PLASMIDS FROM THERMOPHILIC BACILLI

by

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Abstract

A large number (155) of strains of thermophilic bacilli were isolated and fully characterised; strains showing antibiotic resistance or bacteriocin production were examined for the presence of covalently closed circular DNA molecules (plasmids). Six of the strains tested contained plasmids. Strain RS93, a strong bacteriocin producer contained a plasmid (pT93) of molecular weight 4.1 x 10^6 and two streptomycin resistant strains contained the same two plasmids, pAB118A (4.9 x 10^6) and pAB118B (3.0 x 10^6). Two of the tetracycline resistant strains contained the same plasmid (pAB124) of molecular weight 2.9 x 10^6 whilst a third harboured a small plasmid, pAB128 (2.5 x 10^6). These plasmids were digested with 19 different restriction endonucleases and the number of cleavage sites determined. Transformation of Bacillus subtilis 168 (Trp C2) with purified plasmid DNA indicated that pAB124 and pAB128 conferred tetracycline resistance on the host.

Plasmid pAB124 was further characterised with restriction endonucleases and a cleavage-site map constructed. Tetracycline resistance was associated with a 1.95 x 10^6 region of pAB124 lying between two EcoRI sites, and this region was circularised to produce a viable tetracycline resistance plasmid (pAB224). A second plasmid (pAB524) containing an additional EcoRI fragment of pAB124 was constructed. Restriction endonuclease cleavage maps of pAB224 and pAB524 were then constructed.

A chimeric plasmid of pAB124 and an Escherichia coli plasmid, pROG29 (Ap^r) containing the yeast leu2 gene was constructed and expression in E. coli and B. subtilis examined. Similarily a chimeric plasmid of pAB124 and a Staphylococcal plasmid, pUB110 (Neo^r) was constructed and the resulting deletion varient pAB324 characterised.

A method was developed for the preparation of Bacillus stearothermophilus protoplasts and their regeneration to the bacillary form. Transformation of these protoplasts with pAB124 plasmid DNA was achieved and expression of the plasmid in the new host examined.

Contents

Abbreviations

A:	Adenosine
A^{260}:	Absorbance at 260 nanometers
ATP:	Adenosine-5'-triphosphate
Ap^r:	Ampicillin resistance
Ad2:	Adenovirus 2
BSA:	Bovine Serum Albumin
C:	Cytidine
Cm^r:	Chloramphenicol resistance
cpm:	counts per minute
DEAE:	Diethylamino-ethyl
DE52:	Diethylaminoethyl (No52) cellulose
DTT:	Dithiothreitol
DNA:	Deoxyribonucleic acid
DNase:	Deoxyribonuclease
cDNA:	chromosomal Deoxyribonucleic acid
dA:	deoxyadenosine
dC:	deoxycytidine
dG:	deoxyguanosine
dT:	deoxythymidine
dATP:	deoxyadenosine-5'-triphosphate
dCTP:	deoxycytidine-5'-triphosphate
dGTP:	deoxyguanosine-5'-triphosphate
dTTP:	deoxythymidine-5'-triphosphate
dNTP:	deoxynucleoside-5'-triphosphate
Em^r:	Erythromycin resistance
G:	Guanosine
Km^r:	Kanamycin resistance
λ:	Lambda phage
MIC:	Minimal Inhibitory Concentration
Md:	Megadaltons (10^6 daltons)
mRNA:	messenger Ribonucleic acid
Na_2.EDTA:	Diaminoethane-tetraacetic acid disodium salt
Neo^r/Neo^s:	Neomycin resistance/sensitivity
OD:	Optical Density
pdC:	poly. deoxycytidine
pdG:	poly. deoxyguanosine
PEG:	Polyethylene glycol
PEI:	Polyethyleneimine
PMSF:	Phenylmethyl-sulphonyl fluoride
RNA:	Ribonucleic acid

RNase:	Ribonuclease
SDS:	Sodium dodecyl sulphate
Sm^r/Sm^s:	Streptomycin resistance/sensitivity
SV40:	Simian virus 40
T:	Thymidine
Tc^r/Tc^s:	Tetracycline resistance/sensitivity
TCA:	Tri-chloroacetic acid
Tris:	Tris (hydroxymethyl) methylamine
UV:	Ultra Violet
v/v:	volume to volume
w/v:	weight to volume

Restriction endonucleases are named according to the nomenclature of Smith & Nathans (1973); see table 2.2.

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CHAPTER ONE

INTRODUCTION

Plasmids are extrachromosomal autonomously replicating genetic elements found in a wide variety of naturally occurring Gram-positive and Gram-negative bacteria (Broda, 1979). The first plasmids isolated were from species of Shigella and were associated with multiple drug resistance that could be transferred simultaneously to other bacteria (Ochiai et. al., 1959; Akiba et. al., 1960). Such infectious multiple drug resistance was then reported for a strain of Salmonella typhimurium (Datta, 1962). The medical importance of infectious multiple drug resistance and the use of some Escherichia coli plasmids (F factors) in the development of molecular biology has resulted in most research being carried out on plasmids in Gram-negative bacteria, in particular Pseudomonas species and Escherichia coli.

1.1 Isolation of plasmid DNA

Plasmid DNA was first isolated by Marmur et. al., (1961) and involved the transfer of an E. coli plasmid (F') containing the lactose operon to Serratia marcescens. Since Serratia has DNA containing a higher proportion of guanosine-cytosine base pairs, the DNA is more dense than the F'lac plasmid. Thus the Serratia DNA and plasmid DNA could be separated in caesium chloride density gradients, so providing evidence for the extra-chromosomal state of plasmids. This approach was also used to confirm the autonomous nature of Col-plasmids (DeWitt & Helinski, 1965) and R-plasmids (Falkow et. al., 1966; Rownd et. al., 1966).

The first plasmids isolated were identified as linear DNA molecules but as gentler methods of cell lysis became available plasmids were shown by electron microscopy to be circular molecules, in fact covalently closed circular (CCC) supercoiled molecules (Roth & Helinski, 1967; Hickson et. al., 1968; Freifelder, 1968). This property has been used to separate and purify plasmids to great effect.

After lysis, usually with lysozyme and detergent (Clewell & Helinski, 1970; Frazer & Curtis, 1973), the removal of cell debris and chromosomal DNA can be achieved in either of two ways. Either by precipitation of the chromosome by prior treatment with high concentrations of NaCl (Guerry et. al., 1973) or by alkali denaturation, rapid renaturation, followed by removal of single stranded DNA with nitrocellulose (Sharp et. al., 1972)

or phenol (Currier & Nester, 1976). This latter step relies on the fact that since plasmid DNA is present as CCC supercoils the two strands of the duplex do not separate and therefore reassociate readily during the rapid renaturation step. Linear and open circular DNA molecules remain as single-stranded molecules.

The remaining DNA can then be concentrated by precipitation with either ethanol (Currier & Nester, 1976) or polyethylene glycol (Humphreys et. al., 1975) prior to further purification of the plasmid. There are three main procedures for resolving plasmid DNA and the first method reported, and the most commonly used, is ethidium bromide-caesium chloride density gradient centrifugation (Radloff et. al., 1967). This method relies on the CCC supercoiled nature of plasmids, as do the other two procedures. Radloff et. al., (1967) noted that linear and open circular DNA molecules bind more of the intercalating dye, ethidium bromide, than supercoiled DNA. In caesium chloride solutions, Cs^+ ions are displaced and the DNA becomes less dense. This difference in density allows the resolution of CCC supercoils from other DNA species. A second method involves the sedimentation of DNA in sucrose gradients (Hughes and Meynell, 1977). Here resolution of the CCC species occurs because CCC molecules sediment more rapidly than open circles of the same mass due to their more compact form as a result of supercoiling. The final method of plasmid resolution uses electrophoresis of plasmid DNA through an agarose gel (Meyers et. al., 1976). Again, CCC molecules migrate faster than open circular or linear molecules of the same mass. This latter method is not easily applied on a large scale and is predominantly used as a means of plasmid detection in cell extracts.

More recently several screening methods for detecting and sizing plasmid DNA from small bacterial cultures have been reported; by Meyers et. al., (1976) and Hansen & Olsen (1978a) for 30 ml - 50 ml cultures and by Barnes (1977), Telford et. al., (1977) and Eckhart (1978) for single colonies. All these procedures use electrophoresis through agarose gels as the method of detecting plasmid DNA.

The development of these simple techniques for plasmid isolation has resulted in a large number of plasmids being detected in bacteria (Bukhari et. al., 1977); ranging in size from 1.5 megadaltons (plasmid from E. coli 15) Cozzarelli et. al., (1968) to over 200 megadaltons (Duggleby et. al., 1977; Fennewald et. al., 1978; Hansen & Olsen, 1978 a, b), and has allowed detailed characterisation of plasmids in bacteria.

1.2. Characterisation of Plasmids

The early studies on plasmids established two major types on the basis of overall genetic content (Helinski, 1976).

The first major class of plasmids are the conjugative or self-transmissible plasmids that are also termed sex factors. These plasmids have functional genes for autonomous replication and conjugal mating. If these sex factors also possess defined genetic regions of the chromosome they are designated F-prime plasmids (Lederberg et. al., 1952) and were originally identified in E. coli strains (Hayes, 1953). If additional genes are present this group can be subdivided into Col plasmids, those that produce a bacteriocin, or R-plasmids responsible for antibiotic resistance. These conjugative plasmids are most commonly found among the enteric and related Gram-negative bacteria (Helinski, 1973). However, they have also been identified in the Gram-positive genera Streptomyces (Hopwood, 1967) and Streptococcus (Jacob and Hobbs, 1974).

The second major class of plasmids are the non-conjugative or non-self transmissible plasmids, having in common, genes for autonomous replication but no genes promoting conjugal transfer of the plasmid or host chromosome. If these plasmids, in addition, contain genes involved in bacteriocin production (eg ColE1 · Hershfield et. al., 1974) or antibiotic resistance (pI258, Novick et. al., 1979) they are subdivided into Col and R-plasmids respectively.

The mode of replication of plasmids in the host cell is analagous to that of the chromosome (Meselson & Stahl, 1958). Replication is semi-conservative (Bazaral & Helinski, 1970) i.e. a complete new strand is made as the complement of each old strand, so that daughter plasmid molecules each consist of an old strand and a newly synthesised one. In addition it has been shown that most plasmids replicate from one origin eg. Col E1 (Lovett et. al., 1974) although RSF1040 was shown to have two independent origins (Crosa et. al., 1975; 1976); and that replication can proceed both unidirectionaly (eg ColE1 ; Lovett et. al., 1974) or bidirectionaly (eg R6K; Lovett et. al., 1975).

The base sequences at the origins of replication of ColE1 (Tomizawa et. al., 1977) and pBR345 (Bolivar et. al., 1977a) have been determined, although it is not yet known how initiation of replication occurs at these points in the plasmid.

All the evidence suggests that plasmids depend largely on their host

for their replication, since replication mutants affecting chromosome replication generally also affect plasmid replication. For example, dnaA mutations prevent initiation of replication while dnaB and dnaE mutations affect chain growth (Broda, 1979) of both chromosomal and plasmid DNA. There is some evidence, although not conclusive, that plasmids do specify a few proteins concerned in their replication. These are, an enzyme that nicks the double strand of DNA close to the origin of replication, proteins that regulate the number of copies of plasmid per cell and attachment of plasmids to cell structures (Broda, 1979). One further plasmid-specified replication function is incompatibility. This is observed if replication of two plasmids is subject to common regulation and if both are present in the same cell. One or other will then be lost eg between incoming F' factors and the integrated F factors of Hfr strains (Scaife & Gross, 1962; Maas & Maas, 1962). This incompatibility exhibited by some plasmids has allowed their classification into groups, i.e. incompatibility groups (Chabbert et. al., 1972; Datta, 1975; Novick et. al., 1976).

Several of the non-conjugative plasmids isolated from bacteria are present in multiple copies per chromosome per cell (10-15 in the case of ColE1) whilst most conjugative plasmids are present as only 1-2 copies per chromosome per cell. This suggests a different mode of replication control. Plasmids present in only a few copies per cell are thought to replicate such that each copy is replicated once per generation so all plasmid DNA in a cell population would be replicated once before any is replicated a second time (Broda, 1979). This is analogous to chromosome replication (Meselson & Stahl, 1958) and plasmids replicating this way are termed to be under stringent control. In contrast, the multicopy plasmids have been shown to replicate some plasmid DNA molecules twice in the time it takes to replicate only half the chromosome, while a third of the plasmid molecules had not been replicated in 1.5 generations. This mode of replication is termed relaxed control (Novick et. al., 1976) and by definition such replication is not coupled to chromosome replication (the relaxed and stringent control of plasmid replication is not the same as relaxed and stringent control of protein biosynthesis). The initial basis of this definition was the discovery that ColE1) can replicate in the absence of chromosome replication after the addition of chloramphenicol. One plasmid, NR1, is unusual in that it is under stringent control in E. coli but relaxed control in Proteus mirabilis (Rownd, 1969). The mechanism of control of replication is still unclear although two models have been postulated. The model of Jacob, Brenner & Cuzin (1963) states that regulation is

achieved by means of the availability of membrane attachment sites and competition between incompatible plasmids for available sites. Whilst the model of Pritchard, Barth & Collins (1969) postulates that control is through a repressor; the number of repressor molecules would be determined by the number of plasmid copies.

The classification of plasmids using genetic content and incompatibility is a convenient and commonly used system for plasmids from Gram-negative bacteria. Incompatibility groups, although an index of common replication functions, also reflect substantial homology between plasmids. Such sequence homology, determined by heteroduplex analysis, was found between several F, Col V and 3 R-plasmids, all specifying different phenotypes, and this homology was of large DNA segments (Sharp et. al., 1973a). Heteroduplex analysis has been used extensively to examine the sequence homology within a plasmid.

When single-stranded plasmid DNA is submitted to renaturing conditions designed to promote anealing within a single strand an unusual structure was observed under the electron microscope with plasmids R6, R100-1, Col V and F8(N33) (Sharp et. al., 1972). That is, single stranded loops on a short duplex stalk, caused by each strand carrying one sequence complementary to another sequence on the same strand ie an inverted repeat. It was discovered that some mutations in operons carried by λ transducing phages were due to insertion of alien DNA (Shapiro, 1969) and expression 'downstream' from the point of insertion was blocked (polar mutations). The termini of these insertion sequences can also serve as the end points in the formation of deletions (Reif & Saedler, 1975). Five, apparently unrelated, insertion sequences have been described in E. coli (chromosome and plasmids, eg F, R6, R100) and λ. It is thought that insertion sequences are involved in two kinds of recombination. Firstly between pairs of homologous sequences eg Hfr and F' (Guyer, 1978; Broda, 1979) and secondly translocation leading to polar mutations.

Recently an insertion sequence, common to several quite unrelated plasmids, was identified that contained an inverted repeat at each end ie, a transposon. (Heffron et. al., 1975;). The insertion sequence coded for the enzyme β-lactamase, responsible for penicillin resistance, and was bound by an inverted repeat of about 40 nucleotides (Kleckner, 1977). This transposable element (TnA) can be transferred between plasmids (Datta et. al., 1971; Hedges & Jacob, 1974) and between the chromosome and plasmid DNA (Richmond & Sykes, 1972).

Since insertion sequences and transposons are means of joining unrelated segments of DNA it is likely that they have played a major

role in plasmid evolution (Cohen, 1976).

1.3. Uses of plasmids

One property that plasmids possess that gives them a great value as experimental tools in genetics is their ease of transfer between bacteria. This can be effected by transformation sensitising the cell to uptake of DNA, eg $CaCl_2$ treatment of E. coli, or by conjugation (eg F plasmids) involving the conjugative plasmids. The conjugation process is complex depending on about 20 genes which specify the sex-pilus, transfer of the DNA and the overall control of conjugation. The conjugative plasmids as a result tend to be quite large. The smallest known is 17 megadaltons (R6K derivative; Crosa et. al., 1975) while non-conjugative plasmids can be as small as 1 megadalton (Broda, 1979). The conjugative plasmids can promote transfer of non-conjugative plasmids in the cell and of the chromosome. This latter property has been used to map the E. coli chromosome and is a result of the insertion of the entire chromosome into the F plasmid which is then transferred into an F^- host (Jacob & Wollman, 1958).

Plasmids can also be transferred between different genera of the Enterobacteriacea group eg, the nitrogen fixation genes from Klebsiella have been transferred to E. coli using F (Cannon et. al., 1976) and to Azotobacter using RP4 (Cannon & Postgate, 1976).

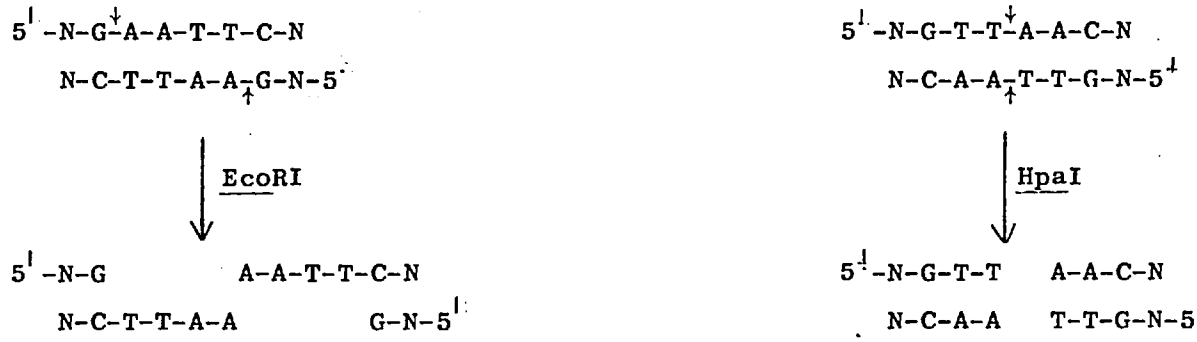
Plasmid DNA can also be joined with fragments of foreign DNA in vitro. The foreign DNA can be isolated from any source, natural or synthetic, making new hybrid or recombinant molecules which can then be introduced into bacterial cells (Cohen, 1975). Therefore large amounts of recombinant DNA can be easily purified (especially if a multi-copy plasmid is used eg pBR322; Bolivar et. al., 1977b), and any transcription or translation products identified and isolated.

Three basic steps can be identified in carrying out such experiments. Firstly, the isolation of DNA fragments from the donor organism; secondly, insertion of the isolated fragment into a vector molecule (eg plasmid); and thirdly, replication and expression of the recombinant DNA molecule in a host organism.

The key to producing DNA fragments rests largely with a group of enzymes called restriction enzymes, which were first identified by their

activity of specifically degrading (restricting) certain types of phage DNA (Arber & Dussoix; 1962). They have been shown to be highly specific endonucleases (Kelly & Smith, 1970) and have been identified in a multitude of bacteria (Roberts, 1980). There are two classes of restriction endonuclease. Those that recognise a DNA nucleotide sequence but then cleave the DNA at random points along its length are known as Class I enzymes and have a requirement for S-adenosyl methionine, ATP and Mg^{2+}. The second group (Class II) recognise a specific nucleotide sequence but cleave the DNA within, or in close proximity to the sequence, so producing a defined and limited number of discrete fragments. Class II enzymes have a requirement for Mg^{2+} only. They are of particular use in molecular genetics since they produce defined fragments.

Class II restriction endonucleases cleave the duplex DNA in one of two ways; either, to produce fully double stranded (flush ended) fragments eg HpaI or, producing fragments with single-stranded self-complementary (cohesive) termini. (eg EcoRI).



The digestion of DNA molecules with Class II enzymes produces a mixture of fragments that are usually fractionated by agarose-gel electrophoresis (Sharp et. al., 1973b) which can separate fragments ranging in size from 10^4 to 3×10^7 daltons. Due to the specificity of Class II enzymes (Roberts, 1980) fragments will be produced with restriction endonucleases that are specific to a given DNA molecule and can therefore be used as a method of physical characterisation (Roberts, 1976; Bingham & Atkinson, 1978).

There are two other methods of producing DNA fragments for in vitro recombination. One method uses hydrodynamic shearing forces that produce randomly sized fragments from a DNA molecule and has been used mainly for constructing E. coli, yeast and Drosophila gene banks (Wensink et. al., 1974; Clarke & Carbon, 1975 and 1976; Struhl et. al., 1976; Carbon et. al., 1977). The second method is necessary for cloning RNA molecules, where reverse transcriptase is used to produce a DNA transcript of the RNA molecule (Temin & Baltimore, 1972) usually mRNA. A number of eukaryotic genes have been

introduced into E. coli by this procedure (Rougeon et. al., 1975; Rabbits, 1976; Maniatis et. al., 1976; Higuchi et. al., 1976).

The choice of a vector molecule into which the isolated DNA fragments can be inserted is restricted by four basic requirements. Firstly, the vector DNA must be able to infect a suitable host organism and replicate within that host. Secondly, the vector must be able to accept foreign DNA, and usually it is advantageous in this context for the vector DNA to contain a single site for a specific restriction endonuclease. Thirdly, insertion of this foreign DNA must not impair essential functions such as control of replication. Fourthly, the vector must have at least one selective marker, eg antibiotic resistance, to enable identification of host cells which have taken up the vector from a mixed population.

The properties of plasmids, described previously, make them ideal vectors. Plasmids are easy to isolate, can infect host cells readily, may carry several selective markers (commonly antibiotic resistance or bacteriocin production) and quite often have suitable restriction endonuclease sites for cloning. A wide variety of plasmid vectors have therefore been isolated and/or constructed for cloning in E. coli (Cohen et. al., 1973; Hershfield et. al., 1974; Tanaka et. al., 1975; Rodriguez et. al., 1976; Boyer et. al., 1977; Bolivar et. al., 1977b). Plasmids have 2 additional properties that make them ideal vectors. They are often present in the cell in multiple copies, 2-30 copies per cell depending on the plasmid. This number can be increased, 100-fold in some cases, by treating the cells with chloramphenicol (Clewell, 1972; Hershfield et. al., 1974; Bolivar et. al., 1977b). The second advantage is derived from the ability of some bacteria to "bud-off" minicells into which plasmid DNA, but not chromosomal DNA, can segregate (Frazer & Curtiss, 1975). This enables the synthesis of plasmid coded gene products to be studied in an environment with a low background of host proteins.

An alternative vector system for E. coli K12 based on the bacteriophage lambda has also been developed (Enquist et. al., 1976; Leder et. al., 1977; Williams et. al., 1977; Murray et. al., 1976; Moir & Brammar, 1976; Donoghue & Sharp, 1977) but will not be discussed here.

The insertion of DNA fragments into a vector molecule relies on the fact that the action of restriction endonucleases producing a cohesive terminus can be reversed by another enzyme DNA-ligase. The complementary single-stranded 'cohesive tails' will readily reassociate by base pairing at temperatures below 15^{o}C and the phosphodiester linkage reformed by DNA-ligase. (Modrich et. al., 1973; Weiss et. al., 1968). Therefore two different fragments of DNA can be readily hybridised and joined providing they were produced by digestion with the same restriction endonuclease.

