB943 cells (Table 2). The parental plasmids pCD1 and pAB224 could only transform competent cells by *Thy*⁺ and *Tc*^r, respectively. However the three hybrid plasmids were able to transform cells to both *Tc*^r and *Thy*⁺. It was found that if selection was first made for *Tc*^r pTT transformants, then all the transformants were also *Thy*⁺. How-



FIG. 2. Photograph of *Eco*RI-digested plasmid DNA after Agarose Gel Electrophoresis: (B) pCD1; (C) pTT3; (D) pTT2; (E) pTT1; (F) pAB224; (A) *Hind*III-digested λ DNA.

ever, if initial selection was first made for Thy⁺ transformants, then some of the pTT transformants were Tc^r. Values ranging from 20 to 90% of the Thy⁺ transformants also Tc^r have been obtained in other experiments. The pTT plasmids therefore can give rise to two

types of transformants, either Thy⁺Tc^r or Thy⁺Tc^s.

Both types of transformant were screened for the presence of extrachromosomal DNA. Cells transformed to Tc^rThy⁺ by the pTT plasmids did not contain extrachromosomal plasmid DNA; gel analysis revealed the presence of only a chromosomal DNA band. Conversely, cells transformed to Tc^s, Thy⁺ always contained extrachromosomal plasmid DNA. As controls, pAB224 (Tc^rThy⁻) transformants always contained extrachromosomal plasmid DNA whereas pCD1 (Tc^rThy⁺) transformants did not.

The most plausible explanation for the Tc^rThy⁺ type of pTT transformants is integration of plasmid DNA sequences into the *B. subtilis* chromosome. If the Tc^r gene of the pTT plasmid molecule becomes integrated into the chromosome then low-level resistance to Tc might result from expression of the integrated Tc^r genes. However, analysis of 12 independently isolated Tc^rThy⁺ (pTT1) transformants failed to detect resistance to Tc even at 2 μ g/ml. All pTT2 and pTT3 transformants of this type (Thy⁺Tc^s) were also sensitive to low levels of Tc. The Tc^rThy⁺ transformants could therefore be the result of integration of only the *thyP3* region of the plasmid into the host chromosome.

TABLE 2
TRANSFORMATION OF COMPETENT QB943 CELLS WITH PLASMID DNA

Transforming plasmid	Initial selection (transformants/ μ g DNA)	Phenotype of transformants		
		Tc ^r Thy ⁺	Tc ^s Thy ⁺	Tc ^s Thy ⁻
pCD1	Thy ⁺ 1.4×10^5	0	100	—
	Tc ^r 0	—	—	—
pAB224	Thy ⁺ 0	—	—	—
	Tc ^r 1.4×10^6	0	0	100
pTT1	Thy ⁺ 5.2×10^6	50	50	0
	Tc ^r 2.1×10^5	100	0	0
pTT2	Thy ⁺ 3.0×10^5	53	47	0
	Tc ^r 3.2×10^6	100	0	0
pTT3	Thy ⁺ 6.4×10^5	54	46	0
	Tc ^r 4.1×10^5	100	0	0

TABLE 3
TRANSFORMATION OF COMPETENT QB943 CELLS WITH *EcoRI*-DIGESTED PLASMID DNA

Transforming plasmid	Restriction enzyme treatment	Initial selection (transformants/ μ g DNA)	Phenotype of transformants
pCD1	none	Thy ⁺ 4.6×10^5 Tc ^r 0	100 Tc ^r Thy ⁺
	<i>EcoRI</i>	Thy ⁺ 6.0×10^5 Tc ^r 0	100 Tc ^r Thy ⁺
pAB224	none	Thy ⁺ 0 Tc ^r 4.2×10^6	100 Tc ^r Thy ⁺
	<i>EcoRI</i>	Thy ⁺ 0 Tc ^r 0	
pTT1	none	Thy ⁺ 4.4×10^5 Tc ^r 1.2×10^5	60 Tc ^r Thy ⁺ 40 Tc ^r Thy ⁺
	<i>EcoRI</i>	Thy ⁺ 4.4×10^5 Tc ^r 0	100 Tc ^r Thy ⁺ 100 Tc ^r Thy ⁺

Effects of Restriction Enzyme Digestion on Plasmid Transformation

In order to determine whether the *thyP3* gene of pCD1 or pTT1 could integrate into the *B. subtilis* chromosome, independently of other plasmid sequences, competent *B. subtilis* QB943 cells were transformed with *EcoRI*-digested plasmid DNA. The results in Table 3 show that, as expected, *EcoRI*-digested pCD1 was capable of transforming cells to Thy⁺ whereas *EcoRI*-digested pAB224 was unable to transform cells to Tc^r. *EcoRI*-digested pTT1 was only capable of transforming cells to Tc^rThy⁺ and not to Tc^rThy⁺. The resultant transformants were found not to contain extrachromosomal plasmid DNA and thus probably result from integration of only the *thyP3* gene into the host chromosome. Thus the Tc^rThy⁺ transformants produced by transformation with undigested pTT plasmid DNA may also contain only the *thyP3* gene integrated into their chromosome.

Transformation with Monomeric Plasmid DNA

To determine whether or not the two types of transformants produced by transformation with the pTT plasmids could be the result of transformation by different molecular species

of plasmid DNA, cells were transformed with ccc monomeric plasmid DNA. About 5 μ g of pTT1, pTT2, and pTT3 plasmid DNA was electrophoresed on an agarose gel and ccc monomeric plasmid DNA was isolated. The purified plasmid DNA was then used to transform competent *B. subtilis* QB943 cells.

Table 4 shows that ccc monomeric plasmid DNA (approximately 1 μ g) could only transform cells to Thy⁺ and not to Tc^r. A sample of Thy⁺ transformants was screened for the presence of extrachromosomal plasmid DNA but were found not to contain any. Thus part of, or all of the transforming plasmids, must have been integrated into the host chromosome. It is likely therefore that upon transformation of Thy⁺ *B. subtilis* cells with an

TABLE 4
TRANSFORMATION OF COMPETENT QB943 CELLS WITH MONOMERIC PLASMID DNA

Plasmid monomer	Initial selection		Phenotype of Thy ⁺ transformants	
	Tc ^r	Thy ⁺	Tc ^r Thy ⁺	Tc ^r Thy ⁺
pTT1	0	3.7×10^6	0	100
pTT2	0	3.9×10^6	0	100
pTT3	0	2.8×10^5	0	100

unfractionated pTT plasmid preparation, some Tc^r Thy^r transformants arise by the transformation of such cells with monomeric plasmid DNA; conversely Tc^r Thy^r transformants are produced by transformation with multimeric plasmid DNA. It is also possible that multimeric plasmid DNA could give rise to similar transformants since such a molecule could be partially degraded during the transformation event, producing a molecule incapable of autonomous replication. If the *thyP3* gene was still intact, however, such a molecule might still be able to integrate into the host chromosome and transform the cells to Thy^r.

Construction and Characterisation of CAT-Containing Plasmids

Lack of functional expression of the Tc^r genes of the pTT plasmids when the *thyP3* gene integrates into the chromosome might occur by several mechanisms, including poor transcription if integrated, or selective deletion from, or nonintegration into, the chromosome. The chloramphenicol acetyltransferase (CAT) gene of pC194 has been shown

to functionally express when integrated into the *B. subtilis* chromosome (Rapoport *et al.*, 1979). This gene was therefore cloned into pTT1 to further investigate whether plasmid integration was total or limited to specific sequences.

pBD64 is a hybrid plasmid containing the pC194 CAT gene (Gryczan *et al.*, 1980). *Bgl*II-*Bam*HI double-digested pBD64 was ligated to *Bgl*II-digested pTT1 and transformed into *B. subtilis* QB943. Cm^rTc^r Thy^r transformants were isolated and screened for the presence of extrachromosomal DNA. The plasmids pTTC1 to pTTC4 were isolated and cleaved with several restriction enzymes, and the digestion products were analysed. Restriction enzyme maps of the pTTC plasmids and pBD64 are shown in Fig. 3.