It is possible to form covalent bonds with blunt-ended fragments using a different DNA-ligase produced by cells infected with the bacteriophage T4 (Sgaramella, 1972; Sgaramella et. al., 1977), although the reaction occurs at low rates and appears to favour small DNA fragments (Sgaramella et. al., 1977).

An example of a cloning experiment using a plasmid vector is outlined in figure 1.1. The plasmid vector contains one site for the restriction endonuclease BamHI and digestion with this enzyme produces a linear molecule. E. coli chromosomal DNA is also digested with BamHI to produce many different sized fragments and these are added to the digested vector. The BamHI activity is destroyed by heat inactivation (66^{o}C for 15 min); the temperature of the mixture reduced to 10^{o}C and E. coli DNA-ligase added. Since digestion of chromosomal DNA with BamHI produces a large number of differently sized fragments, the recombinant molecules will be heterogeneous in respect to size. Since the BamHI site of the plasmid lies within the tetracycline resistance gene, insertion of foreign DNA inactivates this marker. Therefore recombinant molecules will be Tc^{s} and Ap^{r}.

DNA fragments with cohesive termini necessary for efficient ligation can also be obtained using a different procedure involving the enzyme terminal deoxynucleotidyl transferase isolated from calf thymus. This enzyme catalyses the transfer of nucleotides (as dATP, dTTP, dGTP, dCTP) to the 3'-termini of DNA fragments (Chang & Bollum, 1971). In the presence of just one nucleotide a 3'-homopolymeric tail will be synthesised (Jackson et. al., 1972; Lobban & Kaiser, 1973). In a typical genetic manipulation experiment (Fig 1.2) the vector DNA is tailed with a poly.dC tail and the donor DNA with a poly.dG tail. These DNA species now contain homopolymeric complementary tails and can therefore anneal.

An advantage of preparing recombinant molecules by tailing is that only recombinant molecules can be produced; the tailed vector DNA or donor DNA fragments can not associate with themselves by base-pairing since they have non-complementary tails. With restriction endonuclease cloning the vector and donor species of DNA have complementary sequences therefore circularisation of the vector and donor, and donor-donor recombinants occur to a certain extent.

If a restriction endonuclease cleavage site was used for preparing a recombinant molecule, digestion with the same enzyme will excise the inserted DNA fragment. With the terminal transferase method excision is more difficult. However, the endonuclease SI from Aspergillus oryzae under defined conditions (Hofstetter et. al., 1976) cleaves DNA preferentially at poly (dA:dT) sequences, thus if this sequence was used for insertion then excision of the foreign DNA can be achieved with moderate efficiency. An additional disadvantage of the 'tailing' method of gene

Figure 1.1. Insertion of foreign DNA into a vector genome using restriction endonucleases

The plasmid pBR322 (Bolivar et. al., 1977b) is used as an example, and insertion of DNA at the BamHI cleavage site inactivates the tetracycline resistance gene . Recombinant molecules will therefore be Ap^r Tc^s and can be differentiated from the parental plasmid (Ap^r Tc^r).

FIG I.I

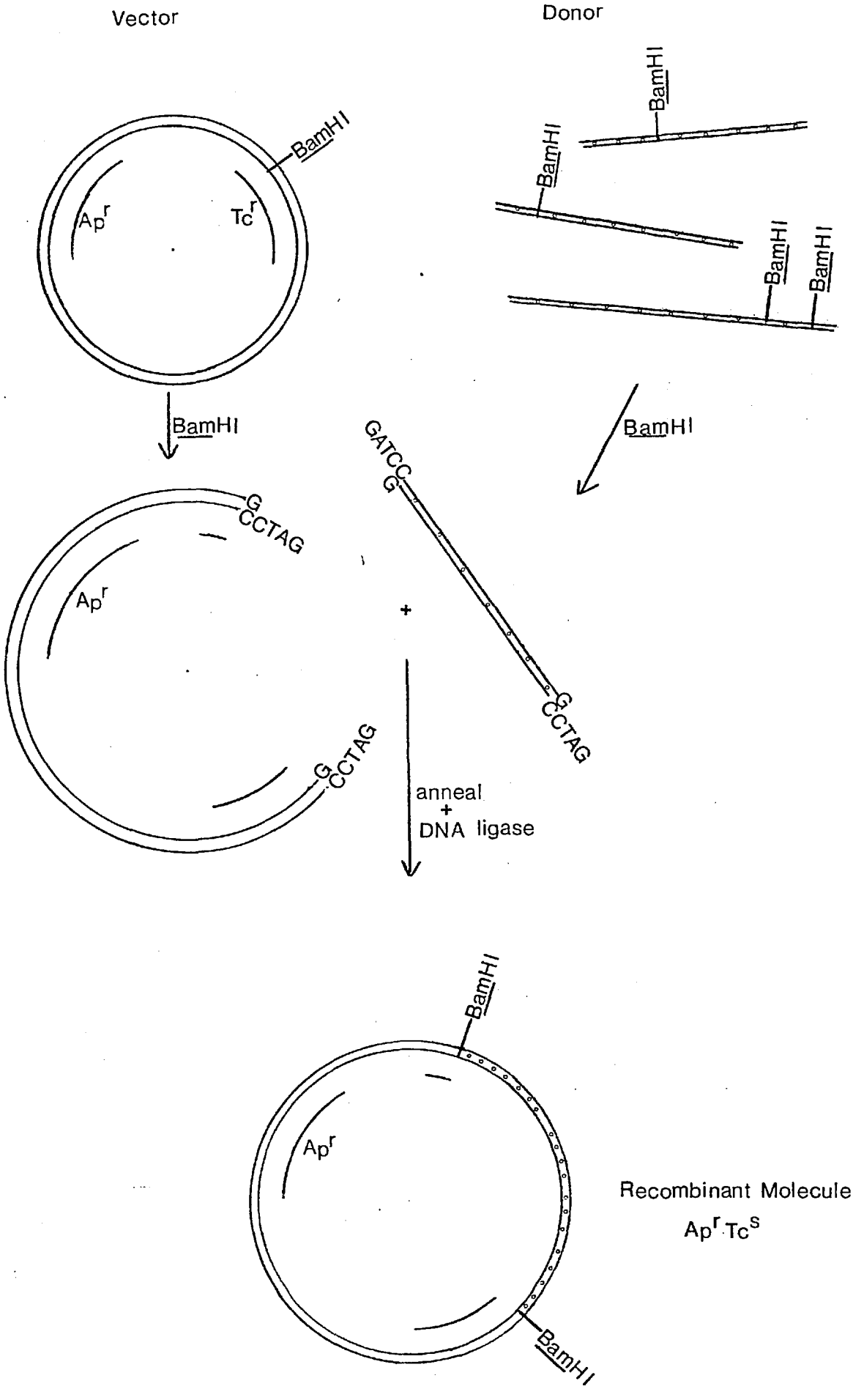
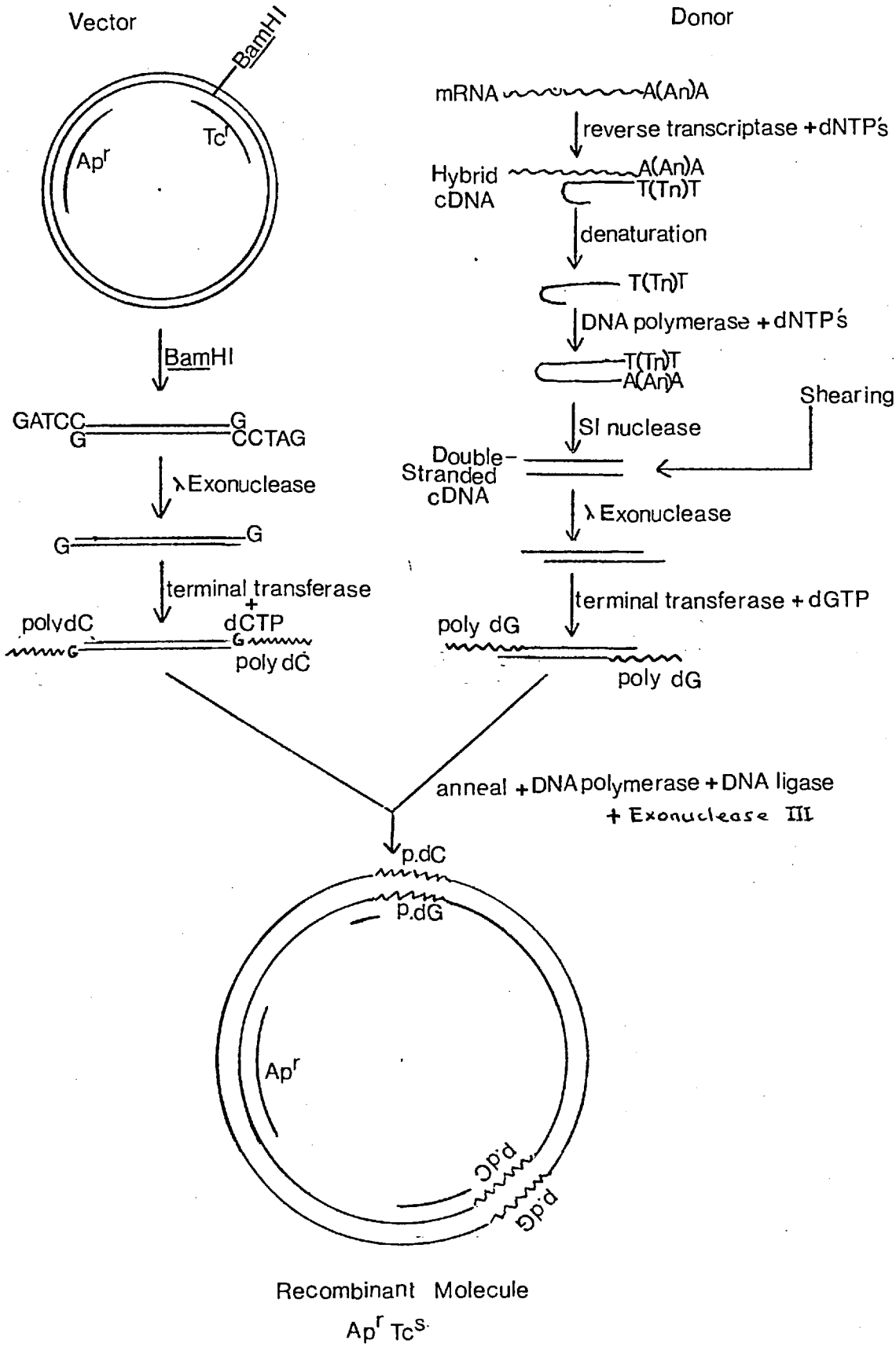

Vector
Donor
BamHI
Ap^r
Tc^r
BamHI
BamHI
BamHI
BamHI
BamHI
BamHI
GATCC
G
G
CCTAG
Ap^r
+
G
CCTAG
anneal
+
DNA ligase
BamHI
Ap^r
BamHI
Recombinant Molecule
Ap^r Tc^s

Figure 1.2. Insertion of foreign DNA into a vector genome by the terminal transferase method

The plasmid pBR322 (Bolivar et. al., 1977b) is used as an example, and insertion of DNA at the BamHI cleavage site inactivates the tetracycline resistance gene . Recombinant molecules will therefore be Ap^r Tc^s and can be differentiated from the parental plasmid (Ap^r Tc^r).

FIG 1.2



Recombinant Molecule

$Ap^r\ Tc^s$

cloning is the necessity for equal length tails on either species of DNA for efficient annealing. This requires very precise and reproducible conditions for the 'tailing' reaction.

The final stage in in vitro genetic manipulation experiments is the transfer of the recombinant molecules to a host organism. The choice of host is based largely on the susceptibility to infection by the vector being used and also on the availability of mutants of the host. Some E. coli strains have been specifically constructed for use as hosts for genetic manipulations (Curtiss et. al., 1977). These generally contain non-revertable mutations which reduce their ability to survive outside of very defined laboratory conditions ('biologically contained' strains).

The recombinant molecules can be transformed into E. coli cells after the exponentially growing cells have been treated with calcium chloride (Mandel & Higa, 1970; Cohen et. al., 1972). The E. coli cells which have the recombinant molecules are identified by selecting for the marker on the vector eg, antibiotic resistance. There are three major ways of identifying foreign DNA in the recombinant vectors selected by antibiotic resistance.

Firstly, one can look for complementation of existing host mutations by the recombinant molecule eg, conversion of trp^- to trp^+. This is dependent on the availability of mutant strains, the donor DNA must contain a gene able to functionally complement a host mutation and the donor DNA must be expressed in E. coli. This method has been used to isolate specific regions of the E. coli chromosome (Clarke & Carbon, 1976; Carbon et. al., 1977) and to show that genes from simple eukaryotes eg, yeast (Struhl et. al., 1976; Ratzkin & Carbon, 1977) and Neurospora (Vapnek et. al., 1977) are expressed in E. coli.

Secondly, one can identify foreign DNA in recombinant vectors by nucleic acid hybridisation (Grunstein & Hogness, 1975; Southern, 1975). Host cells containing possible recombinant vectors are transferred to nitrocellulose filters and then lysed. The released DNA is denatured and then fixed to the filter. A nucleic acid probe (DNA or RNA) radiolabelled to high specific activity is added and in situ hybridisation allowed to take place. Recombinant vectors that contain nucleotide sequences complementary to those of the probe bind the radioactive label and can be detected by autoradiography.

The third method involves immunoassay procedures (Sanzey et. al., 1976; Skalka & Shapiro, 1976; Ehrlich et. al., 1978). This is dependent on the ability of an inserted DNA sequence in a recombinant vector to code for the synthesis of an identifiable protein product in the host. Immune serum directed against a specific protein of a donor organism is first obtained and then incorporated into agar plates on which the

E. coli cells transformed by the recombinants are grown. The normal Antibody-Antigen reaction can be detected visually or by attaching a radio isotope or fluorescent marker to the antibody.

Having identified hosts containing a region of donor DNA of interest, large quantities of the recombinant molecule can be isolated, the expression of the DNA insert examined and a gene product purified.

Several reviews of genetic manipulation have been published (Cohen, 1975; Murray, 1976; Primrose, 1977) and describe the techniques used in more detail.

Due to intensive study over many years knowledge on the genetics and physiology of E. coli is unsurpassed. In comparison, knowledge of the genetics of any other organism is still in a primitive state. For this reason E. coli and its associated plasmids and bacteriophages have been developed as the host-vector system for genetic manipultion experiments. However, E. coli is a normal inhabitant of the alimentary tract of man and domestic animals and also most strains produce an endotoxin.

This endotoxin is a component of the outer membrane of Gram-negative bacteria and consists of a heteropolysaccharide covalently linked to a lipid region (Kabir et. al., 1978). The lipid moiety is responsible for the toxic effects of the endotoxin, which are mainly pyrogenic, although several other toxic effects are known (Kabir et. al., 1978). The production of endotoxin by E. coli makes its use as host in genetic manipulation experiments designed to produce a pharmaceutical product eg vaccines, undesirable - a conclusion recently reported by the MRC Vaccine Committee (J. Melling, pers. comm.).

The problem of endotoxin production linked to the high risk of gut infection, particularly when E. coli is grown on a large scale, and subsequent transfer of the plasmid to another member of the enterobacteriaçae has led to concern over the advisability of using E. coli as a host in genetic manipulation experiments (Berg et. al., 1974). The development of disabled strains eg E. coli X1776 that are sensitive to bile acids (Curtis et. al., 1977) has reduced the risk of infection and proliferation in the gut, but does not avoid the problems associated with endotoxin production.

A possible way of overcoming these problems would be to use a different microorganism as host, which has no association with man and does not produce endotoxins or enterotoxins. For technical reasons (growth characteristics, ease of manipulation, selection etc) bacteria and their plasmids and bacteriophages have the greatest potential as host-vector systems. However, most bacteria are in at least some way, infectious for either man, his domestic animals, or plants. There are,

however, two possible exceptions, Bacillus subtilis and Bacillus stearothermophilus, neither of which are known to produce toxins (Jeljaszewiz & Wadstron, 1978). The available evidence indicates that B. subtilis, although capable of existing on the cutaneous surfaces of man, is totally non-infectious for animals and plants (Kloos & Musselwhite 1975; Le Page, pers. comm.). In addition, some information on the genetics and physiology of this bacterium is available (Lepesant-Kejzlarova et. al., 1975; Young & Wilson, 1975) and possible vectors for this host (bacteriophage and plasmid) have been isolated (Lovett & Bramucci, 1974 and 1975; Lovett et. al., 1976; Young, 1976).

The second exception relates to bacteria which grow only under adverse physiological conditions which are, by definition, non-infectious. The majority of these conditions are incompatible with those used for genetic manipulation research. However, the thermophilic Bacilli (Williams, 1975; Rowe et. al., 1975) which may grow only at elevated temperatures (above 40°C) are potentially useful alternative host-vector systems. These bacteria have not been well characterised genetically, however their physiology, in particular that of B. stearothermophilus and B. caldolyticus is well understood (Atkinson et. al., 1975; Zeikus, 1979). An additional advantage of using thermophilic bacilli as a host in genetic manipulation is their inherent property of producing physically and chemically stable enzymes (Zeikus, 1979). This has resulted in an increased commercial importance of thermophiles. Also, if a thermolabile protein is produced by a thermophilic bacillus, as would be expected when using these strains as hosts for recombinant DNA, the thermophile over-produces the thermolabile protein therefore increasing the possible yield of protein eg, Lactate dehydrogenase in B. caldotenax (A. Atkinson, pers. comm.).

One feature of Bacilli, important when considering the recovery of a product synthesised from a recombinant molecule, is their ability to excrete proteins eg, amylases, β-lactamases, proteases (Glenn, 1976). This property would make isolation and purification of translation products from recombinant molecules relatively easy. An important factor since proteins from Bacilli are becoming increasingly important commercially (Zeikus, 1979).

Although the pyr and leu genes (Chi et. al., 1978) and the thyP3 gene (Duncan et. al., 1977) from Bacillus subtilis have been shown to express quite well when cloned in E. coli, there is evidence that some Bacillus genes may not express well in E. coli; eg, the β-lactamase of B. licheniformis (Dr. W.J. Brammar, pers. comm.) and the Neo^r genes of the plasmid pUB110 (A. Docherty, pers. comm.).

The overall aim of this project, therefore, was to develop a host-

vector system for genetic manipulation in thermophilic bacilli. Since it has been reported that several Staphylococcus aureus antibiotic resistance plasmids express well in another, different, Gram-positive organism, ie B. subtilis (Ehrlich, 1977) it was anticipated that a vector developed for thermophilic bacilli would be useful for genetic manipulation experiments in B. subtilis.

1.4. Plasmids in Bacilli

The development of a host-vector system for genetic manipulation in thermophilic Bacilli can be divided into two main steps. Firstly, the development of a vector, involving isolation and characterisation of plasmids or bacteriophages and secondly, development of the host thermophile, in particular, the transformation of the vector into the host.

A few bacteriophages specific for B. stearothermophilus have been identified (Welker & Campbell, 1965; Egbert & Mitchell, 1967; Epstein & Campbell, 1975) and over 20 lysogenic bacteriophage have been induced from different strains of thermophilic bacilli (R.J. Sharp, pers. comm.). However, these were not examined with a view to developing them as vectors since bacteriophage have a distinct disadvantage over a plasmid vector. Foreign DNA can not just be inserted into a phage genome since the recombinant molecule has to be packaged into the phage protein coat where size is critical (Bellet et. al., 1971). The lambda phage vectors developed for E. coli (Enquist et. al., 1976; Leder et. al., 1977; Williams et. al., 1977; Murray et. al., 1976b; Moir & Brammar, 1976, Donoghue & Sharp, 1977), have large regions (up to 30%) of non-essential DNA deleted. This requires detailed genetic knowledge of the bacteriophage and such knowledge is not available for any of the thermophilic bacteriophages. Plasmids however, have fewer genes, so it is relatively easy to identify non-essential regions and they have no real limitation for the size of DNA fragment that can be inserted.

Unfortunately, no plasmids have been identified in strains of thermophilic bacilli. At the start of this project only a few plasmids had been identified in Bacilli; B. megaterium (Carlton & Helinski, 1969; Henneberry & Carlton, 1973), B. pumilus (Lovett, 1973; Lovett & Burdick, 1973; Lovett & Bramucci, 1974, 1975; Lovett et. al., 1976), B. subtilis (LeHegarat & Anagnostopoulos, 1977; Tanaka & Koshikawa, 1977; Tanaka

et. al., 1977); however none carried any identifiable phenotypic trait (cryptic plasmids) so reducing their potential as vectors.

A screening programme for the presence of plasmid DNA in thermophilic bacilli, limited to antibiotic resistant or bacteriocin producing strains, was initiated. The method of Guerry et. al., (1973) was used, where dection of plasmid DNA relies on separation from the chromosome in ethidium bromide-caesium chloride density gradients. In later experiments the agarose-gel electrophoresis technique of Meyers et. al., (1976) for detecting plasmid DNA was used.

During the course of this work a bacteriocin production plasmid (pBC7) and a tetracycline resistance plasmid (pBC16) were isolated from B. cereus (Bernhard et. al., 1978) and shown to express in B. subtilis. In addition, a large number of antibiotic resistance plasmids from Staphylococci that could express in B. subtilis were identified (Table 1.1). These plasmids may prove useful for genetic manipulation in B. subtilis but may be of limited value for thermophilic bacilli. The extreme temperatures (in excess of 60°C) at which a host-vector system for thermophiles could operate, may well not allow replication and expression of plasmids from mesophiles with a maximum growth temperature of only 45°C (Buchanan & Gibbons, 1974). In addition, the fairly common occurrence of Staphylococcal toxins (Jeljaszewicz & Wadstrom, 1978) raises the possibility that a plasmid derived from Staphylococcus may code for toxin production in addition to an identified antibiotic resistance function.

The final stage in developing genetic manipulation in thermophilic bacilli would be the transfer of the vector (plasmid) into the host thermophile. Genetic transfer within thermophilic bacilli is still in a primitive state. Transformation of B. stearothermophilus has been reported. Isono (1970) reported the transformation, at 37°C, of an amylase deficient mutant of B. stearothermophilus with DNA from an amylase positive strain to the positive wild type. Transformation with DNA from the bacteriophage TP1C and TP84 with the help of intact phage TP-12 has been reported by Welker (1978). However, neither work has been followed by further reports and the irreproducability of the transformation system has been noted (N.E. Welker, pers. comm., A. Atkinson, pers. comm.). There have been no reports of transformation of thermophilic bacilli with plasmid DNA. As a result, a major aim of this project was the development of a transformation system in thermophilic bacilli using any isolated plasmids. This would allow the evaluation of thermophilic bacilli as hosts for recombinant plasmids as the basis for genetic manipulation in these strains.