All four pTTC plasmids have a molecular weight of 6.3 MDa and have identical restriction enzyme maps. The plasmids are smaller than the expected hybrid containing the entire pTT1 plasmid plus the *Bam*HI-*Bgl*II CAT-containing fragment of pBD64. Thus in each case a site-specific deletion presumably occurred. It is interesting to note that the same *Bgl*II site has been regenerated in all four plas-

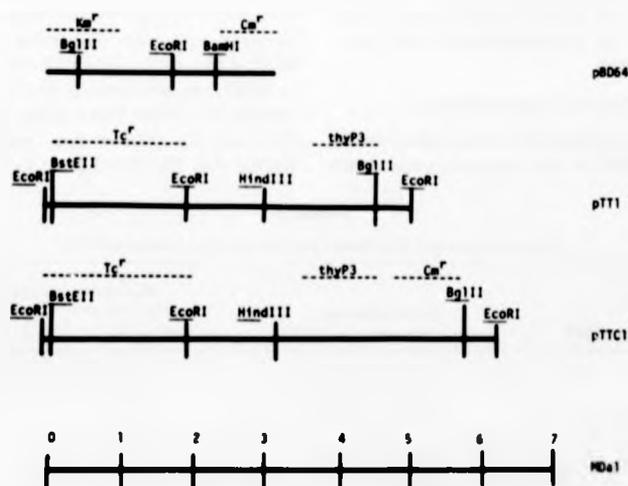


FIG. 3. Restriction enzyme maps of pBD64, pTT1, and pTTC1.

mids, indicating that the pBD64 *Bam*HI-*Bgl*II fragment has been inserted in the same orientation in each case.

Transformation of Competent Cells with pTTC1

The properties of pTTC1 were examined by transforming competent *B. subtilis* BD393 cells with unfractionated pTTC1, monomeric pTTC1, and *Eco*RI-digested pTTC1 (Table 5). Unfractionated pTTC1 gave rise to two types of transformants, the first being Cm^rTe^rThy^r and the second Cm^rThy^r. Monomeric pTTC1 and *Eco*RI-digested pTTC1 could only transform cells to Thy^r and not to antibiotic resistance. A sample of transformants was screened for the presence of extrachromosomal plasmid DNA. It was found that antibiotic-resistant transformants contained extrachromosomal plasmid DNA whereas antibiotic-sensitive transformants did not. CAT assays were performed on 10 antibiotic-sensitive, Thy^r pTTC1 transformants but no CAT activity was detected in these transformants compared with approximately 0.4 IU/ml of culture for antibiotic-resistant transformants. This unexpected finding suggests that the CAT gene is either not integrated into the *B. subtilis* chromosome from these plasmids or, if integrated, is not functionally expressed.

Southern Analysis of Transformants

In order to examine the integration event, chromosomal DNA was isolated from strain

QBT5 (a Te^rThy^r pTT1 transformant of *B. subtilis* QB943) and strain BDTC5 (a Te^rCm^rThy^r pTTC1 transformant of *B. subtilis* BD393). Chromosomal DNA (10 µg) was digested separately with *Eco*RI and *Bgl*II, electrophoresed on agarose gels, and transferred to nitrocellulose membranes. The membranes were then probed using nick-translated plasmid DNA. *Eco*RI- and *Bgl*II-digested *B. subtilis* BD224 chromosomal DNA was used as a control and end-labelled *Hind*III-digested λ DNA was used as a size standard (Fig. 4).

Figure 5 shows the result of a hybridisation using a purified 1.5-MDa *Bgl*II-*Eco*RI fragment of pCD1, containing the *thyP3* gene, as a probe. The probe hybridised to two regions of both *Eco*RI- and *Bgl*II-digested *B. subtilis* BD224 chromosomal DNA. The sizes of the bands are approximately 12 and 1.5 MDa for *Eco*RI-digested chromosomal DNA and 12 and 6.2 MDa for *Bgl*II-digested chromosomal DNA. The ϕ 3T gene has previously been shown to have homology with the *B. subtilis* chromosomal *thyA* gene (Duncan *et al.*, 1978; Galizzi *et al.*, 1981; Stroynowski, 1981). The two bands produced by digestion with either *Eco*RI or *Bgl*II are unlikely to be the result of cleavage within the chromosomal *thyA* gene by these enzymes. Digestion of *B. subtilis* BD224 chromosomal DNA with either *Eco*RI or *Bgl*II does not destroy the Thy^r transforming activity of the DNA (data not presented). Stroynowski (1981) has recently demonstrated that the *thyP3* gene in the phage ϕ 3T