Table 1.1. Plasmid cloning vehicles for use in B. subtilis

Plasmid	Size (Md)	Selective Marker	Restriction endonucleases with 1 site in plasmid	Gene inactivation (enzyme)	Reference	Notes
(I) pBC16	3.00	Tc^r	BamHI	-	Bernhard et. al., 1978	-
(I) pBC16-1	1.90	Tc^r	EcoRI	-	Kreft et. al., 1978	-
(II) pBS161	5.75	Tc^r	EcoRI	-	Kreft et. al., 1978	-
(II) pBS161-1	2.55	Tc^r	EcoRI, HindIII	-		-
(IV) pJK1	6.70	Tc^r	-	-		pJK1 + pJK3 are recombinants of pBR322[a] and pBS161. Both replicate in E. coli.
(IV) pJK3	5.30	Tc^r	-	-		
(III) pT127	2.90	Tc^r	-	-	Ehrlich, 1977	-
(III) pSN-1	2.75	Tc^r	HpaII	-	Shafferman et. al., 1978	-
(III) pTP-2	2.84	Tc^r.Ap^r	EcoRI	-	Kono et. al., 1978	-
(III) pUB110	3.00	Km^r.	BamHI, BglII, EcoRI, XbaI	Km^r (BglII)	Gryczan et. al., 1978	-
(IV) pSL101	5.40	Km^r.trp^+	EcoRI	Km^r (BglII)	Keggins et. al., 1978	B. licheniformis trp cloned in pUB110
(IV) pSL103	5.00	Km^r.trp^+	-	" "		B. pumilus trp cloned in pUB110

Table 1.1 (Cont'd)

Plasmid	Size (Md)	Selective Marker	Restriction endonucleases with 1 site in plasmid	Gene inactivation (enzyme)	Reference	Notes
(IV) pSL106	4.50	$Km^r.trp^+$	EcoRI	Km^r (BglII)	Keggins et. al., 1978	B. subtilis trp cloned in pUB110
(III) pUB1654	4.00	$Km^r.Sm^r$	BamHI, BglII, EcoRI, HindIII, XbaI.	Km^r (BglII) Sm^r (EcoRI, HindIII)	A. Docherty (unpublished)	-
(III) pS194	3.00	Sm^r	EcoRI	-	Lofdahal et. al., 1978a	-
(III) pSC194	7.00	$Sm^r.Cm^r.$	EcoRI	-	Lofdahal et. al., 1978b	-
(IV) pSA0501	2.80	$Sm^r.$	EcoRI, HindIII, XbaI	-	Gryczan & Dubnau, 1978	In vitro recombinants of
(IV) pSA2100	4.60	$Sm.Cm^r$	EcoRI, XbaI	-	" "	Staphylococcal plasmids
(III) pE194	2.40	$Em^r.$	PstI, XbaI	-	Weisblum et. al., 1979	-
(III) pC194	1.80	$Cm^r.$	HindIII	-	Ehrlich, 1977	-
(III) pC221	3.00	$Cm^r.$	HindIII	-		-
(III) pC223	3.00	$Cm^r.$	HindIII	-		-
(III) pUB112	3.00	$Cm^r.$	HindIII	-		-
(IV) pHV11	3.30	$Cm^r.Tc^r$	KpnI, SalI, XbaI	-	Ehrlich, 1978	Recombinant of pC194 + pT127.
(IV) pHV14	4.60	$Cm^r.$	PstI	-	Sgaramella et. al., 1978	Recombinant of pC194 + pBR322[a], expresses in E. coli ($Cm^r.Ap^r$).

Table 1.1 (Cont'd)

	Plasmid	Size (Md)	Selective Marker	Restriction endonucleases with 1 site in plasmid	Gene inactivation (enzyme)	Reference	Notes
(IV)	pCD1	5.40	Thy^+	BamHI	-	Duncan et. al., 1977	Recombinants of pMB9[b] + ϕ3T a B. subtilis phage. Both replicate in E. coli.
(IV)	pCD2	3.90	Thy^+	BamHI, BglII	-	" " "	
(IV)	pBD6	5.80	$Km^r.Sm^r.$	BamHI, BglII, HindIII	Km^r (BglII), Sm^r (HindIII)		
(IV)	pBD8	6.00	$Km^r.Sm^r.$ $Cm^r.$	BamHI, BglII, HindIII EcoRI, XbaI	Km^r (BglII) Sm^r (EcoRI, HindIII)		
(IV)	pBD9	5.40	$Km^r.Em^r.$	BamHI, BglII, BclI, EcoRI, PstI	Km^r (BglII), Em^r (BclI)	Gryczan et. al., 1980	All in vitro recombinants of Staphylococcal Plasmids.
(IV)	pBD10	4.40	$Km^r.Em^r.$ $Cm^r.$	BamHI, BglII, BclI, XbaI	Km^r (BglII), Em^r (BclI)		
(IV)	pBD12	4.50	$Km^r.Cm^r.$	BamHI, BglII, EcoRI HindIII, XbaI	Km^r (BglII)		
(IV)	pBD64	3.20	$Km^r.Cm^r.$	BamHI, BglII, EcoRI, XbaI	Km^r (BglII)		
(IV)	pJK33	4.50	$Tc^r.$	BamHI	-	Goebel et. al., 1979	Deletion of pJG3, see above, replicates in E. coli ($Tc^r.Ap^r.$)
(IV)	pJK201	2.70	$Tc^r.Cm^r.$	BamHI, HindIII, SalI, EcoRI	Cm^r (EcoRI)	" " "	Recombinants of pBS161-1 + pACYC184[c] (Kreft et. al., 1978). Only pJK201 replicates in E. coli.
(IV)	pJK202	2.10	$Tc^r.Cm^r.$	EcoRI	Cm^r (EcoRI)		

Plasmid	Size (Md)	Selective Marker	Restriction endonucleases with 1 site in plasmid	Gene inactivation (enzyme)	Reference	Notes
(IV) pJK502	4.70	Tc^r.	BamHI, HindIII, SalI, PstI	-	Goebel et. al., 1979	Recombinant of pBR322[a] + pBS1 (Bernhard et. al., 1978). Replicates in E. coli (Tc^r.Ap^r).

Legend to Table 1.1

Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Km^r, kamamycin resistance; Sm^r, streptomycin resistance; Tc^r, tetracycline resistance.

Thy^+, thymine complementation in *E. coli* and *B. subtilis*.

trp^+, tryptophan complementation in *B. subtilis*.

a, Bolivar *et. al.*, 1977b.

b, Rodriguez *et. al.*, 1977.

c, Chang & Cohen, 1978.

I, plasmid originally derived from *B. cereus*

II, " " " " *B. subtilis*

III, " " " " *Staphylococcus aureus*

IV, Recombinant plasmid.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Media and Buffers

Unless otherwise state all chemicals were purchased from BDH Chemicals Ltd, Poole, Dorset , and wherever possible were of Analar grade.

2.1.1. Media

1. Tryptone Soya Broth (TSB)

This medium was used for routine culturing of strains and contained the following (gL^{-1}):

Tryptone (Oxoid Ltd)	17
Soya peptone (Oxoid Ltd)	3.0
NaCl	5.0
K_2HPO_4	2.5
Glucose	2.5

Distilled H_2O to 1000 ml, and pH adjusted to 7.3 with 2N HCl. Agar (Oxoid, purified) was added to 1.5% for solid media) (TSBA).

2. BS Broth

This liquid mediumbased on that described by Sargeant et. al., (1971) was used for B. stearothermophilus, the caldo-active bacilli and RS strains of thermophilic bacilli and contained (gL^{-1}):

Bacto-Tryptone (Difco)	20
Yeast extract (Oxoid Ltd)	10
$FeCl_3.6H_2O$	0.007
$MnCl_2.4H_2O$	0.002
$CaSO_4.2H_2O$	1.3
Citric acid	0.32
NaH_2PO_4	3.2
$MgSO_4.7H_2O$	0.27
Sucrose	10

Distilled H_2O to 1000 ml (pH 7.1)

3. TYS Medium

This medium was used for culturing all bacilli for plasmid

isolation, unless otherwise stated, and contained ($g.L^{-1}$)

Bacto-Tryptone (Difco)	20
Yeast extract (Oxoid Ltd)	10
NaCl	10

Distilled H_2O to 1000 ml (pH 7.4)

4. Ramaley and Hixson (1970) Medium (RHM)

This was used for culturing Thermus aquaticus strains and contained ($g.L^{-1}$)

Nitrilotriacetic acid	1.0
$CaSO_4.2H_2O$	0.6
$MgSO_4.7H_2O$	1.0
NaCl	0.08
KNO_3	1.03
$NaNO_3$	6.9
Na_2HPO_4	1.1
$FeCl_3.6H_2O$	0.003
Nitchs trace elements solution	10 ml (see below)
Tryptone (Difco)	1.0
Yeast extract (Oxoid Ltd)	1.0

Distilled H_2O to 1000 ml

The pH was adjusted to 8.2 with 2N NaOH after the addition of Nitches trace elements ($g.L^{-1}$)

H_2SO_4	0.5 ml
$MnSO_4.4H_2O$	2.2
$ZnSO_4.7H_2O$	0.5
H_3BO_3	0.5
$CaSO_4.2H_2O$	0.016
$Na_2MoO_4.2H_2O$	0.026
$CoCl_2.6H_2O$	0.046

Distilled H_2O to 1000 ml.

5. AD Medium

This defined medium was used for culturing the TB strains of thermophilic bacilli and was prepared by adding 1 ml of minimal salts solution and 4 ml glucose, 50% (w/v) to 1 L of minimal base.

(i)	Minimal Salts ($g.L^{-1}$)	$CaCl_2.2H_2O$	5
		$FeCl_3.6H_2O$	0.5
		$ZnSO_4.4H_2O$	5
		$MnCl_2$	0.2
		Distilled H_2O	to 1000 ml.
(ii)	Minimal Base ($g.L^{-1}$)	K_2HPO_4	12.5
		KH_2PO_4	3
		NH_4Cl	1
		NaCl	1
		$MgSO_4.7H_2O$	0.5
		Distilled H_2O	to 1000 ml.

Purified agar (Oxoid) was added to 1·5% (w/v) for solid media.

6. CAL II Medium

This defined medium was used for culturing the caldo-active bacilli and their auxotrophic mutants, and contained ($g.L^{-1}$)

$(NH_4)_2NO_3$	0.5
KH_2PO_4	0.5
NaCl	0.3
$MgSO_4.7H_2O$	0.25
$FeCl_3.6H_2O$	0.05
$CaCl_2.2H_2O$	0.05
Na_2HPO_4	0.05
Sucrose	30
Nitschs trace elements	0.15 ml (see medium 4)
Distilled H_2O	to 1000 ml.

Each constituent was dissolved completely before the addition of a second chemical. The temperature of the medium was brought to 50°C prior to adjusting the pH to 7.5 with sodium silicate. Purified agar (Oxoid) was added to 1·5% (w/v) for solid media.

7. Subtilis Minimal Salts (SMS)

This defined medium was used for B. subtilis and was prepared by the addition of 10 ml glucose 50% (w/v) and 250 ml of SMS X4 concentrate to 740 ml distilled water or distilled water containing 15 g purified agar.

SMS X4 ($g.L^{-1}$)	$(NH_4)_2SO_4$	8
	K_2HPO_4	56

KH_2PO_4	24
$MgSO_4.7H_2O$	0.8
Sodium citrate	4
Distilled H_2O to	1000 ml.

8. Peptone Water Broth

Peptone	10 g
NaCl	5 g
Distilled H_2O to	1000 ml (pH 7.4)

9. Peptone Water Sugars

500 ml of a 2% (w/v) solution of the carbohydrate of choice (filter sterilised) was added to an equal volume of:

Andrade indicator	20 ml
Phenol red solution (0.2%, v/v)	100 ml
Bromthymol blue solution (0.2%, v/v)	20 ml
Bromcresol purple solution (0.4%, v/v)	20 ml

Peptone water broth to 1000 ml.

10. Lambda Medium (LM)

Glycerol	21 g
KH_2PO_4	5 g
$MgSO_4.7H_2O$	1 g
Yeatex (Oxoid Ltd)	1 g
Casein hydrolysate (Oxoid Ltd)	30 g

Distilled water to 1000 ml.

The pH was adjusted to 7.0 with 2N-NaOH.

11. SMM/(SMM6)

0.5 M Sucrose

0.02 M Maleic acid

0.02 M $MgCl_2$ $6H_2O$

0.2% BSA (w/v)

The pH was adjusted with 2 N NaOH to 6.5. (SMM6 contains

sucrose at 0.6 M.)

12. SMMP/(SMM6P)

Was prepared by mixing equal volumes of 4X Penassay broth (Difco Ltd., West Molesley, Surrey) and 2X SMM or 2X SMM6.

13. DM3 regeneration medium

Was prepared by mixing the following solutions:-

(A) Casamino acids (Difco) 5 g
Yeast extract (Oxoid) 5 g
$MgCl_2.6H_2O$ 0.8 g
Purified agar (Oxoid) 8 g
Distilled water to 370 ml.

(B) 1 M sodium succinate pH 7.3, 500 ml.

(C) K_2HPO_4 3.5 g
KH_2PO_4 1.5 g
Distilled water to 100 ml.

(D) 20% (w/v) glucose, 25 ml.

Finally 5 ml of filter-sterilised 2% (w/v) bovine serum albumin was added when the medium had cooled to 50°C.

14. DM4/DM5 regeneration media

These media are composed of the same constituents as DM3 except 0.5 M succinate was replaced by glutamate (0.6 M) for DM4 and 0.6 M succinate for DM5.

All media were autoclaved for 15 min at 121°C.

2.1.2. Buffers

De-ionised water was used for all buffers.

1. TS

50 mM Tris

25% sucrose (w/v)

The pH was adjusted to 8.0 with 4N HCl.

2. TESDS

50 mM Tris

1 mM Na_2.EDTA

2% Sodium dodecyl sulphate (w/v)

The pH was adjusted to 8.0 with 4N HCl.

3. TES

30 mM Tris

50 mM NaCl

5 mM Na_2.EDTA

The pH was adjusted to 8.0 with 4N HCL.

4. TESS

30 mM Tris

50 mM NaCl

5 mM Na_2.EDTA

25% sucrose (w/v)

The pH was adjusted to 8.0 with 4N HCl.

5. TE1

50 mM Tris

1 mM Na_2.EDTA

The pH was adjusted to 7.5 with 4N HCl.

6. TE2

10 mM Tris

0.5 mM Na_2.EDTA

The pH was adjusted to 7.5 with 4N HCl.

7. TSM

10 mM Tris

1 mM $MgCl_2.6H_2O$

100 mM NaCl

The pH was adjusted to 7.5 with 4N HCl.

8. TM

10 mM Tris

1 mM $MgCl_2.6H_2O$

The pH was adjusted to 7.5 with 4N HCl.

9. TEF

200 mM Tris-HCl pH 7.5

10 mM Na_2EDTA

50% (v/v) Formamide (AR)

The pH was adjusted to 8.5 with 4N HCl.

10. Tris-Borate

90 mM Tris

90 mM Boric Acid

3 mM Na_2.EDTA

0.5 $\mu g.ml^{-1}$ ethidium bromide

(Natural pH of 8.0).

11. Tris-Acetate

40 mM Tris

20 mM Sodium acetate

1 mM Na_2.EDTA

0.5 $\mu g.ml^{-1}$ ethidium bromide

The final pH was adjusted to 7.7 with 3M acetic acid

12. ST Buffer

25 mM Tris

250 mM NaCl

10% glycerol (v/v)

10 mM β-mercaptoethanol

0.1 mM PMSF

The final pH was adjusted to 8.0 with 2N HCl.

13. TSG Buffer

25 mM Tris

500 mM NaCl

10% glycerol (v/v)

10 mM β-mercaptoethanol

0.1 mM PMSF

The final pH was adjusted to 7.5 with 2N HCl.

14. KP Buffer

20 mM Potassium phosphate pH 7.4

0.1 mM Na_2.EDTA

10 mM β-mercaptoethanol

0.1 mM PMSF

10% glycerol (v/v)

2.2. Bacterial Strains

A full list of all the strains used in this investigation is given in Table 2.1., and the composition of the media for liquid and plate culture is described in the preceding section.

2.2.1. Isolation of RS strains of thermophilic bacilli at 60°C

Each sample was inoculated into 200 ml of BS broth in 250 ml

bottles and incubated at 60°C for 17 h without aeration. Dilutions in saline (0.9% w/v NaCl) of each bottle that supported growth was made in order to isolate each microorganism present. Different strains in the same sample were identified initially by differing colony morphology and subcultured on TSBA plates until pure cultures were obtained. Only strains identified as Gram-positive rods were included in the RS collection of thermophilic bacilli (Table 2.1). Gram-stains were carried out according to the method of Collins and Lyne (1970).

2.2.2. Isolation of antibiotic resistant strains of thermophilic bacilli at 55°C

Samples of river sludge or silage were inoculated into 200 ml of TSB medium containing one of the following antibiotics (Sigma Ltd., Poole, UK); ampicillin, chloramphenicol, kanamycin, tetracycline hydrochloride (each at 25 $\mu g\ ml^{-1}$) streptomycin sulphate (100 $\mu g\ ml^{-1}$) or heavy metal ions; mercuric nitrate, cadmium nitrate, or sodium arsenate at 0.2 mM or 2 mM; and incubated at 55°C for 24 h or until growth was apparent. Each microorganism present in the media supporting growth was isolated and purified by diluting samples in saline and plating on TSBA plates containing the appropriate antibiotic or heavy metal ion. Only strains identified as Gram-positive rods were included in the TB collection of thermophilic bacilli (Table 2.1).

2.3. Biochemical tests

Sugar fermentations, based on the method of Collins & Lyne (1970), were carried out by inoculating sugars in basal peptone water with a 6 h peptone water broth culture derived from an overnight plate culture. Citrate utilization was tested on Simmon's citrate (Collins & Lyne, 1970) slopes, and gelatine liquefaction, starch hydrolysis, casein hydrolysis, nitrate and nitrite reduction, indole production, acetoin production, growth in 0.02% (w/v) azide (NaN_3), catalase and oxidase production, were all examined by the methods of Cowan & Steel (1974). Tolerance to saline was determined by inoculating bottles containing 10 ml nutrient broth No2 (Oxoid CM67) supplemented with 1 to 10% (w/v) NaCl. Sabouraud dextrose agar plates (Cowan & Steel, 1974) were based on Oxoid agar (CM41). Readings, ie, a colour change, were taken at 1, 2 and 3 day intervals.

Table 2.1. Bacterial strains used in this investigation

Strain		Source
Bacillus stearothermophilus	NCA 1503	NCA.
" "	ATCC 12016	ATCC.
" "	EP 240	Chalky soil in Salisbury area.
" "	AY 174	Ann Yap, CAMR.
" "	LO2	See Chapter 4.
" caldotenax		W. Heinen, Nijmegen, Holland.
" caldolyticus		" " "
" caldovelox		" " "
Thermus aquaticus B		R.A.D. Williams, London.
" " X1		" "
" " YT1		" "
Thermophilic bacillus	RS 1 to 3	Soil at CAMR.
" "	RS 4 to 8	Rhinog Mountains, Wales.
" "	RS 9 to 14	Forge cooling water, Boston.
" "	RS 15 to 17	Contaminants from Thermus cultures.
" "	RS 18	Contaminant from NCA 1503 culture.
" "	RS 19 to 43	Soil from ICI, Bracknel, Berks.
" "	RS 44 to 48	Sand from St. Jean de Monts, France.
" "	RS 49 to 62	Soil " " " "
" "	RS 63 to 69	Soil from Ile de Ré, France.
" "	RS 70	Sand " " " "
" "	RS 71	Water sample from Bath, Somerset.
" "	RS 72	Water bath, CAMR.
" "	RS 73 to 74	Contaminants of NCA 1503 culture.
" "	RS 75 to 84	Soil from La Mont St. Micheal. France.
" "	RS 85 to 92	Soil from Les Eaux (Granville), France.
" "	RS 93	Contaminant of CAMR hot water supply.
" "	RS 94 to 97	Water from River Cam, Cambridge.
" "	RS 98 to 112	Water from Spring near St. Albans.

Table 2.1 (Cont'd)

Strain	Source
Thermophilic bacillus RS 116 to 129	Soil from Kingston, Surrey.
" " RS 144	Compost heap, Porton.
" " RS 161, RS 166	Soil from Greece.
" " RS 173	Water from Greece.
Thermophilic bacillus TB 113 to 119	Streptomycin plate of river sludge sample.
" " TB 120	Penicillin G " " " sludge sample.
" " TB 121	Penicillin G plate of silage sample.
" " TB 122	Penicillin G " of soil sample.
" " TB 123 to 128	Tetracycline plate of river sludge sample.
" " TB 129 to 134	Chloramphenicol plate of river sludge sample.
" " TB 135	Cadmium (0.2 mM) plate of river sludge sample.
" " TB 136 to 138	Arsenate (2 mM) plate of river sludge sample.
" " TB 139 to 142	Kanamycin plate of river sludge sample.
" " TB 143	Mercury (2 mM) plate of river sludge sample.
" " TB 144	Contaminant of TB 124 plate.
" " TB 150	" of TB 118 plate.
Thermophilic bacillus TB 151	Streptomycin plate of silage sample.
" " TB 152 to 153	Tetracycline " " " sample.
" " TB 154	Ampicillin " " " sample.
Bacillus coagulans ATCC 8038	ATTC.
B. subtilis IG 20 (r^-. m^-. *trpC2*)	A. Docherty, Bristol Univ.
B. subtilis BD 224 (*recE*$_4$.*trpC2*.*thr5*)	Bacillus Genetic Stock Centre, Ohio, USA.
" CU 741 (*trpC2*.*leuC7*)	S. Zahler, Cornell University, USA.
" QB 944 (K1) (*pur* A16.*Cys* A14 *trpC2*)	Dedonder *et. al.*, 1977. Paris, France.

Table 2.1 (Cont'd)

Strain	Source
B. subtilis QB 928(K2) (aroI906. purB33 dal.trpC2)	Dedonder et. al., 1977. Paris, France.
" QB 935(K6) (aroD120. lys1 trpC2)	" " " "
" QB 936(K7) (leuA8.aroG932 ald.trpC2)	" " " "
" QB 917(K8) (hisA1. thr5. trpC2)	" " " "
B. subtilis (pUB110)	A. Docherty, Bristol University.
" (pUB1654)	" " "
Staphylococcus aureus (pI 258)	R.P. Novick, Public Health Research Institute, New YOrk, USA.
Escherichia coli C600 (pROG29),	R.F. Sherwood, CAMR, Salisbury.
" " HB101 (pBR322)	P.J. Rigby, Imperial College, London.
" " W3110 (λc_{I857} ts^-. Sam_7)	P.J. Rigby, Imperial College, London.

2.4. Antibiotic Sensitivity

Disposable bioassay plates were prepared with 200 ml Oxoid Sensitest agar (CM 409), with agar at concentration of 3% (w/v), seeded with 3 ml of a 6 h peptone water broth culture and allowed to dry. Antibiotic sensitivity discs (Oxoid Ltd) were then placed on them, a minimum of 12 cm apart, and the plates incubated overnight at 60°C or 50°C. The diameter of the zone of growth inhibition was then recorded.