TABLE 5
TRANSFORMATION OF COMPETENT BD393 CELLS WITH pTTC1

Transforming DNA	Initial selection (transformants/µg DNA)	Phenotype of transformants	
		Te ^r Cm ^r Thy ^r	Te ^r Cm ^r Thy ^r
pTTC1	Thy ^r 2.0 × 10 ⁶	69	31
	Te ^r 1.9 × 10 ⁵	100	0
	Cm ^r 1.5 × 10 ⁵	100	0
Monomeric pTTC1	Thy ^r 2.2 × 10 ⁶	0	100
	Te ^r 0	—	—
	Cm ^r 0	—	—
<i>Eco</i> RI digested pTTC1	Thy ^r 6.0 × 10 ⁵	0	100
	Te ^r 0	—	—
	Cm ^r 0	—	—

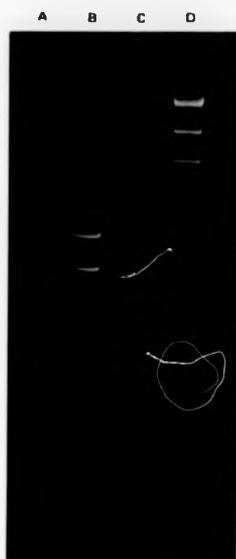


FIG. 4. Photograph of restriction enzyme-cleaved plasmid DNAs after agarose gel electrophoresis: (A) *Bgl*II-*Bam*HI-digested pBD64; (B) *Eco*RI-*Bgl*II-digested pTT1; (C) *Eco*RI-*Bgl*II-digested pTTC1; (D) *Hind*III-digested λ DNA.

is flanked by sequences which are homologous to a region of the *B. subtilis* chromosome not containing the *thyA* gene. This region of homology was shown to be due to the presence of *SP β* prophage sequences, which most strains of *B. subtilis* 168 carry. Thus a probable explanation for the two bands produced from *Eco*RI- or *Bgl*II-cleaved BD224 chromosomal DNA is that the larger band in each case results from hybridisation of the *thyP3* gene to the *B. subtilis* chromosomal *thyA* gene and the smaller band represents hybridisation of *thyP3* flanking sequences to *SP β* prophage DNA.

The tracks containing chromosomal DNA from strain QB15 and strain BDTC5 (both antibiotic-sensitive, Thy⁺ transformants) each have three bands. Two of these bands, of molecular weights approximately 12 and 1.5 MDa for *Eco*RI-digested chromosomal DNA and approximately 12 and 6.2 MDa for *Bgl*II-di-

gested chromosomal DNA, correspond to the bands produced after hybridisation of the probes to *B. subtilis* BD224 digested chromosomal DNA. The third band, which has a molecular weight of approximately 8 MDa after *Bgl*II cleavage of the chromosomal DNA and 4.2 MDa after cleavage with *Eco*RI in both cases, is not present in the *B. subtilis* BD224 control track. The additional band presumably results from hybridisation of the *thyP3* probe to the *thyP3* gene integrated into the *B. subtilis* chromosome. This suggests that a specific integration event has occurred, resulting in the same size fragment from both pTT1 and pTTC1 being integrated into the chromosome.

Figure 6 is an autoradiograph showing the result of hybridising a pC194 probe to *Bgl*II- and *Eco*RI-digested chromosomal DNA. The pC194 probe was a 1.8-MDa *Hind*III restriction fragment of pHV14 isolated from an agarose gel. Only a weak hybridisation band can be seen in each track. The *Eco*RI-digested



FIG. 5. Autoradiograph showing the result of hybridising a labelled *thyP3* probe to (A) *Bgl*II-digested and (B) *Eco*RI-digested BD224 cDNA; (C) *Bgl*II-digested and (D) *Eco*RI-digested QB15 cDNA; (E) *Bgl*II-digested and (F) *Eco*RI-digested BDTC5 cDNA; (G) End-labelled *Hind*III-digested λ DNA.



FIG. 6. Autoradiograph showing the result of hybridising a labelled pC194 probe to (A) *EcoRI*- and (B) *BglII*-digested BD224 cDNA; (C) *EcoRI*- and (D) *BglII*-digested QB15 cDNA; (E) *EcoRI*- and (F) *BglII*-digested BD1C5 cDNA; (G) End-labelled *HindIII*-digested λ DNA.

chromosomal DNAs give a band of about 4.2 MDa and the *BglII*-digested chromosomal DNAs give a band of about 8 MDa. The hybridisation band is not the result of hybridisation to integrated pC194 CAT sequences since the band is also present in the control DNA track. The nature of the pC194 sequences which have weak homology with the *B. subtilis* chromosome is unknown.