2.5. Bacteriocin Sensitivity

The strains under test were grown for 6 h in peptone water broth and serial dilutions prepared in saline and plated on TSBA. After overnight incubation at 60°C or 50°C, the bacteria on plates with 10-50 colonies were killed by exposure to chloroform for 30 min, and then overlaid with 5 ml TSBA containing the indicator strain prepared from a 6 h peptone water broth culture (approximately 10^6 cells). After overnight incubation at 60°C or 50°C the width of the zone of growth inhibition was recorded.

2.6. Screening of thermophilic bacilli for plasmid DNA

2.6.1. Method 1

This method was initially developed for screening of Escherichia coli strains for plasmid DNA, based on the method of Guerry et. al., (1973).

Growth of bacilli strains. An inoculum (0.1 ml) of an overnight culture in BS broth was used to inoculate 20 ml of fresh BS broth containing 0.1 ml (^{3}H) thymidine, 1 mCi.ml^{-1} (45 Ci.m mol^{-1} Radiochemical Centre, Amersham) and 0.25 ml deoxyadenosine (10 mg.ml^{-1}), and incubated overnight at 60°C with gentle shaking.

Growth of Thermus aquaticus strains. Ramaley and Hixon (1970) medium (200 ml) containing 0.2 ml (^{3}H) thymidine (1 mCi.ml^{-1}, 45 Ci.m mol^{-1}) and 0.5 ml deoxyadenosine (10 mg.ml^{-1}) was inoculated with 5 ml of a fresh Thermus aquaticus culture in the same medium and incubated for 4-5 days at 70°C without shaking.

Preparation of lysates. Cells were harvested by centrifugation (6,000 xg for 10 min) 4°C resuspended in 0.25 ml TS buffer and 0.1 ml lysozyme solution (5 mg.ml^{-1} in TS) was added. After 10 min incu-

bation on ice, 0.1 ml Na_2.EDTA pH 8.0 (0.25 M) was added and incubation continued for a further 5 min at 20°C. Lysis was brought about by the addition of 0.5 ml TESDS buffer and 0.6 ml NaCl (4M) was then added and the lysate vortexed for 2 min to produce an homogeneous suspension, which was then left overnight on ice. A cleared lysate was obtained by centrifugation at 45,000 xg for 60 min (4°C). A 20 µl sample of cleared lysate was analysed for the presence of plasmid DNA by agarose-gel electrophoresis (see section 2.) and the remainder subjected to isopycnic centrifugation in caesium chloride-ethidium bromide density gradients.

Isopycnic centrifugation. Caesium chloride (3.8 g) was dissolved in 0.5 ml ethidium bromide (1 $mg.ml^{-1}$ in TES buffer) and 2.5 ml TES, and the cleared lysate (1 ml) was then added. The mixture was transferred to a 10 ml polycarbonate centrifuge tube, and centrifuged in a 10 x 10 ml titanium rotor at 150,000 xg (10°C) for 24 h in an MSE65 ultracentrifuge (MSE Scientific Ltd., Crawley, UK). Each tube was transferred to a tube piercing device (MSE Scientific Ltd., Crawley, UK.) and 5 drop fractions collected from the bottom of the tube under gravity until the complete gradient was collected (about 40 fractions). A sample (25 µl) of fractions was placed on 2.5 cm glass fibre circles (Whatman Ltd., Maidstone, UK) and dried in an oven at 80°C. Each glass fibre circle was then washed with 1 x 10 ml cold (4°C) 5% (w/v) tri-chloroacetic acid, 1 x 10 ml 95% (v/v) ethanol and finally 1 x 10 ml diethyl ether. After drying for 5 min at 80°C each glass fibre circle was transferred to a glass scintillation vial (Searle Ltd., High Wycombe, UK), 6 ml of scintillation fluid NE260 (Nuclear Enterprises Ltd., London) added and counted for (^3H) activity in a LKB 1215 Rackbeta liquid scintillation counter.

The presence of satellite DNA bands in the density gradients was observed by their flourescence under Ultraviolet light using a 320 nm UV source (Ultra Violet Products Ltd., Winchester, UK) and from the ^3H profile.

2.6.2. Method 2

The above method does not work well for most thermophilic bacilli (see Chapter 3) and the growth and lysis conditions were altered to produce a better result.

Growth of bacilli strains. TYS medium (200 ml) was inoculated with each strain from a fresh TSBA plate and incubated at 50°C for 8 to 10 h with vigorous aeration. The medium also contained the appropriate

antibiotic when culturing the TB strains of antibiotic resistant bacilli (Table 2.1).

Preparation of lysates. The cells were harvested by centrifugation at 6,000 xg for 10 min (4°C), washed once in 100 ml TES buffer and then suspended in 3 ml TESS; 0.5 ml 0.2 M-Na_2.EDTA pH 8.0 and 1 ml lysozyme (10 mg. ml^{-1} in TES) were then added. The mixture was vortexed and kept on ice for 10 min, before transferring to a 37°C water bath for a further 5 min. Lysis was accomplished by adding 0.5 ml (10% w/v) sodium dodecyl sulphate (SDS) vortexing and then followed by 1.0 ml NaCl (5 M). After vortexing for 2 min the mixture was left overnight at 0°C. A cleared lysate was obtained by centrifugation at 45,000 xg for 60 min (4°C) and was then extracted with an equal volume of chloroform/3-methylbutan-1-ol (24:1, by vol), the mixture was centrifuged at 6000 xg for 5 min (10°C) and the upper aqueous phase carefully removed from the denatured protein at the interface. After a second extraction, the aqueous phase was centrifuged at 45000 xg for 10 min (4°C) to remove any remaining SDS precipitate. A 20 µl sample of the cleared lysate was analysed for the presence of plasmid DNA by agarose-gel electrophoresis, (see section 2.13) and the remainder subjected to isopycnic centrifugation in caesium chloride-ethidium bromide density gradients.

Isopycnic centrifugation. Caesium chloride (3.8 g) was disolved in a mixture of 2.0 ml chloroform extracted cleared lysate, 0.5 ml ethidium bromide (1 mg.ml^{-1} in TES) and 1.5 ml TES buffer. Centrifugation was again carried out at 150000 xg for 24 h at 10°C in a MSE65 ultracentrifuge (see method 1). Satellite DNA bands were visualised by fluorescence under UV light and the gradients collected via a density gradient scanner (model 2580) coupled to a Gilford 250 spectrophotometer (Gilford Instruments, Oberlin, Ohio, USA). Absorbance at 260 nm was recorded and 100 µl fractions were collected with a Redirac 2112 fraction collector (LKB, South Croydon, Surrey).

2.7. Large scale Purification of Plasmid DNA from thermophilic Bacilli

This is a modification of the above method (2.6.2.).

2.7.1. Culture media and conditions

A fresh TSBA plate of each strain was used to inoculate 5 ribbed, 1L, conical flasks each containing 400 ml TYS plus any appropriate

antibiotics at 12 $\mu g.ml^{-1}$, and incubated at 55°C for 10-12 h.

2.7.2. DNA Extraction

Cells from each flask were harvested independently by centrifugation at 6000 xg for 10 min (4°C), washed in 200 ml TES and suspended in 15 ml TES. To each of the 5 cell suspensions, 2.5 ml Na_2.EDTA (0.25 M), and 5 ml lysozyme (10 $mg.ml^{-1}$ in TES) were added. The mixture was vortexed and kept on ice for 10 min, before transferring to a 37°C water bath for a further 5 min. Lysis was accomplished by adding 2.0 ml SDS (10% w/v), vortexing and followed by 4.0 ml 5 M NaCl. The lysate was vortexed for 2 min and left overnight at 0°C. A cleared lysate was obtained by centrifugation at 45000 xg for 60 min (4°C), and each of the 5 lysates extracted twice with chloroform as described above (2.6.2).

2.7.3. Alkali denaturation of Chromosomal DNA

The chloroform extracted cleared lysates were pooled, and the pH adjusted slowly to 12.2 ± 0.1 with 200 µl additions of 3 M NaOH while mixing gently with a magnetic stirrer. After 10 min at pH 12.2 the pH was reduced to 8.5 with 500 µl additions of 2 M Tris-HCl pH 7 taking about 2-3 min. An equal volume of chloroform 3-methylbutan-1-ol (24:1) was then added and mixed vigourously for 2 min to produce a good emulsion. After 5 min on ice the emulsion was separated by centrifugation at 6000 xg for 5 min, (4°C) and the aqueous phase carefully removed avoiding the precipitated single stranded chromosomal DNA at the interface. After a second chloroform extraction any remaining precipitate in the aqueous phase was removed by centrifugation at 45000 xg for 10 min (4°). This process also removes the linear and open circular species of plasmid DNA.

2.7.4. Concentration by polyethylene glycol

Polyethylene glycol (PEG) 6000 was added to the cleared lysates after alkali denaturation to give a final concentration of 10% (w/v); the mixture was stirred gently to dissolve the PEG (prior grinding of the PEG in a pestal and mortor to a fine powder aided solution)

and then left on ice for 3 h. The precipitated DNA was removed by centrifuging at 12000 xg for 15 min (4°C) and gently suspended in a small volume TES buffer (maximum 14 ml). The solubilization of the pellet was aided greatly by using a spatular as a baffle on the side of the centrifuge tube whilst vortexing. The resuspended DNA was then extracted 3 times with chloroform/3-methylbutan-1-ol as in 2.7.3. to remove the residual PEG, and then centrifuged at 45000 xg for 10 min (4°C) to remove any remaining precipitate in the aqueous phase.

2.7.5. Isopycnic centrifugation

Caesium chloride (20 g) was dissolved in 4 ml TESS, 2.5 ml ethidium bromide (1 $mg.ml^{-1}$ in TES) and all the PEG concentrated DNA extract. The final volume of all the additions was made up to 2.0 ml with TES. Diethylpyrocarbonate, 5 µl (Sigma Ltd., London) was added and the solution mixed gently for 5 min, to inactivate nuclease activity. The solution was then divided equally between 4 x 10 ml polycarbonate centrifuge tubes and centrifuged for 24 h at 150000 xg (10°C) in a MSE65 ultracentrifuge (2.6.1).

The DNA bands were visualised in the gradients by fluorescence with UV light and the top of gradient including the chromosomal DNA band was carefully removed using a piece of thin tubing connected to a peristaltic pump (LKB, South Croydon, Surrey). After rinsing the tubing with distilled water, the plasmid band was then removed in a similar manner, and dialysed against 5 L of TE1 buffer for 17 h (4°C). The DNA was then precipitated by adding 1/10th volume of 4 M sodium acetate and 1 volume propan-2-ol, and leaving on solid CO_2 for 5 min. The DNA was collected by centrifugation for 2 min in a microcentrifuge (Quick Fit Instrumentation, UK) and the supernatant discarded. Residual liquid in the tubes was removed by blowing N_2 over the sample, and the plasmid DNA was then suspended in a small volume of TE1 buffer.

2.7.6. Phenol extraction and ethanol precipitation of DNA

Plasmid DNA was treated twice with an equal volume of buffer-saturated phenol (freshly distilled over N_2) equilibrated in TE1 and mixed gently for 5 min at room temperature. The phenol phase

was removed after centrifugation at 10000 xg for 5 min (4°C) and the aqueous phase extracted twice with chloroform (2.7.3) and four times with 10 volumes diethyl ether to remove trace amounts of phenol. The aqueous phase was adjusted to 0.4 M sodium acetate with a 4 M solution and 2 volumes of cold (-20°C) ethanol added. After 17 h at -20°C, or 5 min on solid CO_2, the plasmid DNA was removed by centrifugation at 40000 xg for 15 min (-10°C), suspended in a small volume of TE2 buffer and dialysed for 24 h against 2 changes of 3 L TE2 buffer (4°C).

2.8. Small scale isolation of plasmid DNA from thermophilic bacilli

This follows the same basic procedure as on the large scale (2.7) and was used for the detection of plasmid DNA in transformants of B. stearothermophilus.

50 ml cultures in TYS medium were used, and cleared lysates prepared as described in section 2.6, but with 1/10th volume additions. After extraction with chloroform/3-methylbutan-1-ol the alkali denaturation&PEG concentration steps were omitted and the cleared lysates treated twice with phenol (2.7.6). Residual phenol was removed with chloroform and diethyl ether as described above and samples (10 µl - 20 µl) analysed by agarose-gel electrophoresis (2.13).

2.9. Isolation of plasmid DNA from Bacillus subtilis

The lysozyme/EDTA and SDS lysis method described in 2.7 worked well with B. subtilis, however a more rapid procedure for purifying plasmid DNA based on Triton lysis was also shown to be effective and is described below. (A. Docherty; pers. comm.)

2.9.1. Large scale isolation

Growth and Lysis: Cultures (200 ml) in TYS were prepared and the cells harvested by centrifugation (6000 xg for 10 min at 4°C), washed in 100 ml TES buffer and suspended in 5 ml TESS. Lysozyme, 1.5 ml (50 $mg.ml^{-1}$ in TES) and 3 ml Na_2EDTA (0.25 M pH 8.0) were added and the mixture incubated on ice for 5 min. 12 ml of 1% (V/V) Triton

X100 in 50 mM-Tris-HCl pH 8.0, 50 mM-Na_2.EDTA and 0.1 ml of RNaseI (Sigma Ltd., Poole, UK) 5 mg.ml^{-1} in H_2O, pre-heated to 80°C for 30 min, were added, and incubation continued for a further 25 min on ice. Lysis was accomplished by the addition of 3 ml 5 M-NaCl and incubation at 4°C for about 10 min. A cleared lysate was obtained by centrifugation at 45000 xg for 60 min (4°C) and then extracted twice with chloroform/3-methylbutan-1-ol (see 2.6.2). The plasmid DNA was then concentrated with PEG 6000 as previously described (2.7.4) and subjected to isopycnic centrifugation.

Isopycnic centrifugation: Caesium chloride (6 g) was dissolved in 5.8 ml of the plasmid preparation from above, and 0.2 ml ethidium bromide (10 mg.ml^{-1} in TES buffer) and centrifuged at 150000 xg for 24 h (10°C) as described previously (2.6). The DNA bands were visualised under UV light and the plasmid band removed and dialysed as described in 2.7.5. After precipitation with propan-2-ol (2.7.5) the plasmid DNA was extracted with phenol and precipitated with ethanol as described in section 2.7.6.

2.9.2. Small scale isolation

This method was developed from the above procedure to facilitate the rapid detection of plasmid DNA in large numbers of transformants of B. subtilis.

Growth and Lysis: Each clone under test was inoculated into 15 ml TYS medium (containing any appropriate antibiotic) in universal bottles and incubated overnight at 37°C. The cells were harvested by centrifugation (6000 xg for 10 min at 4°C), washed in 10 ml TES buffer, suspended in 100 µL TES and the following solutions added; 50 µL Na_2.EDTA pH 8.0 (0.25 M), 100 µL lysozyme (10 mg.ml^{-1} in TES). After incubation on ice for 30 min, 10 µL RNaseI, (Sigma Ltd., Poole, UK) 5 mg.ml^{-1} in water, pre-heated to 80°C for 30 min, 125 µL 2% (V/V) Triton in 50 mM-Tris-HCl pH 8.0, 5 mM-Na_2.EDTA were added and incubation on ice continued for 30 min or until lysis was complete.

Purification: The DNA preparation from above was centrifuged at 45000 xg for 60 min (4°C) and the supernatant extracted twice with chloroform/3-methylbutan-1-ol, twice with 2 volumes phenol followed by 2 chloroform and 4 diethyl ether extractions, precipitated with ethanol and dialysed against TE2 as described in sections 2.7.3 and 2.7.6. The resulting plasmid DNA preparation was substantially

free of chromosomal DNA and suitable for restriction endonuclease analysis on agarose-gels.

The method described in 2.8 for thermophilic bacilli was also suitable for B. subtilis.

2.10. Isolation of plasmid DNA from Escherichia coli

2.10.1. Large scale method

This is a modification of the method for thermophilic bacilli (2.7).

Growth: A fresh overnight culture (10 ml) in TSB was used to inoculate 1L of fresh medium and the culture incubated at 37°C until the OD_{550} reaches 1.0. The cells were harvested by centrifugation (6000 xg for 10 min at 4°C), resuspended in fresh medium containing 100 $\mu g.ml^{-1}$ chloramphenicol and incubated overnight at 37°C. This chloramphenicol enrichment of plasmid DNA (Clewell, 1972) was only used for producing pBR322 (Bolivar et. al., 1977b); all other E. coli strains were grown in TSB for 16 h.

DNA extraction: Cells were harvested by centrifugation (6000 xg for 10 min at 4°C) suspended in 30 ml TS buffer and 30 mg lysozyme added. After 5 min on ice, 6 ml Na_2EDTA pH 8.0 (0.5 M) was added and incubation continued on ice for a further 5 min. Lysis was achieved by the addition of 6 ml 20% (w/v) SDS (in H_2O) followed by 12 ml 5 M-NaCl. After vigorous mixing the lysate was left at 0°C overnight, and then centrifuged at 45000 xg for 60 min (4°C). The supernatant was extracted twice with chloroform/3-methylbutan-1-ol, twice with phenol and 4 times with diethyl ether as described previously (2.7). The plasmid DNA was further purified by alkali denaturation and concentrated with polyethylene glycol (2.7) prior to isopycnic centrifugation as described in section 2.7.5.

2.10.2. Small scale method

This method was derived from the above for the detection of plasmid DNA in E. coli transformants. 10 ml cultures in TSB were grown for 16 h at 37°C, the cells harvested and DNA extracted as

described above using 1/100th volume additions. The alkali denaturation, polyethylene glycol and isopycnic centrifugation steps were omitted.

2.11. Isolation and Purification of Bacilli Chromosomal DNA

2.11.1. Growth and Lysis

An overnight culture (10 ml) in TYS was used to inoculate 400 ml fresh medium and the culture incubated at 55°C until the OD_{550} was about 1.5. The cells were harvested by centrifugation (6000 xg for 10 min at 4°C), washed in 100 ml TES buffer, suspended in 20 ml TES and 100 mg lysozyme added. After 5 min incubation on ice, 2 ml Na_2EDTA pH 8.0 (0.25 M) was added and incubation continued on ice for 30 min followed by 10 min at 37°C. Lysis was completed by the addition of 0.5 ml 10% SDS (w/v) pre-warmed to 50°C, and the lysate then cooled rapidly to 4°C in an ice/water bath.

2.11.2. Isopycnic centrifugation

Caesium chloride (20 g) was dissolved in 4 ml TESS, 2.5 ml ethidium bromide (2 $mg.ml^{-1}$ in TES), and 13.5 ml lysate with 5 µL diethyl pyrocarbonate added finally. The mixture was divided into 4 x 10 ml polycarbonate centrifuge tubes and centrifuged at 150000 xg for 24 h at 4°C (2.6.1). The chromosomal DNA bands were removed with a peristaltic pump (2.7.5), pooled and dialysed for 24 h (4°C) against 2 changes of 5 L TES buffer and then subjected to a second isopycnic centrifugation. The chromosomal DNA band was again dialysed against TES buffer.

2.11.3. Phenol extraction and Ethanol precipitation of DNA

The DNA pool from the caesium chloride gradients was treated twice with phenol, twice with chloroform/3-methylbutan-1-ol and 4 times with diethyl ether as previously described (2.7.6). Two volumes of cold (-20°C) ethanol was then added and the chromosomal DNA precipitated at the interface wound onto a spatula, washed

twice by immersion in 10 ml ethanol, dried by blowing N_2 over the pellet and then suspended in a small volume of TE2 buffer. The DNA preparation was extensively dialysed against TE2 buffer ($4^{o}C$) before being stored at $-20^{o}C$.

2.12. Purification of Lambda DNA from an E. coli lysogen

2.12.1. Growth of E. coli W3110 ($\lambda Cl_{857} ts^{-}$. Sam_7)

This procedure was developed for the large scale production of E. coli lambda lysogens on a commercial basis (Bingham and Atkinson, unpublished), and a small scale method is described below.

A fresh culture (10 ml) in LM medium grown at $30^{o}C$ was used to inoculate 2 x 2L flasks each containing 1L of LM medium and incubated at $30^{o}C$ with vigorous aeration. The optical density was monitored (550 nm) at 30 min intervals and when it reached 1.0-1.2 the flasks were transferred to a $55^{o}C$ water bath and mixed continuously until the temperature reached $43^{o}C$ when they were transferred to a $43^{o}C$ incubating shaker. After 20 min shaking at $43^{o}C$ to induce autonomas replication of the phage the flasks were transferred to an iced water-bath to reduce the temperature to $37^{o}C$ and then incubated for 2.5 - 3 h at $37^{o}C$ to allow complete replication of the phage. Lysis of the E. coli cells is prevented by the Sam_7 mutation and the cells were therefore harvested by centrifugation at 6000 xg for 10 min ($4^{o}C$).

2.12.2. Purification of Lambda bacteriophage

The cells obtained from the above were suspended in 100 ml TSM buffer and 10 ml chloroform added and mixed vigorously at $37^{o}C$ for 10 min to release the phage from the cells. 50 µL DNase (5 $mg.ml^{-1}$) and 100 µL RNase (5 $mg.ml^{-1}$), both from Sigma Ltd., Poole, UK, were then added and incubation at $37^{o}C$ continued for 5 min. The cell debris was removed by centrifugation at 10000 xg for 15 min ($4^{o}C$) and the phage in the supernatant pelleted at 45000 xg for 3 h ($4^{o}C$). The phage were suspended in a small volume of TM buffer (maximum

10 ml) and saturated caesium chloride solution added in the ratio of 1.25 ml per ml phage suspension. The mixture was then divided into 2 x 10 ml polycarbonate centrifuge tubes and centrifuged for 17 h at 150000 xg ($5^{o}C$) as described previously (2.6). The phage band was removed with a thin piece of tubing connected to a peristaltic pump (LKB, South Croydon, Surrey) and then dialysed against 2L of TM buffer for 17 h ($4^{o}C$). Proteinase K, 1 mg, (Boehringer Ltd., Lewes, Sussex) pre-incubated at $60^{o}C$ for 30 min was then added and the phage dialysed for a further 17 h ($4^{o}C$) against 1L TM buffer.

2.12.3. Isolation of Lambda DNA

The dialysed phage from above was then dialysed against two changes of 500 ml TEF buffer for 24 h at room temperature to disrupt the phage and release the DNA. The formamide was removed by dialysis against 5L TE2 buffer overnight ($4^{o}C$) and the suspension extracted twice with phenol, then twice with chloroform and 4 times with diethyl ether as described in section 2.7.6. The lambda DNA was precipitated with ethanol (2.7.6.) and the dried pellet suspended in a small volume TE2 buffer. The DNA preparation was dialysed against 2L of TE2 ($4^{o}C$) overnight and then stored at $-20^{o}C$.

The resulting lambda DNA was of sufficient purity to be digested with restriction endonucleases and the resulting fragments used as molecular weight markers in agarose gels (see 2.13). About 20(mg purified λ DNA was obtained with this method.

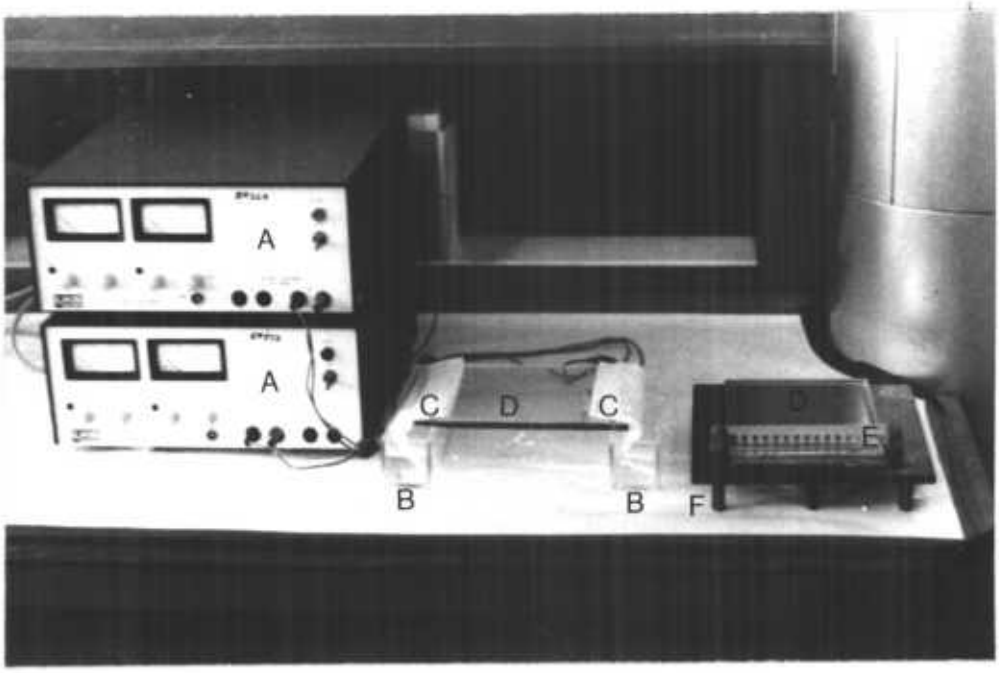
2.13. Agarose-gel electrophoresis

2.13.1. Method

A glass plate of 14 x 18 cm with a 0.5 cm wall was used to prepare slab gels of 0.8% (w/v) agarose (BioRad standard low M_r, Watford, Herts) in Tris-borate buffer. The following aluminium well-formers were used:

5 mm x 1 mm - 14 wells (analytical)
20 mm x 1 mm - 5 wells (preparative)
115 mm x 2 mm - 1 well (preparative)

Plate 2.1. Agarose-gel Electrophoresis System



A: Power Pack (LKB, South Croydon, Surrey).

B: Perspex Troughs.

C: Filter Paper 'wicks' (Whatman, Maidstone, Kent).

D: Glass Plate containing agarose gel (14 x 18 x 0.5 cm).

E: Well Former (14 wells, analytical).

F: Gel pouring table.

The agarose-gel electrophoresis system is described in detail in Section 2.13.

The glass plate containing the gel was then placed between two perspex troughs (19 cm x 4 cm x 4.5 cm deep) containing Tris-borate buffer and a piece of platinum wire, running the length of the trough as the electrode. Electrical connection was completed by using Whatman chromatography paper No1 as a wick between the gel and buffer in the reservoir. The complete agarose-gel system is shown in plate 2.1.

Gels were normally run for 3-4 h at 150 volts (35-40 mA) or until the bromophenol blue front had travelled at least 11 cm. They were then photographed under UV light with a transluminator, 254 nm (Ultra Violet Products Ltd., Winchester, UK) with either a Polaroid Land Camera and orange filter; or with a Practica super TL 35 mm camera and Soligor ϕ49G orange filter with Ilford FP4 film.

2.13.2. Molecular weight determination of DNA fragments in agarose gels

Two sets of molecular weight standards were included in the agarose gels. Lambda DNA digested with restriction endonuclease HindIII, producing fragments of (10^6 daltons) 14.47, 6.23, 4.18, 2.82, 1.58, 1.39, 0.31; and a double digest with HindIII and EcoRI producing fragments of 13.4, 3.35, 3.20, 2.80, 2.32, 1.33, 1.27, 1.05, 0.89, 0.59, 0.47, 0.31 (Murray & Murray, 1975). An enlargement (10X) of a 35 mm photograph of the gels was used to measure the distance travelled from the well for each DNA fragment and a graph constructed of Log molecular weight against distance travelled. Unknown molecular weights of fragments were then determined from their distance travelled from the well using this calibration curve. An average of 2 or 3 estimations was used.

2.13.3. Removal of DNA from agarose gels

The following two methods were used:

(a) Freeze-squeeze method: This is based on the method of Thuring et. al., (1975) using a Tris-acetate buffer system. The DNA bands were visualised under UV light and the gel containing the DNA fragment of interest cut out, wrapped in parafilm and frozen at $-20^{o}C$ for 1 h. The parafilm bag containing the gel

was then squeezed to exude the liquid from the gel containing the DNA. Due to contamination from the agarose the DNA must be extensively dialysed against TE2 buffer (4°C). The recovery of DNA from the gel using this method was only about 50%.

(b) Electrophoretic elution: Using the preparative agarose-gel system, gels were run for about 2½ h, left in the glass plate and viewed under UV light from above. A well was cut in the gel just in front of the DNA band of interest, filled with buffer and electrophoresis continued. As the DNA moved into the well, visualised by an overhead UV light source, the buffer was removed, and replaced with fresh buffer repeatedly until all the DNA had travelled into the buffer. The buffer fractions were pooled, dialysed extensively against TE2 buffer (4°C), and the DNA precipitated with ethanol (2.7.6) and resuspended in a small volume of TE2.

2.14. Restriction Endonucleases

A full list of all the restriction endonucleases used in this investigation and their source is given in Table 2.2, and the isolation and purification of enzymes carried out in the laboratory is described in the Appendix, and includes the detection of new restriction endonucleases in thermophilic microorganisms.

The buffers used for digesting DNA varies with the restriction endonuclease used and therefore the composition of the buffers is summarised in Table 2.3. Each constituent was made as independant ten times concentrated solutions in double distilled water and autoclaved (DTT was filter sterilized). Assay buffers were then made by appropriate dilutions in sterilised double distilled water containing 1 mg.ml^{-1} gelatine, where endonucleases required dilution before use 50% (v/v) glycerol containing 1 mg.ml^{-1} gelatine was used for the dilutions. Triton X100 (0.1% w/v) was included when diluting EcoRI and PstI.

DNA (0.5 µg - 1.0 µg) was incubated in 20 µl assay buffer containing the restriction endonuclease (0.5 - 1.0 unit) for 60 min at 37°C, except for CauI, CauII (50°C) and BclI, BstEII, TacI, TaqI (60°C). One unit is defined as the amount of restriction endonuclease required to completely digest 1 µg DNA in 60 min at the relevant temperature (37°, 50° and 60°C). Reactions were

Table 2.2. Restriction endonucleases used in this investigation

A	B	C	D				E	F
Microorganism	Enzyme[a]	Recognition Sequence[b]	No. of cleavage sites[c]				Source	Ref.
			λ	Ad2	SV40	φx174		
Acinetobacter calcoaceticus	AccI	GT/$^{AG}_{CT}$AC	7	8	1	2	P.L. Biochemicals Inc.	1.
Anabaena variabilis	AvaI	C/PyCGPuG	8	15	0	1	Uniscience Ltd.	2, 3.
	AvaII	G/G$^{A}_{T}$CC	>17	>30	6	1	" "	4.
Arthrobacter luteus	AluI	AG/CT	>50	>50	35	24	" "	5.
Bacillus amyloliquefaciens H	BamHI	G/GATCC	5	3	1	0	Dr. R. Flavell	6, 7.
Bacillus caldolyticus	BclI	T/GATCA	7	5	1	0	A.H.A. Bingham[d]	8.
Bacillus globigii	BglI	GCCN_5GGC	22	12	1	0	Uniscience Ltd.	9, 1.
	BglII	A/GATCT	6	12	0	0	Dr. R. Flavell	9, 1.
Bacillus stearothermophilus ET	BstEII	G/GTNACC	11	8	0	0	Uniscience Ltd.	1.
Brevibacterium albidum	BalI	TGG/CCA	15	17	0	0	C.P. Laboratories Ltd.	10.
Chloroflexus aurantiacus	CauI	GG$^{A}_{T}$CC	>30	>30	6	1	A.H.A. Bingham[d]	11.
	CauII	CC$^{C}_{G}$GG	>30	>30	0	?	A.H.A. Bingham[d]	11.
Escherichia coli RY13	EcoRI	G/AATTC	5	5	1	0	A.H.A. Bingham[d]	12, 13.
Escherichia coli R245	EcoRII	/CC$^{A}_{T}$GG	>35	>35	16	2	Uniscience Ltd.	1, 14.
Haemophilus aegyptius	HaeII	PuGCGC/Py	>30	>30	1	8	Dr. R. Flavell	15, 16.
	HaeIII	GG/CC	>50	>50	19	11	Uniscience Ltd.	17, 18.
H. galinarum	HgaI	GACGC[e]	>50	>50	0	14	C.P. Laboratories	19, 20.
H. haemolyticus	HhaI	GCG/C	>50	>50	2	18	A.H.A. Bingham[d]/ Uniscience Ltd.	21.
H. influenzae Rc	HincII	GTPyPuAC	34	>20	7	13	Uniscience Ltd.	22.

Table 2.2 (Cont'd)

A	B	C	D				E	F
H. influenzae Rd	HindII	GTPyPuAC	34	>20	7	13	A.H.A. Bingham[d]/	23, 24.
	HindIII	A/AGCTT	6	11	6	0	Uniscience Ltd.	25.
H. influenzae Rf	HinfI	G/ANTC	>50	>50	10	21	A.H.A. Bingham[d]/ Uniscience Ltd.	1.
H. parahaemolyticus	HphI	GGTGA[f]	>50	>50	4	9	C.P. Laboratories	1, 26.
H. parainfluenzae	HpaI	GTT/AAC	13	6	4	3	A.H.A. Bingham[d]/	27, 28.
	HpaII	C/CGG	>50	>50	1	5	Uniscience Ltd.	27, 28.
Herpetosiphon giganteus	HgiAI	$G^{A}_{T}GC^{A}_{T}/C$	20	?	0	3	C.P. Laboratories	1.
Klebsiella pneumoniae OK8	KpnI	GGTAC/C	2	8	1	0	A.H.A. Bingham[d]	29, 30.
Moraxella bovis	MboII	GAAGA[g]	>50	>50	16	11	C.P. Laboratories	1, 31.
Moraxella nonliquefaciens	MnlI	CCTC	>50	>50	51	34	P.L. Biochemicals Inc.	1.
Proteus vulgaris	PvuI	CGATCG	4	7	0	0	C.P. Laboratories	1.
	PvuII	CAG/CTG	15	22	3	0		
Providencia stuartii	PstI	CTGCA/G	18	25	2	1	A.H.A. Bingham[d]	29, 32.
Rhodopseudomonas spheroides	RspI	?	4	7	0	0	A.H.A. Bingham[d]	33.
Staphylococcus aureus 3A	Sau3A	/GATC	>50	>50	8	0	Uniscience Ltd.	34.
Staphylococcus aureus PS96	Sau96I	G/GNCC	>30	>30	11	2	C.P. Laboratories	1.
Streptomyces achromogenes	SacI	GAGCT/C	2	7	0	0	P.L. Biochemicals	1.
Streptomyces albus	SalI	G/TCGAC	2	3	0	0	Uniscience Ltd	35.
Streptomyces stanford	SstII	CCGC/GG	3	>25	0	1	Dr. P.J. Rigby	1.

Table 2.2 (Cont'd)

A	B	C	D				E	F
Thermoplasma acidophilum	*Tac*I	CG/CG	>50	>50	0	14	Uniscience Ltd.	36.
Thermus aquaticus	*Taq*I	T/CGA	>50	>50	1	10	A.H.A. Bingham[d]/ Uniscience Ltd.	1, 37.
Xanthomonas badrii	*Xba*I	T/CTAGA	1	4	0	0	A.H.A. Bingham[d]/ Uniscience Ltd.	38.
X. holicola	*Xho*I	C/TCGAG	1	6	0	1	Uniscience Ltd.	1.
X. malvacearum	*Xma*I	C/CCGGG	3	12	0	0	A.H.A. Bingham[d]	39

Footnotes to Table

a. The enzymes are named in accordance with the proposal of Smith & Nathans (1973).

b. Recognition sequences are written from $5' \longrightarrow 3'$, only one strand being shown, and position of cleavage denoted by (/). Pu, any purine; Py, any pyrimidine, and N, any nucleotide, replace a specific nucleotide in the recognition sequence.

c. The number of cleavage sites on 4 viral geneomes are given; in Lambda (λ), Adenovirus-2 (Ad2), Simian virus 40 (SV40) and ϕx174 Rf DNA (ϕx174).

d. See Appendix.

e. *Hga*I cleaves as indicated:

$$5' \text{ GACGCNNNNN}^{\downarrow}$$
$$3' \text{ CTGCGNNNNNNNNNN}_{\uparrow}$$

f. *Hph*I cleaves as indicated:

$$5' \text{ GGTGANNNNNNNN}^{\downarrow}$$
$$3' \text{ CCACTNNNNNNN}_{\uparrow}$$

g. *Mbo*II cleaves as indicated:

$$5' \text{ GAAGANNNNNNNN}^{\downarrow}$$
$$3' \text{ CTTCTNNNNNNN}_{\uparrow}$$

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Enzyme	Concentration of constituent (mM)			
	Tris-HCl pH 7.5	$MgCl_2$	NaCl	DTT
SacI	10	10	10	0.5
SalI	10	10	10	0.5
SstII	14	6	90	0.5
TacI	10	10	-	0.1
TaqI	10	6	6	0.5
XbaI	10	10	100	0.5
XhoI	6	6	150	0.5
XmaI	10	10	10	0.5

Assay buffers were made from 10x stock solutions as described in 2.14.1.

Table 2.3. Constituents of assay buffers for restriction endonucleases

Enzyme	Concentration of constituent (mM)			
	Tris-HCl pH 7.5	$MgCl_2$	NaCl	DTT
AccI	6	15	60	0.5
AvaI	20	10	30	-
AvaII	80	10	30	-
AluI	6	6	-	0.5
BamHI	10	10	10	0.5
BclI	10	10	10	0.5
BglI	20	7	-	0.5
BglII	20	7	-	0.5
BstEII	6	6	-	0.5
BalI	10	10	6	1.0
CauI	10	10	10	0.5
CauII	10	10	10	0.5
EcoRI	100	20	50	0.5
EcoRII	100	5	-	-
HaeII	50	5	-	0.5
HaeIII	50	5	-	0.5
HgaI	6	15	60	0.5
HhaI	20	20	50	0.5
HincII	20	20	50	0.5
HindII	20	20	50	0.5
HindIII	20	20	50	0.5
HinfI	20	20	50	0.5
HphI	10	10	6	1.0
HpaI	20	20	50	0.5
HpaII	20	7	-	1.0
HgiAI	10	10	100	-
KpnI	10	10	10	0.5
MboII	10	10	6	0.5
MnlI	10	10	100	-
PvuI	10	6	15	0.5
PvuII	10	10	6	1.0
PstI	10	10	10	0.5
RspI	6	6	-	0.5
Sau3A	6	15	60	0.5
Sau961	6	15	60	0.5

terminated by the addition of 2 µl, 0.5 M-Na_2.EDTA pH 8 containing 0.03% (w/v) bromophenol blue when samples were to be subjected to agarose-gel electrophoresis.

Where a plasmid was digested with two enzymes having different buffer requirements, it was first digested with the enzyme requiring the buffer of lower ionic strength, and then the buffer was adjusted with ten times concentrated components prior to addition of the second endonuclease.

2.15. Mapping of Restriction Endonuclease cleavage sites

Each species of plasmid DNA was initially digested with the full range of restriction endonucleases (Table 2.2) to determine the number of cleavage sites present for each enzyme. The molecular weights of the fragments produced were determined graphically using λ DNA molecular weight standards as described in section 2.13. Since the fluorescence of DNA fragments in agarose-gels is directly proportional to the size of the fragment, when using 1 µg DNA large fragments produce a very wide band in the gel making a molecular weight estimation inaccurate. As accuracy is essential for constructing restriction endonuclease maps, this problem was overcome by using only 0.5 µg DNA in the digests and using 2 loadings in the gels, 5 µl of the mixture and 15 µl. The former allows the large fragments to be sized accurately and the latter allows detection and sizing of the small fragments. Molecular weight values were taken as the average of at least 2 estimations.

Firstly, all the restriction endonucleases with just one cleavage site were positioned in relation to each other using a double digest technique, where the plasmid was digested in turn by two of the enzymes. All the possible combinations of pairs of restriction endonucleases were used.

Secondly, using these single sites as reference points, the location and orientation of 2 or more fragments produced by a particular endonuclease were determined, using double digests where, in turn, each of the enzymes with 1 cleavage site was used as one of the enzymes in a double digest.

2.16. Ligations

DNA (0.5 µg) was digested with 1.5 units of restriction

endonuclease for 60 min and the enzyme then denatured by heating to 66°C for 15 min. BclI, BstEII, ThaI and TaqI are active at this temperature and the restriction endonuclease was removed by phenol extraction followed by ethanol precipitation (section 2.7.6).

The species of DNA to be ligated were then mixed, precipitated with ethanol (2.6.6), suspended in 20 µl ligase buffer (66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, 0.4 mM ATP) at 4°C and 0.05 units T_4-DNA ligase (Uniscience Ltd., Cambridge) added. Incubations were carried out at 4°C for at least 17 h.

2.17. Plasmid Copy Number

2.17.1. Growth of cells

A fresh overnight culture in TYS (10 ml) was used to inoculate (0.1 ml) fresh medium (200 ml) containing 0.1 ml (^{3}H) thymidine, (1 $mCi.ml^{-1}$; 45 $mCi.mmol^{-1}$) and 0.5 ml deoxyadenosine (10 $mg.ml^{-1}$) and incubated until the culture was in mid-log phase growth, as determined by the OD_{550}.

2.17.2. Lysis

This follows the procedure described in 2.5.2 except that a small sample (10 µl) of the lysate before the clearing spin was retained, spotted onto a glass fibre circle, washed with 5% (w/v) TCA, 95% (v/v) ethanol and diethyl ether and counted for ^{3}H activity as previously described (2.6.1). This gave total ^{3}H cpm for chromosomal + plasmid DNA.

2.17.3. Isolation of (^{3}H) plasmid DNA by CsCl gradients

The cleared lysate from above was extracted twice with chloroform (2.6.2) and 2 ml subjected to isopycnic centrifugation as described in 2.6.2. The plasmid band was removed from the gradient (2.7.5) and a small sample counted for ^{3}H activity as described in 2.6.1. This method of determining the proportion of ^{3}H in plasmid DNA did not work for pAB124 or derivatives as

separation of chromosomal and plasmid bands is poor in caesium chloride-ethidium bromide density gradients, therefore an alternative method was used.

2.17.4. Isolation of ^{3}H plasmid DNA by agarose-gel electrophoresis

A sample (100 µl) of the chloroform treated cleared lysate was subjected to electrophoresis in a 0.8% (w/v) agarose gel in Tris-acetate buffer. The DNA was visualised by fluorescence under UV light and the plasmid band (covalently closed circular species) cut out of the gel and the gel dissolved in saturated potassium iodide solution, (Blin et. al., 1975). An equal volume of cold, 10% (w/v) TCA solution was added; the precipitated DNA was collected on a 2.5 cm glass-fibre circle using a Millipore filtration apparatus, washed with 5 ml 5% (w/v) TCA, 5 ml 95% (v/v) ethanol, 5 ml diethyl ether and then counted for ^{3}H activity in a scintillation counter (2.6.1).

Using the total cpm in chromosomal plus plasmid DNA and the total cpm of plasmid DNA the copy number of the plasmid was calculated (see Chapter 3).

2.18. Curing of plasmids from TB strains of thermophilic bacilli

It was shown that sub-culture in the absence of antibiotic in TYS and AD minimal medium consistently would not promote plasmid expulsion. Therefore chemicals known to stimulate plasmid expulsion were used (Hirota, 1956; Hirota, 1960; Gale et. al., 1972). The MIC of each strain (TB 150, Sm^{r}; TB144, Tc^{r}, TB 128, Tc^{r}) to acridine orange , ethidium bromide, and mitomycin C was determined. Each strain was cultured in TYS medium containing a level of mutagen that would just allow growth at 50°C, then sub-cultured a second time in the same liquid medium and any antibiotic sensitive strains in the population identified by plating serial dilutions (in saline) of the culture on TSBA plates, then replica-plating (Collins & Lyne, 1970) onto TSBA plates containing the appropriate antibiotic, and screening for the presence of plasmid DNA (2.6.2).

2.19. Electron Microscopy

Dr. P.W.J. Rigby, Mr. W. Chia and Mr. S. Kidd gave assistance in preparation and shadowing of grids and operation of the electron microscope at Imperial College.

The spreading of DNA for electron microscopy was performed as described by Davis et. al., (1971).

2.19.1. Preparation of Grids

(1) Parlodion film. Copper grids were placed on filter paper in a water filled buchner funnel and the surface of the water cleaned by a first Parlodion film. This was prepared by putting one drop of water free Parlodion solution in i-amyl acetate (3% v/v) onto the water surface in the middle of the funnel. After evaporation of the solvent (5-6 min) the thin membrane of Parlodion formed was removed with a needle. A second Parlodion film was prepared in the same way and allowed to sink onto the grids by draining away the water. The grids coated with the Parlodion membrane were dried in air.

(2) Carbon film. Copper grids were placed on a glass slide and covered with a Neopren solution (0.3% v/v in toluene). Excess Neopren was removed with filter paper and the grids air-dried. Pure carbon was evaporated onto a glass slide in the vacuum evaporator and the carbon film then floated onto a water surface. Each Neopren coated grid was submerged into the dish of water containing the carbon film and lifted through the film so that the carbon film covers the grid. The grids were then dried in air.

2.19.2. Aqueous spreading

A solution of ammonium acetate pH 7.5 (0.25 M) contained in a petri dish acted as the hypophase and a glass slide (washed in the hypophase solution) supported by a teflon bar (1 x 1 x 10 cm) served as a ramp. 50 µl of spreading solution (0.5 $\mu g.ml^{-1}$ DNA, 0.1 $mg.ml^{-1}$ cytochrome C, 0.5 M NH_4.acetate, 1 mM Na_2.EDTA pH 7.5) was spread with a micro-pipette on the glass slide about 1 cm from the hypophase surface and allowed to run onto the hypophase. The

film was picked up from the hypophase surface onto a prepared grid and stained for 30 sec in staining solution (5×10^{-5} M uranyl acetate in 90% (v/v) ethanol from a 5×10^{-3} M stock solution in 50 mM HCl) and rinsed in isopentane. The grids were then rotary shadowed with platinum/paladium.