No hybridisation was detected when labelled pAB224 DNA was hybridised to *BglII*- and *EcoRI*-digested chromosomal DNAs. This

demonstrates that pAB224 sequences have not been integrated into the chromosomes of the *Thy*⁺, antibiotic-sensitive pTT1 and pTTC1 transformants.

The results from the Southern analysis strongly imply that upon transformation with either pTT1 or pTTC1, *Thy*⁺, antibiotic-sensitive transformants are produced by an integration event resulting in a specific fragment, containing the *thyP3* gene, being inserted into the host chromosome. Although the actual site of *thyP3* integration into the chromosome was not determined, it is probably at or near the *thyA* locus; probably into SP β prophage sequences.

Stability of Hybrid Plasmids

The stability of the hybrid plasmids pTT1, pTT2, pTT3, and pTTC1 was investigated by growing batch cultures of the plasmid-bearing strains. Table 6 shows that in the absence of antibiotic selection pressure, plasmid markers were lost from all strains, though at markedly different rates. Strains containing pTT1 and pTT2 lost both the *Thy*⁺ and *Tc*^r phenotypes simultaneously. This suggests that cells transformed to *Thy*⁺*Tc*^r by either of these two plasmids do not contain an integrated copy of the *thyP3* gene. The reason why pTT2-free strains were detected at a higher frequency than pTT1-free strains is unknown, but could be related to the larger size of pTT2. pTT3-containing cells lost the *Tc*^r marker but retained the *Thy*⁺ phenotype in the absence of selection. Such *Tc*^r*Thy*⁺ clones did not contain extrachromosomal plasmid DNA and there-

TABLE 6
STABILITY OF HYBRID PLASMIDS

Plasmid	Phenotype of transformants selection pressure			
	None	<i>Thy</i> ⁺	<i>Tc</i> ^r	<i>Cm</i> ^r
pTT1	<i>Tc</i> ^r <i>Thy</i> ⁺ (4)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	—
pTT2	<i>Tc</i> ^r <i>Thy</i> ⁺ (23)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	—
pTT3	<i>Tc</i> ^r <i>Thy</i> ⁺ (44)	<i>Tc</i> ^r <i>Thy</i> ⁺ (20)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	—
pTTC1	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (41)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)



FIG. 6. Autoradiograph showing the result of hybridising a labelled pC194 probe to (A) *EcoRI*- and (B) *BglII*-digested BD224 cDNA; (C) *EcoRI*- and (D) *BglII*-digested QBT5 cDNA; (E) *EcoRI*- and (F) *BglII*-digested BDTC5 cDNA; (G) End-labelled *HindIII*-digested λ DNA.

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pTT2	<i>Tc</i> ^r <i>Thy</i> ⁺ (23)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Thy</i> ⁺ (100)	—
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pTTC1	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (41)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)	<i>Tc</i> ^r <i>Cm</i> ^r <i>Thy</i> ⁺ (100)

fore probably contain an integrated copy of the *thyP3* gene. Integration of the *thyP3* gene into the chromosome could have occurred either upon initial transformation of the strain with plasmid DNA or during subsequent vegetative growth of the cells.

The Thy^r, Tc^r, Cm^r genes of pTTC1 were lost simultaneously from plasmid-containing cells. This suggests that cells transformed to Tc^rCm^rThy^r by pTTC1 also do not contain an integrated copy of the *thyP3* gene. The large size of pTTC1 may account for the high frequency of plasmid-free cells detected.

When plasmid-containing strains were grown under selection pressure a different pattern of plasmid stability was observed. If selection for antibiotic resistance was applied no plasmid markers were lost. However, if selection was made for retention of the *thyP3* gene, pTT1, pTT2, and pTTC1 also retained their antibiotic resistance markers. However, with pTT3-containing cells Tc^rThy^r colonies were detected at a frequency of 20%.