2.20. Transformation of Bacillus subtilis with plasmid DNA

2.20.1. Transformation of competent B. subtilis cells

This is based on the method of Stacey (1968). A single colony from a fresh TSBA plate was used to inoculate 200 ml of SMS containing 0.5% glucose and 20 $\mu g.ml^{-1}$ of any amino acid required by the B. subtilis auxotrophic mutant being used. After incubation overnight at 35°C the culture was diluted into fresh medium to give an optical density (550 nm) of 0.8 and shaken vigorously at 35°C. The OD_{550} was monitored at 30 min intervals, and when the increase in OD decreased to 50% of that observed during logarithmic growth the culture was diluted with an equal volume of pre-warmed starvation medium, which lacks any amino acids required by the strain. Vigorous aeration was continued for 90 min, after which time the cells were maximally competent.

In later experiments the dilution into a starvation medium was not carried out, since it was found that cells became competent when left in the original medium (see Chapter 5).

1 ml of the competent cell culture was then added to pre-warmed plasmid DNA (0.5 µg - 5 µg) in 50 µL polyethylene glycol 6000 (50% w/v) and incubated for 1 h at 35°C with vigorous shaking. Pre-warmed TYS medium (5 ml) was then added and incubation continued for a further 1 h prior to plating on selective plates.

2.20.2. Transformation of B. subtilis protoplasts

This is based on the method of Chang & Cohen (1979).

A fresh overnight culture (37°C) in TYS medium was used to inoculate 100 ml of fresh medium and incubation at 37°C continued with vigorous aeration. The Optical Density (550 nm) of the

culture was monitored at 30 min intervals and when it reached 1.2, 40 ml of the culture was centrifuged at 6000 xg for 10 min (25°C). The cells were resuspended in 3 ml of SMM (Wyrick & Rogers, 1973), lysozyme added (600 µg) and incubated at 42°C for 30 min (Gabor & Hotchkiss, 1979).

Plasmid DNA (1 ng to 5 µg) was mixed with an equal volume of 2X SMM and 0.5 ml of protoplast solution from above added followed immediately by the addition of 1.5 ml 40% (w/v) PEG in SMM and gentle mixing of the contents of the tube. After 2 min exposure to PEG, 5 ml of SMMP was added to dilute out the PEG and the protoplasts were recovered by centrifugation for 10 min at 5000 xg (25°C). The protoplasts were resuspended in 1 ml SMMP and incubated for 1.5 h at 37°C to enable phenotypic expression of genetic determinants carried on the plasmid before plating on selective media (DM3). Dilutions were carried out in SMM containing 1% (w/v) BSA. Plates were incubated at 37°C for 2-3 days to allow regeneration of the protoplasts to the bacillary form.

2.21. Transformation of E. coli with Plasmid DNA

This is based on the method of Cohen, Chang and Hsu (1972).

A fresh overnight culture (10 ml) was prepared in TYS medium (2.1) and used to inoculate 100 mls of the following medium; 50 ml TYS + 50 ml AD minimal + 1 ml 50% (w/v) glucose. The culture was incubated at 37°C with rapid shaking for 90 min and then chilled on ice. The cells were harvested by centrifugation at 6000 xg for 10 min (4°C), washed once in 10 mM $CaCl_2$ (80 ml) once in 75 mM $CaCl_2$ (5 ml) and suspended in 5 ml 75 mM $CaCl_2$.

Cells (0.4 ml) were then added to 30 µl DNA solution (0.5 - 5 µg) and incubated at 0°C for 45 min. The suspension was then incubated at 37°C for 10 min and then chilled on ice. Pre-warmed (37°C) TYS medium (5 ml) was then added and the cells incubated at 37°C for 2-3 h to allow phenotypic expression of genetic determinants on the DNA. Samples (0.1 ml) were then diluted in saline and plated on selective plates.

2.22. Transformation of B. stearothermophilus LO2 with Plasmid DNA

A fresh plate was used to inoculate 100 ml of TYS medium

(2.1) and the culture incubated at 55°C for 17 h with vigorous aeration. The culture was used to inoculate 200 ml of pre-warmed TYS to give an OD_{550} of 0.3 - 0.4 and incubation at 55°C continued. The optical density (550 nm) was monitored at 20 min intervals and when it reached 0.7 - 0.9, 40 ml samples were centrifuged at 6000 xg for 10 min (20°C). The cells were resuspended in 2 ml SMM6 (after all excess liquid had been drained from the centrifuge pot), 4 mg lysozyme added and incubated at 42°C for 30 min with occasional shaking.

Plasmid DNA (0.01 - 2 µg) was mixed with an equal volume of X2 SMM6 and added to 0.5 ml protoplast suspension in a small (10 ml) centrifuge tube (see above) followed immediately by the addition of 1.5 ml 40% PEG (w/v) in SMM6. The contents of the tube were mixed gently, incubated at 42°C for 2 min and then 5 ml of SMM6P (pre-warmed) added to dilute out the PEG. The suspension was centrifuged at 5000 xg for 15 min (20°C) and the pelleted protoplasts resuspended gently in 2 ml of SMM6P and incubated for 2 h at 55°C with occasional mixing. Dilutions were made in SMM6 + 1% BSA (w/v) and plated on DM4 or DM5 plates (2.1) containing antibiotic. Plates were taped up with plastic tape to reduce evaporation and incubated at 55°C for 36-48 h.

2.23. DNA-DNA hybridisation on nitrocellulose filters

This is based on the method of Denhardt (1966).

2.23.1. Labelling of DNA with ^{32}P by "nick translation"

A method communicated by Dr. A. Akrigg based on the method of Rigby et. al., (1977) was used.

Labelled dTTP (α ^{32}P) (2000 - 3000 $Ci.mmol^{-1}$), 20 µl (Radiochemical Centre, Amersham), was dried down under vacuum in a small tube and 20 µl X5 reaction buffer added (on ice) composed of the following;

1 µL 1 M $MgCl_2$

1 µL 0.1 M K. phosphate buffer (pH 7.4)

1 µL 15 mM ATP

7 µL 1 M Tris-HCl pH 7.4

1 µL 0.1 M Na_2.EDTA

1 µL 2 mM dATP

1 µL 2 mM dGTP

1 µL 2 mM dCTP

1 µL 0.1 M DTT

5 µL Distilled water.

1 pg . DNaseI (grade DN-EP, Sigma Ltd., Poole, UK) was then added, followed by 45 µl distilled water, 1 µg DNA (2 µL) and 25 µL DNA polymerase I, in 10 mM Tris-HCl pH 7.0 (A.H.A. Bingham) containing 1 unit of activity. The mixture was then brought to room temperature, held for 2 min and then incubated at 10°C for 60 min. The reaction was stopped by the addition of a drop (0.05 µL) of phenol, equilibrated in 20 mM-Tris-HCl pH 7.5. The phenol was removed with a pasteur pipette and a drop of Orange G (Sigma Ltd., Poole, UK) solution (1% w/v) added to the aqueous phase as a marker.

The mixture was then applied to a Sephadex G50 column (10 ml) equilibrated in 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 2 mM EDTA to separate labelled DNA from unincorporated nucleotides. Fractions (250 µL) were collected and 1 µL samples counted for ^{32}P activity in a scintillation counter (see 2.6.1). Two peaks of activity eluted from the column, fractions showing most of the activity eluting from the column first were pooled, and the DNA denatured by boiling for 15 min and rapid cooling to 0°C. A specific activity of 2.5×10^6 cpm per µgDNA was obtained.

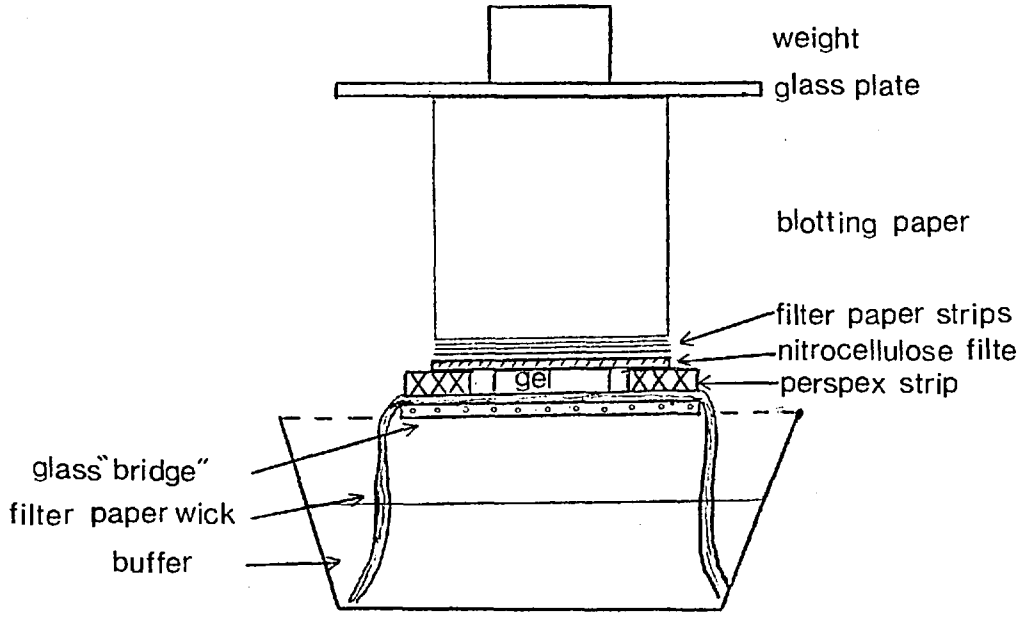
2.23.2. Agarose gels and Southern "blotting"

Samples of DNA were subjected to agarose-gel electrophoresis as described in 2.13. The gel was then soaked in denaturing solution for 45 min (0.2 M NaOH, 0.6 M NaCl) and then in neutralising solution for 45 min (1 M-Tris-HCl pH 7.4, 1.5 M NaCl). The DNA was then transferred onto nitrocellulose strips (Whatman Ltd., Maidstone) as illustrated in Fig 2.1. SSC (X20) buffer was used (3 M NaCl, 0.3 M Na. citrate), and 24 h was allowed for DNA transfer to the nitrocellulose filter.

The gel was peeled away from the nitrocellulose filter which was then washed in 0.3 M NaCl, 0.03 M Na.citrate for 15 min, dried in a 37°C incubator followed by baking at 80°C for 3 h in a vacuum oven (Gallenkamp Ltd., UK).

The amount of DNA remaining in the gel was determined by

FIG 2.1 'Southern' blotting device



restaining with ethidium bromide and photographing under UV light as described in section 2.13.

2.23.3. DNA-DNA Hybridisation (Denhardt, 1966)

The nitrocellulose filter was placed in a plastic bag and a sufficient quantity of buffer (0.3 M NaCl, 0.03 M Na.citrate, 50% formamide) added to just cover the filter. The labelled DNA (1-2 x 10^6 cpm) was then added and the bag sealed. The bag was then incubated at 37°C for 24-36 h.

Following hybridisation, the nitrocellulose filter was removed from the bag and washed in 0.3 M NaCl, 0.03 M Na. citrate for 4 x 20 min, replacing buffer after each period. The filters were then air dried on Whatman 3 MM paper for 1-2 h.

2.23.4. Autoradiography (Laskey & Mills, 1975)

This was kindly carried out by Dr. A. Akrigg. The filter was placed on X-ray film (Kodak Ltd. X Omat-R) and kept at -70°C for 1-3 days. The film was then developed as recommended by the manufacturer.

APPENDIX

ISOLATION AND PURIFICATION OF RESTRICTION ENDONUCLEASES

Appendix Contents

At the outset of this work only a few restriction endonucleases were available commercially, and were of questionable quality. Therefore procedures for the laboratory scale purification of the more useful restriction endonucleases, 14 in all, were developed to provide a wider range of enzymes particularly for plasmid characterisation.

In addition a screening programme of thermophiles for the presence of Class II restriction endonucleases was initiated, as no enzyme had been isolated from a thermophile at that time. It was hoped these new enzymes would be useful for plasmid characterisation on the basis that most extreme thermophiles have significantly more G:C base-pairs than mesophilic bacteria (Sharp, et. al., 1980); thus a restriction endonuclease from a thermophile may recognise a base sequence containing several G:C bases and might therefore have cleavage sites within a G:C rich plasmid from a thermophile.

A.1. Isolation of new Restriction Endonucleases

A.1.1. Screening of Thermophiles for Restriction Endonucleases

Strains were grown to stationary phase in 5 x 2L flasks each containing 1L of TYS medium, except for Thermus aquaticus (Ramaley & Hixson's medium) and the photosynthetic bacteria, Chloroflexus aurantiacus and Rhodopseudomonas spheroides (not grown photosynthetically) which were kindly supplied by Dr. J. Darbyshire. Cells were harvested by centrifugation at 6000 xg for 10 min at 4°C.

A sample of cell-paste (10 g) was suspended in 30 ml ST buffer and the cells disrupted by sonication (4 x 1 min) at 20 KH_z, 5 Amp, while maintaining the temperature below 10°C. Cell debris was removed by centrifugation (40000 xg, 60 min at 4°C) and the supernatant adjusted to pH 7.4 (2N NaOH). The extract (20 ml) was then loaded onto a 1.8 L (55 x 6.4 cm) Sephacryl S-200 (Pharmacia, Uppsala, Sweden) gel-filtration column equilibrated in TSG buffer and eluted with the same buffer at 100 $ml.h^{-1}$, 12 ml fractions were collected.

Since most Class II restriction endonucleases have a molecular weight within the range 50000 - 90000 (Bingham & Atkinson, 1978) only fractions from the Sephacryl column corresponding to this range were assayed for restriction endonuclease activity using λ DNA and Adenovirus 2 DNA (kindly provided by Dr. P. Gallimore, Birmingham University).

The results of the initial screen of thermophiles is given in Table 1 and show that 6 new restriction endonucleases were detected. Larger scale purifications of each were then carried out to obtain the endonucleases free of contaminating non-specific nucleases. The cell-pastes were kindly provided by Mr. M.J. Comer.

Appendix Table 1. Detection of restriction endonuclease activity in thermophilic bacteria

		Restriction Endonuclease	No. cleavage sites λ	Ad2	SV40
Bacillus stearothermophilus	EP262	-	-	-	-
" "	ATCC 12016	-	-	-	-
" "	EP240	+ (BstA1)[a]	?	?	?
" "	AY174	-	-	-	-
Bacillus caldotenax		-	-	-	-
" caldolyticus		+ (BclI)	>3[b]	5	1
" caldovelox		-	-	-	-
Thermus aquaticus	B	-	-	-	-
" "	YT1	+ (TaqI)	>50	>50	1
" "	X1	-	-	-	-
Chloroflexus aurantiacus		+ (CauI/II)[c]	>50	>50	6
Rhodopseudomonas spheroides[d]		+ (RspI)	4	7	0

Each strain was screened for restriction endonuclease activity as described in Appendix . (A.1.1.).

a, This enzyme was very unstable and difficult to purify.

b, BclI sites in lambda DNA are extensively modified.

c, Two endonucleases are present in all extracts.

d, This bacterium is not a thermophile.

A.1.2. The BclI Restriction Endonuclease

Purification

200 g cell paste was suspended in 500 ml of TSG buffer at 4°C and passed twice through a Manton-Gaulin homogenizer (500 $Kg.cm^{-2}$) to disrupt the cells. Cell debris was removed by centrifugation at 25000 xg for 60 min (4°C) and the pH of the supernatant adjusted to 7.4(2N NaOH).

The extract (550 ml) was loaded onto a 13L Sephacryl S200 (Pharmacia, Uppsala, Sweden) gel-filtration column (85 x 14 cm) equilibrated in TSG buffer and eluted with the same buffer at 660 $ml.h^{-1}$. Fractions (330 ml) were collected and those showing endonuclease activity pooled (2.5 L) and concentrated to 250 ml with an Amicon CH_3 hollow fibre concentrator (Amicon, High Wycombe) containing a PM10 membrane.

The concentrated pool was dialysed against 40 L of KP buffer for 17 h (4°C) and loaded onto a 600 ml DE-52 cellulose column (Whatman Ltd., Maidstone), 19 x 6.4 cm, equilibrated in the same buffer. BclI was eluted by a 4 L linear gradient of 0-600 mM NaCl in KP buffer (250 $ml.h^{-1}$) after washing the column with 1L KP buffer. Fractions (110 ml) showing BclI activity were pooled (1.5L) and concentrated to 250 ml as before.

The concentrated pool was then diluted 5-fold with KP buffer and added to 150 g (wet wt.) phosphocellulose P11 (Whatman Ltd., Maidstone) pre-cycled (Greene et. al., 1974) and equilibrated in KP buffer. After stirring for 1 h at 4°C, the resin was collected in a column (9 x 6.4 cm) washed with 400 ml KP containing 50 mM NaCl and BclI eluted with a 2L linear gradient (125 $ml.h^{-1}$) of -50 mM-1 M NaCl in KP. The active fractions (10 ml) showing BclI activity were pooled (400 ml) and concentrated to 40 ml as before. At this stage the endonuclease was largely free of non-specific nucleases and suitable for cleavage analysis. For cloning work involving ligations with BclI, the enzyme was further purified by a second gel-filtration step on a Sephacryl S200 column (1.8L) to give better results. A summary of the results of the purification protocol is given in Table 2.

Characterisation

Calibration of the final Sephacryl column with aldolase

(MW; 156000 daltons), bovine serum albumin (68000), ovalbumin (45000), chymotrypsinogen A (24,700) and cytochrome C (12400) gave an estimate of the native molecular weight of BclI as 52000 and SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) of the peak faction revealed one major band at 25000. Thus it would appear the enzyme is a dimer of 25000 sub-units.

Throughout the purification of BclI, Ad2 DNA was used for assays, since λ DNA was only partially digested and gave a poor result on gels due to the extensive modification of bacteriophage λ against BclI in the E. coli host it was produced in (see Chapter 2.12). Such modification in E. coli, due to dam methylation is also known for several other restriction endonucleases (Roberts, 1980). Using λ DNA produced in an E. coli dam$^-$ strain, BclI was shown to have 7 cleavage sites. This enzyme also has 5 sites in adenovirus 2 DNA and 1 in simian virus 40 DNA. The recognition sequence of BclI was determined by R.J. Roberts & D. Sciaky (Bingham, et. al., 1978) as:

$$5'\text{-T}\downarrow\text{G-A-T-C-A-}3'$$
$$3'\text{-A-C-T-A-G-T-}\uparrow 5'$$

with the position of cleavage denoted by the arrow.

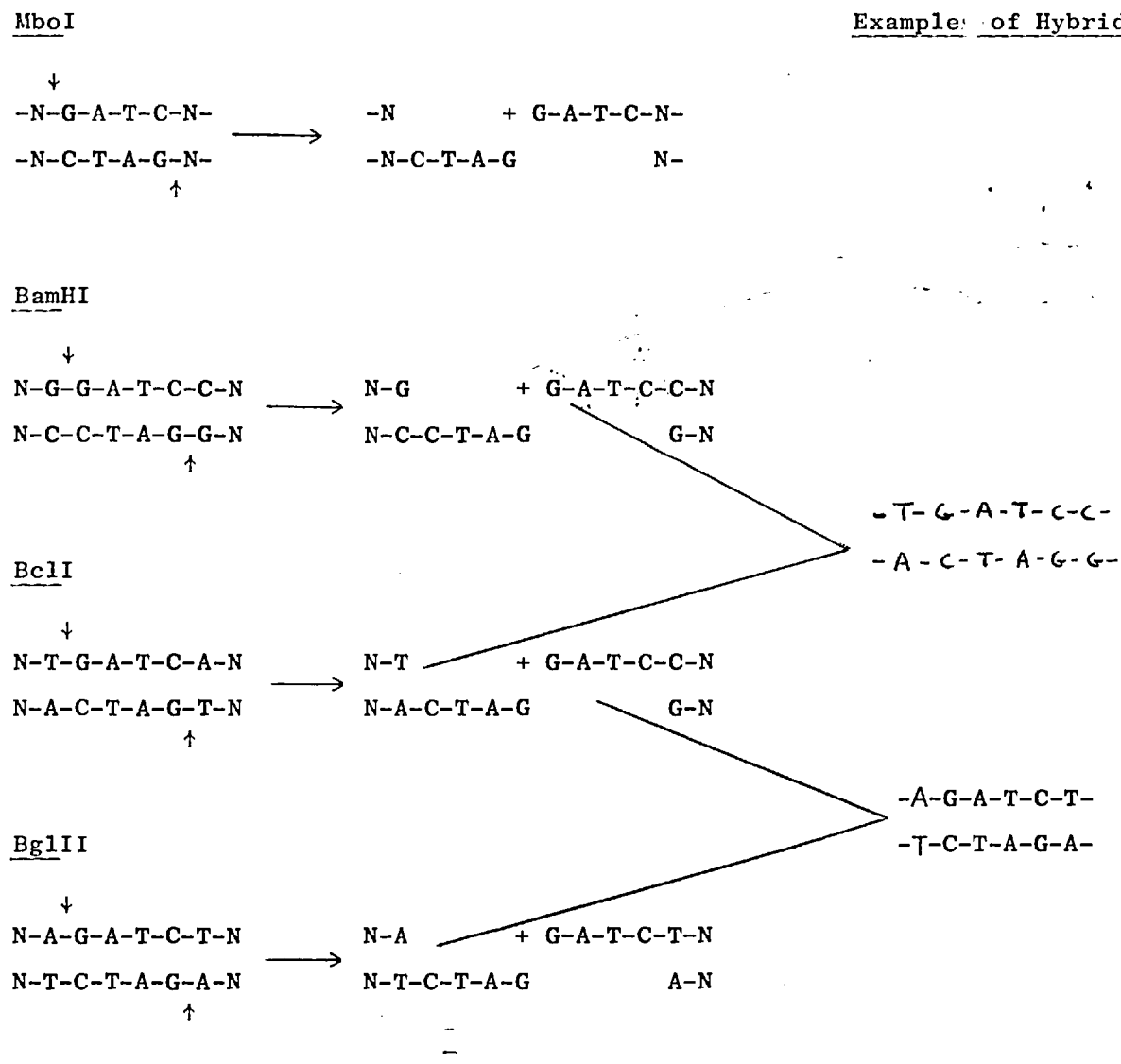
Cleavage with BclI therefore produces a CTAG extension identical to that produced by MboI, BglII, and BamHI (Roberts, 1980) so it is possible to ligate together DNA fragments produced from digestion with any of the four enzymes (Fig 1). Excepting MboI fragments this generates a new hexanucleotide sequence which will not be recognised by either of the 2 endonucleases used to initially produce the fragments (Fig 1, Table 3) and can be used to great effect in genetic manipulation experiments to select for recombinant molecules in vitro prior to transformation/transfection.

Appendix Table 2. Purification of BclI restriction endonuclease

	Protein (mg)	Enzyme (Total Units x 10^{-6})	Specific Activity (Units mg^{-1})	Recovery (%)
Extract	4,200	41	9,760	100
Sephacryl Pool (A)	1,600	38	23,750	93
DEAE-Cellulose Pool	800	29	36,250	71
Phosphocellulose Pool	40	14	350,000	34
Sephacryl Pool (B)	7	8	1,140,000	19

BclI was purified as described in Appendix (A2.1.2.) from 200 g cell-paste. Protein was estimated by the method of Lowry *et al.* (1951) and endonuclease activity as described in Methods (2.14.). One unit of enzyme is defined as the amount of enzyme that will completely digest 0.5 µg Ad2 DNA in 30 min at 50°C.

Appendix Fig 1. Annealing of restriction endonuclease produced fragments with a CTAG extension



Appendix Table 3. 5' Recognition sequences produced by annealing DNA fragments produced by MboI, BamI, BglII and BclI.

	MboI (N/GATC)	BamI (G/GATCC)	BglII (A/GATCT)	BclI (T/GATCA)
MboI (N/GATC)	N/GATC	N/GATCC	N/GATCT	N/GATCA
BamI (G/GATCC)		G/GATCC	G/GATCT	G/GATCA
BglII (A/GATCT)			A/GATCT	A/GATCA
BclI (T/GATCA)				T/GATCA

A.1.3. The CauI and CauII Restriction endonucleases

Purification

Large scale extractions of C. aurantiacus were effected by suspending 200 g cell paste in 600 ml TSG buffer and passing once through a Manton-Gaulin homogeniser at 500 Kg/cm^2; the homogeniser was washed with 400 ml TSG buffer to remove the remaining disrupted cells. Cell debris was removed by centrifugation at 25000 x g for 60 minutes (4^{o}C) and the supernatant adjusted to pH 7.4 (2N NaOH). PEI (2.5% solution (v/v), prepared as described by Bingham et. al., 1977) was added slowly to the extract (1000 ml) to a final concentration of 0.1% v/v (40 ml) and gently stirred for 15 min on ice. The precipitate was removed by centrifugation at 25000 x g for 60 min (4^{o}C). This step removes the heavy pigmentation as well as nucleic acids.

DE-52 cellulose (400 ml settled volume = 200 g wet wt.) equilibrated in TSG buffer at pH 7.4, was added to the supernatant (975 ml) and stirred slowly at 4^{o}C for 30 min. The resin was removed by centrifugation at 25000 x g for 30 min at 4^{o}C, and the supernatant diluted 1:4 with KP buffer.

Phosphocellulose (200 g wet wt. = 60 g dry wt.) equilibrated in KP buffer was added to the DEAE-cellulose treated extract and stirred for 60 min at 4^{o}C. The resin was collected in a column (8 x 6.4 cm), washed with 500 ml KP buffer and eluted with a 3 l linear gradient of 0-900 mM NaCl in KP buffer at 200 ml.hr^{-1} (4^{o}C). 150 ml fractions were collected. Upon assaying the fractions for the restriction endonuclease (50^{o}C) it was clear that two activities eluted. One, termed CauI, between fractions 6-12; and a second (CauII) between fractions 21-28 (Fig 2). There is about four times as much CauII present as CauI at this stage. Each endonuclease was then further purified independently.

The CauI phospocellulose pool (750 ml) was adjusted with 1M HCl to pH 7.0 and a suspension of hydroxyapatite equilibrated in KP buffer pH 7.0 containing 400 mM NaCl, was added to give a settled bed volume of 40 ml. After gentle stirring at 4^{o}C for 1 hour the hydroxyapatite was allowed to settle and the supernatant decanted. The resin was suspended in 50 ml KP buffer pH 7.0, 400 mM NaCl, collected in a column (2.5 x 4.4 cm), washed with 30 ml of the same buffer and eluted with a 250 ml linear gradient of 20 mM potassium phosphate, pH 7.0, 400 mM NaCl, 10% glycerol (v/v) 10 mM β-mercaptoethanol to 200 mM potassium phosphate pH 7.0,

400 mM NaCl, 10% (v/v) glycerol, 10 mM β-mercaptoethanol at 15 ml.hr^{-1} (4°C). Fractions (10 ml) were collected and those containing enzyme activity pooled (50 ml) and concentrated to 10 ml with an Amicon PM10 membrane.

The concentrated pool was loaded onto a 1.8 L (55 x 6.4 cm) Sephacryl S-200 gel filtration column, equilibrated in TSG buffer, and eluted with the same buffer (100 ml.hr^{-1}), 12 ml fractions were collected, the active fractions pooled (120 ml) and concentrated to 10 ml against an Amicon PM10 membrane. Gelatine was added to a final concentration of 0.5 mg.ml^{-1} and the pool dialysed against 20 vol. 25 mM Tris-HCl pH 7.4, 400 mM NaCl, 10 mM β-mercaptoethanol, 50% (v/v) glycerol and stored at -20°C.

The CauII phosphocellulose pool (1350 ml) was further purified using hydroxyapatite and Sephacryl S200 columns as described for CauI above.

A summary of the results of the purification is given in Table 4. The specific activity of the final CauI pool is much lower than that of CauII (Table 4) and this is reflected by the presence of slight amounts of non-specific nuclease in the final CauI pool.

Characterisation

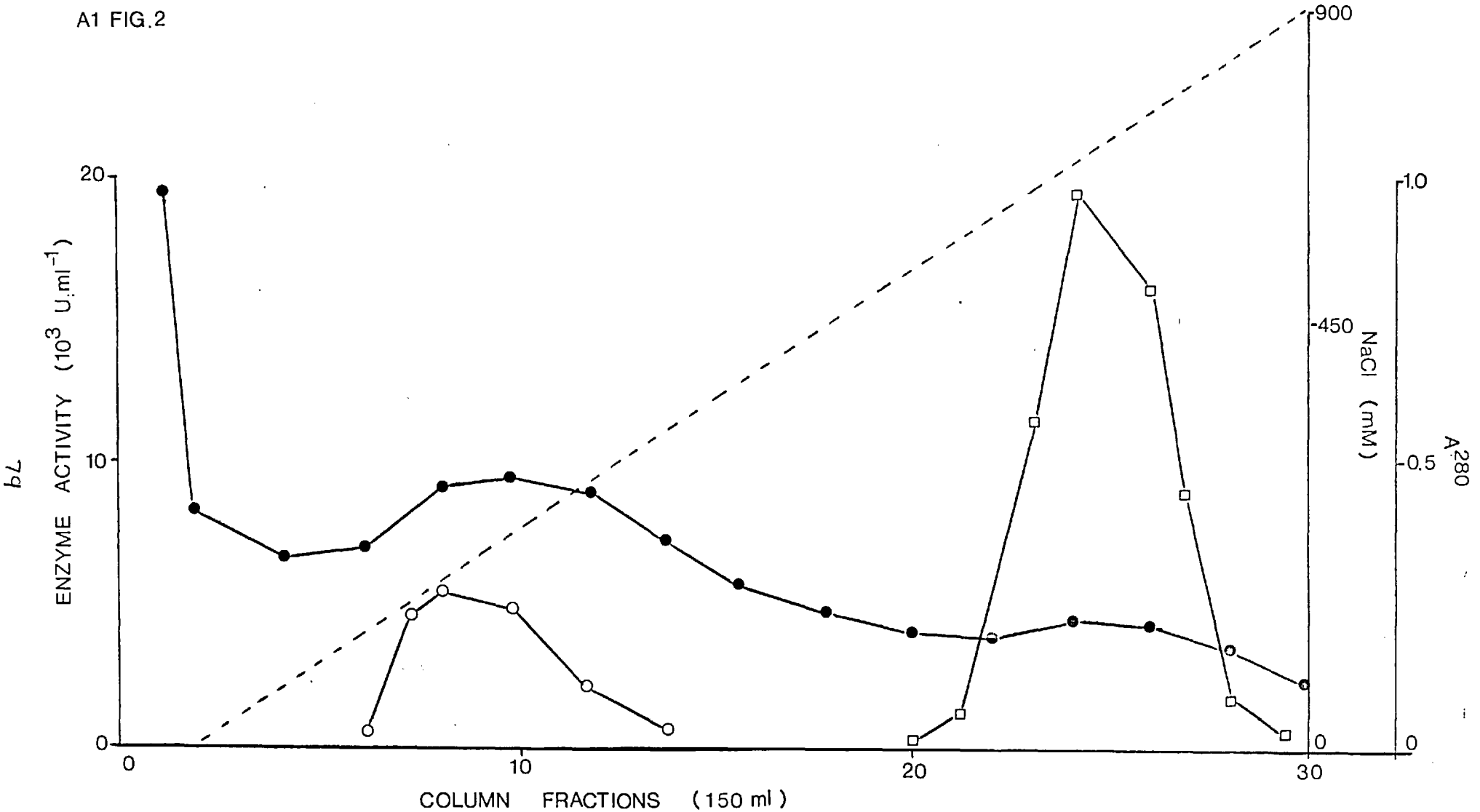
Calibration of the Sephacryl S200 column as described for BclI, enabled an estimation of the native molecular weights of the endonucleases as 66000 for CauI and 42000 for CauII. SDS polyacrylamide gel-electrophoresis (Laemmli, 1970) of the two pools showed that neither was homogeneous, and the CauI preparation contained substantially more contamination. However both enzyme preparations were suitable for cleavage analysis and it was shown that λ DNA and AD2 DNA both contained more than 50 cleavage sites while SV40 had 6 sites for CauI but no CauII sites.

The recognition sequence of the two endonucleases were determined by Prof. W. Fiers (unpublished) to be:-

CauI	CauII
$GG^{A}_{T}CC$	$CC^{C}_{G}GG$

Both enzymes recognise sequences of predominantly G : C nucleotides, an intersting observation since they come from a photosynthetic thermophile. The position of cleavage within the

A1 FIG.2


ENZYME ACTIVITY (10^3 U.ml^{-1})
20
10
0
0
10
20
30
COLUMN FRACTIONS (150 ml)
900
450
0
NaCl (mM)
1.0
0.5
0
A_{280}

Appendix Figure 2. Elution of CauI and CauII restriction endonucleases from phosphocellulose

An extract of Chloroflexus aurantiacus (200 g cell paste) was prepared, treated with PEI &DEAE cellulose and loaded on to a Phosphocellulose column (8 x 6.4 cm) as described in Appendix (A1.3).

CauI (o——o) and CauII (□——□) activities were eluted from the column with a 500 ml linear gradient of 0-900 mM NaCl (------) in KP buffer. Protein was monitored by absorbance at 280 nm. 1 unit is defined as 0.5 µg Ad2 DNA completely digested in 30 min at 50°C.

Appendix Table 4. Summary of the purification of CauI and CauII endonucleases

	Protein (mg)	Enzyme (Total units x 10^{-6})	Specific Activity (Units. mg^{-1})
Extract	7,100	5.5*	774*
PEI pool	2,945	5.5*	1,870*
DEAE-Cellulose pool	1,760	5.2*	2,950*
CauI Phosphocellulose pool	200	0.5	2,500
Hydroxyapatite pool	50	0.3	6,000
Sephacryl S-200 pool	7.5	0.17	22,670
CauII Phosphocellulose pool	231	2.1	9,100
Hydroxyapatite pool	25	1.0	40,000
Sephacryl S-200 pool	5.5	0.7	127,300

An extract of C. aurantiacus (200 g) was purified as described in Appendix (A.1.3). Protein was estimated by the method of Lowry et al. (1951) and endonuclease activity (50°C) as described in Methods (2.14.). One unit of enzyme is defined as the amount that will completely digest 0.5 µg Ad2 DNA in 30 min at 50°C. (*Denotes a mixture of CauI, CauII activities.)

sequences has not been determined.

A.1.4. The TaqI Restriction endonuclease

Purification

Cell paste (200 g) was suspended in 500 ml TSG buffer (4°C) and cells disrupted by passing twice through a Manton-Gaulin homogeniser (500 $Kg.cm^{-2}$) and cell debris removed by centrifugation (25000 xg for 10 min at 4°C). The extract was subjected to Sephacryl S200 gel-filtration (90 x 14 cm) in TSG buffer and the pool containing TaqI concentrated and dialysed against KP buffer as described for BclI (A.1.2).

Phosphocellulose (300 g wet wt.) was then added and the suspension stirred for 1 h (4°C), collected in a large column (5 x 12 cm) and washed in turn with 1L KP buffer containing 100 mM NaCl and 1L KP buffer + 500 mM NaCl. The TaqI endonuclease elutes in the final wash, which was then concentrated and dialysed against KP buffer (A.1.2), and the wash then loaded onto a 100 ml phosphocellulose column (12 x 3.2 cm) equilibrated in KP buffer. The column was washed with 200 ml KP + 50 mM NaCl and TaqI eluted with a 800 ml linear gradient of KP + 50 mM NaCl to KP + 800 mM NaCl. Fractions (10 ml) were collected and those showing maximal TaqI activity were pooled, concentrated and dialysed against TSG containing 50% (v/v) glycerol.

A summary of the results of the purification procedure are given in Table 5.

Characterisation

Digestion of λ DNA and Ad2 DNA showed that TaqI had at least 50 cleavage sites for these DNA molecules, whilst SV40 contained only 1 site. The recognition sequence of TaqI was shown to be T/CGA (Sato et. al., 1977).

Appendix Table 5. Summary of the purification of TaqI Restriction Endonuclease

	Protein (mg)	Enzyme (Total Units x 10^{-6})	Specific Activity (Units mg^{-1})	Recovery (%)
Extract	5,200	2.4	460	100
Sephacryl Pool	1,940	1.5	770	62
Phosphocellulose Pool (A)	180	0.55	3,050	23
(B)	14.5	0.21	14,500	9

TaqI was purified from 200 g cell paste as described in Appendix (A.1.4). Protein was estimated by the method of Lowry et al (1951) and endonuclease activity (60°C) as described in Methods (2.14). One unit of enzyme is defined as the amount that will completely digest 0.5 µg λ DNA in 30 min at 60°C.

A.1.5. The RspI Restriction endonuclease

Purification

A cell-free extract from 200 g cell-paste was prepared and treated with PEI./DEAE-cellulose as described for CauI/II (A.1.3). The extract was subjected to phosphocellulose chromatography ($4^{o}C$) on a 150 ml column (8 x 4.4 cm) and RspI eluted by a 1.5 L linear gradient of 0 to 1 M NaCl in KP buffer (100 $ml.h^{-1}$). Fractions (100 ml) showing major RspI activity were pooled and 100 ml hydroxyapatite added. After stirring for 1 h ($4^{o}C$) the resin was collected in a column (4 x 6.4 cm), washed with 200 ml KP buffer + 400 mM NaCl and RspI eluted with a 750 ml linear gradient of 20 - 200 mM potassium phosphate pH 7.0 in a buffer containing 400 mM - NaCl, 10 mM β-mercaptoethanol and 10% (v/v) glycerol. The fractions (10 ml) showing RspI activity were pooled, concentrated and subjected to Sephacryl S200 gel-filtration as described for CauI/II (A.1.3).

A summary of the results of the purification are given in Table 6.

Characterisation

Calibration of the Sephacryl S200 column (A.1.2.) allowed an estimate of the native molecular weight of RspI as 72000 daltons. It was shown to have 4 cleavage sites in λ DNA, 7 sites in Ad2 DNA but none in SV40 DNA. The recognition sequence of RspI has not, as yet, been determined but it may be an isoschizomer of two enzymes from different strains of R. spheroides that recognise the sequence CGATCG andhave the same cleavage pattern (Roberts, 1980).

A summary of the different extraction procedures and chromatography steps for the new restriction endonucleases is given in Table 7.

Appendix Table 6. Summary of the purification of RspI Restriction Endonuclease

	Protein (mg)	Enzyme (Total Units x 10^{-6})	Specific Activity (Units. mg^{-1})	Recovery (%)
Extract	2,960	1.0	340	100
PEI/DE52 pool	1,980	0.98	490	98
Phosphocellulose pool	180	0.6	3,300	60
Hydroxyapatite	40	0.36	9,000	36
Sephacryl S-200	26	0.28	10,700	28

RspI was purified from 200 g cell paste as described in Appendix (A.1.5). Protein was estimated by the method of Lowry et al (1951) and endonuclease activity as described in Methods (2.14.). One unit of enzyme is defined as the amount that will completely digest 0.5 µg Ad2 DNA in 30 min at 37°C.

Appendix Table 7. Summary of the purification of new Restriction Endonucleases

	Nucleic acid removal		Column chromatography				
	Sephacryl S-200 (13L column)	PEI/DEAE-Cellulose	DEAE-Cellulose	Phosphocellulose I	Phosphocellulose II	Hydroxyapatite[a]	Sephacryl S-200
BclI	+	-	A 0-600	-	50-1000	-	-
			B 0.6 (4.5L)		0.25 (2L)		1.8
			C 250-400		450-600		52,000[b]
CauI	-	+	A -	-	0-900	20-200	-
			B		0.25 (2L)	0.04 (0.3L)	1.8
			C		200-300	70-110	66,000[b]
CauII	-	+	A -	-	0-900	20-200	-
			B		0.25 (2L)	0.04 (0.3L)	1.8
			C		650-800	80-120	42,000[b]
TaqI	+	-	A -	500*	50-800	-	-
			B	0.5 (1L)*	0.1 (0.8L)		
			C	500*	400-500		
RspI	-	+	A -	-	0-1000	20-200	-
			B		0.25 (2L)	0.1 (0.8L)	1.8
			C		400-550	100-125	72,000[b]

Legend to Table 7.

All the restriction endonucleases were purified from 200 g cell-paste as described in the text to appendix.

A: Column gradient; mM-NaCl except for (a) hydroxyapatite where it is mM K.phosphate.

B: Column size (Litre); Size of gradient given in brackets.

C: Elution of enzyme; mM-NaCl except for (a) hydroxyapatite where it is mM-K.phosphate,
(b) sephacryl, where elution is expressed in terms of estimated molecular weight.

*Elution was by means of a column wash of 500 mM-NaCl in KP buffer.

A.2. Isolation of other Restriction Endonucleases

The following enzymes were purified, EcoRI, HhaI, HindII, HindIII, HpaI, KpnI, PstI, XbaI, XmaI using the procedures developed in section A.1 for the restriction endonucleases from thermophiles. The method of purification is summarised in Table 8, and any deviations from the methods described in A.2.1. listed in the notes to the table.

I would like to thank Mr. A.F. Sharman for providing excellent technical assistance when purifying the above 9 enzymes.

Appendix Table 8. Summary of the purification of Restriction Endonucleases

	Nucleic acid removal		Column chromatography					
	Sephacryl S200 (13L)	PEI/DEAE Cellulose	DEAE-Cellulose	Phosphocellulose I	Phosphocellulose II	DEAE-Cellulose	Hydroxyapatite[a]	Sephacryl[b] S200 (1.8L)
EcoRI	-	+	A -	300 + 600*	250-800	-	50-500	+
			B -	1.0*	0.1 (0.8L)		0.02 (0.2L)	
			C -	600*	550-650		200-280	
HhaI	+	-	A 0-500	-	100-1000	-	-	+
			B 0.5 (3.2L)		0.1 (0.8L)			
			C 150-250		550-650			
HindII	-	Streptomycin	A 0-400	-	50-800	-	-	+
		sulphate	B 1.0 (8L)		0.2 (1.5L)			
		(1%)	C 100-200		450-550			
HindIII	-	Streptomycin	A -	-	50-800	-	-	+
		sulphate	B 1.0		0.2 (1.5L)			
		(1%)	C -		500-600			
HpaI	-	Streptomycin	A 0-500	100 + 500*	50-800	-	-	-
		sulphate	B 1.0 (8L)	0.3*	0.2 (1.5L)			
		(1%)	C 100-200	500*	400-500			

Appendix Table 8. (Cont'd)

	Nucleic acid removal		Column chromatography					
	Sephacryl S200 (13L)	PEI/DEAE Cellulose	DEAE-Cellulose	Phosphocellulose I	Phosphocellulose II	DEAE-Cellulose	Hydroxyapatite[a]	Sephacryl[b] S200 (1.8L)
KpnI	+	-	A 0-600	-	150-800	-	-	+
			B 1.0 (8L)		0.2 (1.5L)			
			C 200-300		450-550			
PstI	-	+	A -	200 + 600*	100-800	0-500	-	+
			B	0.3*	0.2 (1.5L)	0.1 (0.8L)		
			C	600*	450-600	125-175		
XbaI	-	+	A -	-	100-800	0-500	20-200	+
			B		0.2 (1.5L)	0.1 (0.8L)	0.02 (0.2L)	
			C		450-600	200-350	45-70	
XmaI	-	+	A -	100 + 500*	100-600	-	-	-
			B	0.3*	0.1 (0.8L)			
			C	500*	350-400			

Legend to Table 8.

All restriction endonucleases were purified from 200 g cell paste except EcoRI (1 Kg) and HhaI (50 g) using methods described in section A.1.1.

A: Column gradient; mM-NaCl except for Hydroxyapatite (a) where K. phosphate was used (mM).

B: Column size (L); size of gradient is given in brackets.

C: Elution of enzyme; mM-NaCl except for Hydroxyapatite (a) where it is mM-K. phosphate, or Sephacryl (b) which involves gel-filtration only.

*: Elution was by means of a column wash of NaCl (500 mM or 600 mM) preceded by a wash of 100 mM-300 mM NaCl in Kp buffer.

Notes

(1) EcoRI. All buffers contained 0.2% (v/v) Triton X100 and disruption of cells took place in a TSG buffer with reduced NaCl (300 mM). A pre-wash of the first phosphocellulose column with 300 mM NaCl in Kp buffer removed a large proportion of non-specific nucleases.

(2) HhaI. Haemophilus haemolyticus cells contain 2 restriction endonucleases, HhaI and HhaII (Roberts, 1980); and these were separated on the DEAE-cellulose column. HhaI eluted at 150-250 mM-NaCl while HhaII eluted later at 300-400 mM-NaCl.

(3) HindII/HindIII. These two enzymes are present in Haemophilus influenzae Rd cells and were separated by the DEAE-cellulose step after streptomycin sulphate (added to 1%) precipitation of nucleic acids. Under conditions of low ionic strength (Kp buffer) HindIII does not bind to DEAE-cellulose whilst HindII does and was eluted with a NaCl gradient.

(4) HpaI. A pre-wash of the first phosphocellulose column with 100 mM-NaCl (in Kp buffer) removed a substantial quantity of the non-specific nucleases.

(5) PstI. All buffers contained 0.1% (v/v) Triton X100 and a pre-wash of the first phosphocellulose column was used to remove most of the non-specific nucleases.

(6) XmaI. This endonuclease was very unstable and could not be easily stabilised with Triton X100 as for EcoRI, PstI and had a very short shelf-life.