DISCUSSION

Instability of hybrid plasmids has been a major problem when using *B. subtilis* as a host for molecular cloning. Several workers (Ehrlich *et al.*, 1976; Jayaraman *et al.*, 1981; Rapoport *et al.*, 1979) have found that cloned *B. subtilis* chromosomal DNA fragments are unstable in *recE*₄ backgrounds. The hybrid plasmids constructed by these workers often underwent gross genetic rearrangements or became integrated into the host chromosome. Some instability problems have been overcome through the use of *recE*₄ host cells (Rapoport *et al.*, 1979; Tanaka & Sakaguchi, 1978). However, even in a *recE*₄ background molecular rearrangements have been observed (Duncan *et al.*, 1978; Fujii & Sakaguchi, 1980; Tanaka, 1979; Uhlen *et al.*, 1981). The pTT series of plasmids appear to be markedly stable even in *recE*₄ hosts; deletions have not been observed.

Previous work by Canosi *et al.* (1978) and Mottes *et al.* (1979) established that monomeric forms of plasmids were not active in

transformation of competent cells. Recently Canosi *et al.* (1981), Bensi *et al.* (1981), and Iglesias *et al.* (1981) constructed a series of hybrid plasmids that contained DNA sequences homologous to regions of the *B. subtilis* chromosome. Monomers of such plasmids were active in transformation only in *recE*₄ hosts. The transformants contained extrachromosomal plasmid DNA; plasmid sequences were not integrated into the host chromosome. These workers concluded that monomeric plasmid DNA could be activated for transformation through integration of any sequences which have homology with the *B. subtilis* chromosome, into the plasmid.

In our experiments transformation with monomeric pTT and pTTC plasmids produced transformants which did not contain extrachromosomal DNA but instead the *thyP3* gene alone was inserted into the host chromosome. In these cases perfect plasmid-chromosome homology did not exist and this could therefore affect the ability of monomeric plasmid DNA to transform competent cells to antibiotic resistance. In order to test this, cells already carrying a chromosomally integrated *thyP3* gene were transformed with monomeric pTT1 and pTTC1. However, even in this case, with the same gene present on both chromosome and plasmid, monomeric pTT1 or pTTC1 could not transform cells to Tc^r.

Integration of the *thyP3* gene alone from the pTT and pTTC plasmids into the host chromosome closely agrees with the finding of Ehrlich *et al.* (1976) and is in contrast to the total plasmid integration reported by Duncan *et al.* (1978) and Galizzi *et al.* (1981). This infers that integration of plasmid sequences into the host chromosome may be dependent, in some way, on the nature of the plasmid vector itself as well as the cloned homologous sequences. Also the lack of transformation of competent *B. subtilis* cells, containing an integrated *thyP3* gene, to antibiotic resistance by monomeric pTT or pTTC plasmid DNA contrasts with the recent work of Canosi *et al.* (1981) and strongly suggests that chromosomal homology alone is not sufficient to activate monomeric plasmid DNA

for transformation. Several of the above workers have used pC194 in their constructions and the influence of the weak homology reported in this paper between sequences from that plasmid and the *B. subtilis* chromosome may make interpretation of integration events more difficult.

Stability analysis of the plasmids revealed that in the case of pTT1, pTT2, and pTTC1, if an autonomously replicating plasmid is present within the cell, then the *thyP3* gene does not integrate into the chromosome. However, cells harbouring pTT3 may, in addition to the extrachromosomal plasmid-borne *thyP3* gene, also contain an integrated copy of this gene. Alternatively the *thyP3* gene of pTT3 may become integrated into the chromosome as plasmid is lost from the cell. Integration of the *thyP3* gene thus may cause or result in plasmid loss. The reason why the *thyP3* gene of pTT3 becomes integrated into the host chromosome while the *thyP3* gene of pTT1, pTT2, and pTTC1 does not, is unknown. It must clearly be due to differences in the *thyP3* flanking sequences. This demonstrates how sequences surrounding a cloned gene may influence its stability and integration properties.

The plasmids pTT1, pTT2, and pTTC1 are not lost from the cell if selection for a plasmid-borne marker is applied. Derivatives of these *thyP3*-containing plasmids could prove to be useful for the production of cloned gene products on an industrial scale in the absence of added antibiotics. When cells containing these plasmids are grown in complex broth without added thymine and with no antibiotic present, there is a selective pressure against cells which lose plasmid DNA since such cells would undergo thymineless death.

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