CHAPTER THREE

ISOLATION AND CHARACTERISATION OF THERMOPHILIC BACILLI AND SCREENING FOR PLASMID DNA

3.1. Isolation and characterisation of thermophilic bacilli

This was carried out in conjunction with R.J. Sharp. The isolation of different strains was based on the different colony morphology of colonies grown on complex medium (TSBA) at 60°C. A full list of the environmental samples used to isolate thermophilic bacilli, and the resulting strains isolated are given in Table 2.1. The strains were characterised using both biochemical tests and growth characteristics, eg. the ability to grow in 5% (w/v) saline or 0.02% (w/v) azide. The results of the strain characterisation are shown in Table 3.1.

Thermophilic bacilli can be classified as either strains of *B. stearothermophilus* or *B. coagulans* based on growth in 0.02% (w/v) azide and their range of growth temperature (Buchanan & Gibbons, 1974). The former is unable to grow in the presence of azide and has a maximum growth temperature between 65°C and 75°C, and a minimum of 30°-45°C; whilst the latter can grow in azide and has lower maximum and minimum growth temperatures, 55°-60°C and 15°-25°C respectively.

The range of growth temperature for the strains of thermophilic bacilli was determined (Table 3.1). About half had a maximum growth temperature above 70°C and of these 65% had a minimum growth temperature above 37°C. This group can therefore be classified as strains of *B. stearothermophilus*. Most of these strains could not grow in the presence of 0.02% (w/v) azide, confirming the classification. The majority (80%) of the strains not able to grow at 70°C (50% of the total) had a lower minimum growth temperature, 25°-30°C, more characteristic of *B. coagulans*. However, this classification could not be confirmed by growth in azide since only a few strains (20%), eg RS44, could grow in 0.02% (w/v) azide.

The biochemical tests carried out showed there to be a wide variety of strains in the collection; only in a few cases did two strains appear identical eg RS25 and RS26 (Table 3.1).

The results shown in Table 3.1 indicated that spores could only be detected microscopically in 60% of the strains. Since *Bacilli* by definition are Gram-positive spore-forming rods (Buchanan & Gibbons, 1974) this may indicate that spore-formation does not occur on the medium used or that some strains are not true *Bacilli*.

It was hoped that the large number of apparently different thermophilic bacilli strains would contain a few plasmid-bearing strains. Rather than screening all the strains isolated for the presence of CCC

plasmid DNA, they were first tested for antibiotic resistance and for bacteriocin production. A selective marker on a plasmid would greatly facilitate development of a vector for gene-manipulation in thermophilic bacilli as described in Chapter 1.

The testing of the strains for antibiotic resistance and bacteriocin production is described in methods (2.4, 2.5) and the results given in Tables 3.2 and 3.3. The majority of the strains had a natural resistance to Naladixic acid at the level used, only 25 strains being sensitive. Resistance to chloramphenicol and erythromycin was quite common, 7 Cm^r 8 Em^r, and 4 $Em^r.Cm^r$. strains were identified. Only one ampicillin resistant strain was identified, and no resistance to tetracycline, streptomycin or gentamycin was shown. When growing these strains for plasmid screening it was found that the Ap^r, Cm^r and Em^r strains did not grow well in liquid media (BS broth) containing the antibiotic even at a low concentaration (2 $\mu g.ml^{-1}$), suggesting antibiotic tolerance rather than true resistance.

The results of the bacteriocin production tests (Table 3.3) indicated that the majority did produce some bacteriocin activity. The sum of inhibitory zone width (SZW) for all the indicator strains provides a measure of strong bacteriocin producers. About half the strains tested showed no or only slight bacteriocin production. Of the remainder about 20% were extremely potent bacteriocin producers, eg RS93, RS88, with an SZW of over 50.

Since the antibiotic resistant RS strains were only resistant to very low levels of antibiotic, possibly accounted for by a permeability alteration, a second isolation procedure was adopted. Thermophilic bacilli resistant to high levels of antibiotic (25 $\mu g.ml^{-1}$) were directly isolated by inoculating an environmental sample into liquid media containing the antibiotic (2.2.2). The different strains isolated by this method (TB strains) are listed in Table 2.1, and 8 Sm^r, 3 Ap^r, 7 Tc^r, 6 Cm^r, and 4 Km^r strains were isolated. In addition 4 strains tolerant to heavy metal ions were also isolated; TB135 (Cadmium, 0.2 mM), TB136 + TB138 (Arsenate, 2 mM) and TB143 (Mercury, 2 mM).

Table 3.1. Characterisation of RS strains of thermophilic bacilli

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
A	Growth at 70°C	+	+	B	-	-	-	+	W	+	W	B	+	W	W	+
B	Growth at 37°C	+	-	+	+	-	-	-	+	+	+	+	+	+	+	-
C	Growth at 30°C	+	-	+	+	-	-	-	+	+	+	+	-	+	+	-
D	Growth at 25°C	-	-	W	+	-	-	-	+	W	W	W	-	W	+	-
E	Growth in azide	W	+	-	-	-	-	-	-	-	-	-	-	-	-	-
F	Growth in 5% saline	+	-	+	+	-	-	-	+	+	+	W	+	+	+	-
G	Starch hydrolysis	-	R	-	+	-	-	+	+	+	-	-	+	-	W	R
H	Casein hydrolysis	-	W	-	+	+	+	+	+	+	-	-	-	-	+	W
I	Gelatine hydrolysis	-	+	-	+	-	+	+	+	+	W	+	+	-	+	-
J	NO_3 reduction	+	+	-	+	W	+	+	+	+	-	-	+	-	+	-
K	NO_2 reduction	+	+	-	+	-	-	W	+	W	-	-	+	-	+	W
L	Catalase	+	+	+	+	+	-	-	+	+	+	+	-	+	+	-
M	Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N	Dextrin	W	W	-	-	-	-	W	-	W	-	-	+	-	-	W
O	Arabinose	+	+	W	-	-	-	-	-	-	+	+	-	+	+	-
P	Fructose	-	-	-	-	-	-	-	-	-	-	-	+	W	+	+
Q	Mannitol	W	W	-	-	-	-	+	+	-	-	-	-	-	-	-
R	Starch	+	+	-	-	-	-	+	-	+	-	-	+	-	-	-
S	Xylose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T	Lactose	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
U	Glycerol	-	-	-	-	W	-	+	W	+	-	-	+	-	-	+
V	Spore (Position)	-	T	-	-	T	T	C	-	-	T	T	-	T	-	C
W	(Swelling	-	+	-	-	+	+	+	-	-	S	-	-	-	-	-

Table 3.1 (Cont'd)

	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31
A	+	W	+	W	+	W	W	–	–	–	–	W	W	W	W	+
B	–	+	+	+	–	+	–	+	+	+	+	+	+	+	+	+
C	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+	+
D	–	+	W	W	–	–	W	W	W	W	W	W	–	W	W	–
E	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	–	+	+	W	–	+	+	W	+	+	+	W	W	W	W	W
G	–	+	–	–	R	–	–	–	–	–	–	–	–	–	–	–
H	+	W	–	–	W	–	–	–	–	–	–	–	–	–	–	–
I	+	W	–	W	W	–	–	–	W	W	W	W	W	–	W	W
J	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–	+
K	W	+	W	–	+	–	–	–	–	–	–	–	–	–	–	W
L	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N	W	–	–	–	W	–	–	–	–	–	–	–	–	–	–	–
O	W	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P	+	W	+	–	W	+	+	–	W	–	–	–	–	–	–	–
Q	–	+	W	–	W	W	W	–	–	–	–	–	–	–	–	–
R	–	–	–	–	W	–	–	–	–	–	–	–	–	–	–	–
S	W	–	–	–	W	–	–	–	–	–	–	–	–	–	–	–
T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
U	+	–	W	–	–	+	+	–	–	–	–	–	–	–	–	–
V	–	C	–	–	T	T	T	T	–	T	T	–	C	C	T	T
W	–	–	–	–	+	+	+	+	–	+	+	–	+	+	–	–

Table 3.1 (Cont'd)

	32	33	34	35	36	37	38	39	40	42	43	44	45	46	47	48
A	–	W	W	–	–	+	+	–	–	W	W	–	–	–	B	+
B	+	+	+	+	+	–	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
D	W	W	W	W	–	W	W	W	W	+	W	+	+	W	W	–
E	–	–	–	–	–	–	–	–	–	–	–	+	–	+	–	+
F	+	+	+	+	+	+	+	+	+	W	+	+	+	–	+	–
G	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	–
H	–	–	–	–	–	–	–	–	–	–	–	–	–	W	–	W
I	W	W	W	–	W	–	W	–	W	W	W	+	+	–	–	–
J	–	–	–	–	–	–	–	–	–	–	–	+	+	+	–	+
K	–	–	–	–	–	–	–	–	–	–	–	W	+	W	–	+
L	+	+	+	+	+	–	–	–	+	–	+	+	+	+	–	+
M	+	+	+	+	+	+	+	+	+	+	+	+	+	W	+	+
N	–	–	–	–	–	–	–	–	–	–	–	–	–	W	–	W
O	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
P	–	–	–	–	–	+	–	+	–	–	–	+	–	+	+	+
Q	–	–	–	–	–	+	–	+	–	–	–	+	–	–	+	+
R	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–
S	–	–	–	–	–	W	–	–	–	–	–	–	–	W	–	–
T	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
U	–	–	–	–	–	+	–	W	–	–	–	–	–	–	+	–
V	–	T	T	T	T	CT	T	CT	T	T	C	–	–	T	–	C
W	–	S	S	–	+	+	+	+	S	S	S	–	–	+	–	+

Table 3.1 (Cont'd)

	49	50	51	52	53	54	55	56	57	58	59	60	62	63	64	65	66
A	B	–	+	W	+	B	–	+	+	+	+	–	W	B	B	+	–
B	+	–	–	+	+	+	+	–	–	–	–	+	–	+	+	+	+
C	+	–	–	+	W	+	W	–	+	W	–	+	–	+	+	+	+
D	+	–	–	+	W	+	–	–	W	–	–	+	–	+	+	W	+
E	+	–	W	+	–	W	–	–	–	–	–	–	–	–	+	–	+
F	+	–	–	+	W	+	W	–	–	–	–	+	–	+	W	W	–
G	+	+	R	+	–	+	–	–	–	–	+	–	–	R	+	+	–
H	–	+	W	–	–	+	–	W	–	W	–	W	–	–	+	–	+
I	+	+	W	+	–	+	–	W	–	+	–	–	W	+	–	–	–
J	+	–	+	+	–	+	–	+	–	–	–	+	+	+	W	–	–
K	+	W	+	+	–	+	–	+	–	–	–	–	–	+	–	–	–
L	+	+	W	+	+	+	+	+	–	+	+	–	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	W	–	+	+	+	+	–
N	–	W	W	W	–	W	–	W	W	+	+	–	W	–	–	–	–
O	–	–	–	–	–	+	–	–	–	–	+	–	–	+	+	–	–
P	+	+	+	+	+	+	+	+	–	+	+	–	–	+	+	–	+
Q	+	+	+	+	+	+	+	+	W	+	+	–	–	+	+	–	+
R	–	+	W	–	–	–	–	–	–	+	+	–	–	–	–	–	–
S	–	W	W	–	W	–	W	–	–	–	W	–	–	–	–	–	W
T	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–
U	–	+	W	W	+	–	+	+	+	W	–	–	–	–	–	–	–
V	–	T	–	–	C	–	T	T	T	T	–	–	C	–	T	–	T
W	–	+	–	–	+	–	+	+	+	+	–	–	S	–	+	–	–

Table 3.1 (Cont'd)

	67	68	69	70	71	73	74	75	76	77	78	79	80	81	82	83	85
A	+	+	–	+	+	+	+	W	+	B	+	+	–	+	+	+	+
B	+	–	+	+	–	–	–	–	–	–	–	–	+	–	+	+	–
C	+	–	+	+	–	–	–	–	–	W	–	–	+	–	+	+	–
D	W	–	–	W	–	–	–	–	–	–	–	–	–	–	–	–	–
E	W	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
F	–	–	W	+	–	–	W	–	–	+	W	–	–	–	W	W	–
G	+	–	R	–	–	R	R	R	–	R	R	R	–	R	–	–	+
H	W	–	–	–	+	W	+	W	W	W	W	W	W	–	–	W	W
I	–	–	–	–	W	W	+	+	W	–	–	–	W	–	–	–	+
J	–	+	–	–	+	+	+	+	+	–	+	+	W	+	–	–	+
K	–	W	–	W	W	W	–	–	+	–	+	+	W	W	–	–	+
L	+	–	+	+	–	–	W	+	+	+	W	–	–	–	+	+	+
M	W	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N	–	W	W	W	W	–	+	–	+	+	+	+	W	+	–	–	+
O	–	W	–	–	–	+	+	–	–	–	–	–	–	+	–	–	–
P	+	W	–	+	W	W	+	–	+	+	+	+	W	+	+	+	+
Q	W	W	–	+	–	–	+	W	+	+	W	W	W	+	+	–	+
R	–	W	–	–	–	–	–	–	+	+	+	+	–	+	–	–	+
S	+	–	–	–	–	–	W	–	–	–	–	–	–	–	–	+	–
T	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–
U	–	W	–	+	+	+	+	–	–	W	+	+	–	+	W	+	+
V	–	T	–	T	C	–	C	T	C	–	–	–	C	C	C	C	–
W	–	+	–	+	–	–	S	+	S	–	–	–	–	+	+	+	–

Table 3.1 (Cont'd)

	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101
A	+	+	+	+	+	+	+	N	W	-	-	+	+	+	+	W
B	-	-	-	-	-	-	-	N	+	+	+	W	-	W	+	+
C	-	-	-	-	-	-	-	N	+	+	+	-	-	-	-	+
D	-	-	-	-	-	-	-	N	+	+	+	-	-	-	-	+
E	-	-	W	W	W	W	-	N	+	+	+	-	-	-	-	-
F	-	-	-	-	-	-	-	+	W	+	+	W	+	+	+	+
G	+	R	-	R	R	-	+	N	W	+	+	R	-	-	-	+
H	W	-	W	W	W	+	+	N	-	W	W	-	-	-	N	-
I	+	W	+	-	W	+	+	N	-	-	+	-	-	-	-	W
J	+	+	+	+	-	+	+	-	+	+	+	+	-	-	-	+
K	+	W	-	W	-	W	+	-	+	W	+	+	-	-	-	W
L	+	+	+	+	+	+	+	+	+	+	+	W	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N	+	+	-	+	+	-	+	N	-	W	-	+	-	-	W	+
O	-	+	-	+	-	-	-	N	-	-	-	-	-	-	-	-
P	+	+	-	+	+	-	+	N	+	+	+	-	-	-	-	+
Q	+	-	-	-	-	-	-	N	W	-	-	-	-	-	-	-
R	+	W	-	+	-	-	+	N	-	-	-	W	-	-	-	+
S	-	+	-	+	+	-	+	N	+	-	-	W	+	+	-	+
T	-	-	-	-	-	-	-	N	-	-	-	-	-	-	-	-
U	-	+	-	-	-	-	-	N	-	-	-	-	-	+	+	-
V	-	-	-	T	-	-	T	-	-	-	T	T	C	T	CT	-
W	-	-	-	+	-	-	S	-	-	-	+	+	+	+	+	-

Table 3.1 (Cont'd)

	102	103	104	105	106	107	108	109	110	111	112
A	W	W	W	W	W	W	+	+	+	W	W
B	+	+	+	+	+	+	+	+	+	+	+
C	+	+	+	+	+	+	W	W	W	W	W
D	+	+	+	+	+	+	-	-	-	-	-
E	+	+	+	W	W	W	-	-	-	-	-
F	+	+	+	+	W	+	W	W	+	+	+
G	+	-	+	+	+	+	-	R	-	-	-
H	+	+	+	+	+	W	-	-	-	-	-
I	W	-	-	-	W	W	-	-	-	-	-
J	+	+	+	+	+	+	+	+	-	-	-
K	+	+	+	+	+	+	+	+	-	-	-
L	+	+	+	+	+	+	+	+	+	+	+
M	+	+	+	+	+	+	+	+	+	+	+
N	-	-	-	-	-	-	+	+	-	-	-
O	-	-	-	-	-	-	+	-	-	-	-
P	+	+	+	+	+	+	+	+	-	-	-
Q	-	-	-	W	+	W	-	+	-	-	-
R	-	-	-	-	-	-	-	+	-	-	-
S	-	-	-	-	-	-	+	+	-	-	-
T	-	-	-	-	-	-	-	-	-	-	-
U	-	-	-	-	-	-	-	W	-	-	-
V	T	T	T	T	-	-	T	C	T	T	T
W	+	+	+	-	-	-	+	-	+	S	+

Legend to Table 3.1.

All tests were carried out on fresh cultures grown at 60°C as described in Methods (2.2). Readings were taken at 1, 2 and 3 day intervals and a positive result was denoted by (+), a weak result by (W), and a negative result by (-), B denotes good growth at 70°C in broth but not on plates; R denotes zone of starch hydrolysis restricted.

The position of the spore was either central (C) or terminal (T) and slight swelling denoted by (S). (N) denotes not tested.

Table 3.2. Antibiotic Sensitivity of RS strains of Thermophilic bacilli

RS	Em	Cm	Tc	Na	Sm	Ap	Gm
1	30	20.5	30	0	19	45	26.5
2	26.5	20.5	29	0	20	45	28
3	14.5	24	25	8	20	21.5	25
4	28.5	24	28	0	23.5	35	25
5	7.5	28	42.5	0	26	53.5	30.5
6	12	22.5	40	0	25	53.5	30
7	27	24.5	29.5	8	20	65	26
8	14	26	29.5	0	26	25	30
9	25	16.5	24	0	14	39	19
10	0	0	24	0	20	36	22.5
11	0	20	25.5	0	19.5	36	25
12	26	20	24	9	14	41.5	23
13	9	10	29	0	21	44.5	24
14	0	0	30	0	32	50	37
15	32	28	36	11	26	56	30.5
16	27	23	28.5	0	23.5	55	28
17	32.5	34.5	24.5	9	29	18.5	40
18	21.5	22	23.5	12	19	46	22
19	25.5	15	25	0	19	38	24
20	33	27	36	0	26.5	54.5	34
21	27.5	26.5	29	0	28	46	32.5
22	21.5	23	27	0	26	46	28
23	34	14.4	28	0	18	46	17
24	30	23.5	27.5	0	20	41	23
25	27.5	25.5	25.5	0	20	34.5	23
26	0	0	25	0	19	40	22
27	32.5	23.5	32	0	22.5	44	23.5
28	27	0	28	0	21	38.5	23
29	0	0	25.5	0	18	38	23
30	0	22.5	26	0	15	42.5	22
31	13.5	12.5	28.5	0	21	34	24
32	33	0	29	0	22	38	25
33	31	0	29	0	22	38	24.4
34	30	0	30	0	21	38	25
35	28	24	27.5	0	21	37.5	23
36	28	25	28	0	21	33	24

Table 3.2 (Cont'd)

RS	Em	Cm	Tc	Na	Sm	Ap	Gm
37	18	26	40	16	29	54.5	36
38	30	0	26	0	20	37	24
39	18.5	29	26	0	25.5	47.5	33
40	29	0	26	0	21.5	35.5	19.5
42	0	22	24	0	23	42	24
43	0	22	25	0	21.5	40	25
44	24	22	25	0	19	15	25
45	32	41.5	39	10	28	16.5	38
46	32	26	32.5	0	22	46	32
47	40	33	37	0	24	58	33.5
48	30	24.5	32.5	0	20	47.5	25.5
49	8.5	22.5	23.5	0	20	26	25.5
50	26	27	32.5	0	25	47.5	31.5
51	32	23	28	0	16.5	42.5	24.5
52	25	28	26	0	18	18	23.5
53	16.5	27.5	27.5	13	21	44	26
54	0	23	25	0	19	25	24
55	17.5	28	30	15	33	50	36
56	34	31	33.5	0	28	62	36
57	24	25	28	11	22	42.5	28.5
58	25	28	33.5	8	23	50.5	30
59	40.5	36	40	0	32	63	41
60	N	N	N	N	N	N	N
62	38	31.5	37	0	23	63	34
63	0	21.5	30	0	18	15.5	3.5
64	23	21	20	0	16	0	19.5
65	31.5	21	25	0	22	38.5	33
66	0	29	21.5	0	21	12	27.5
67	34.5	32	38	0	33	53	41.5
68	35.5	28.5	35	0	22	54	28
69	31.5	25	26	0	23.5	40.5	24.5
70	20	28.5	27	0	19.5	43.5	30
71	39.5	34	37	0	28.5	61	36
73	41	38	38	0	28	62	40
74	39	36	34	0	26	58	36

Table 3.2 (Cont'd)

RS	Em	Cm	Tc	Na	Sm	Ap	Gm
75	40.5	33.5	38	0	25.5	59	34
76	42	38	41	0	33	65	44
77	22.5	27.5	26.5	15	23.5	44.5	30.5
78	36	26.5	29.5	0	26	48.5	32
79	34	25	30	0	25	45.5	32
80	11	18	29	0	20	52	25
81	33	27	32.5	0	19.5	46	26.5
82	30.5	29	29	16	22.5	46	32.5
83	29.5	29	29	15	21	44	29
85	39	32.5	35	0	31.5	56	39
86	37.5	32	35	0	32	55	41
87	41	37	42	0	37	69	39
88	35.5	31	35.5	0	29.5	56	34
89	40	37	41	0	34	64	42
90	36	34	39	0	28.5	61	36
91	35	30	33	0	26	52	33
92	39.5	36	39	16	31	70	36
93	23.5	20.5	30	0	27	46	31
94	22	25	24	11	16	17	21
95	22.5	26	25	12.5	16	18.5	19
96	22	26	24.5	11.5	15.5	18	18
97	32	26.5	32	0	22	49	26
98	17	29	37	14	24	48	30.5
99	17.5	28	36	16	23	47.5	30
100	12	29	23	19.5	18	54	23
101	32	32	38	0	24	58	29.5
102	21.5	24	23.5	0	16.5	18	19
103	23	25	24	0	17	19	19
104	11	16.5	13	13.5	18	18.5	21
105	23	24.5	24.5	12	17	20	20
106	24	22	16	0	19	20.5	25
107	12.5	25	19.5	10.5	18	19.5	20
108	34.5	0	30.5	0	24.5	51	29.5
109	28	23	30	0	19.5	41	22.5
110	28.5	22.5	26	0	18	35.5	20.5
111	0	24.5	27.5	0	19	35	21
112	28	25	26	0	19	45	21

Legend to Table 3.2

Sensitivity of the thermophilic bacilli to the antibiotics was carried as described in Methods (2.4). Results were recorded after overnight incubation (60°C) as inhibitory zone radius (mm). Em, Erythromycin (E10); Cm, Chloramphenicol (C30); Tc, Tetracycline (TE30); Na, Nalidixic acid (NA30); Sm, Streptomycin (S25); Ap, Ampicillin (PN10); Gm, Gentamycin (CN10).

Table 3.3. Production of bacteriocin by RS strains of thermophilic bacilli

INDICATOR STRAIN (IS)	1	2	3	5	6	7	9	10	11	13	15	16	17
15	0	0	0	2	0	6	0	0	0	0	0	0	0
48	0	2	0	2	0	13	0	0	0	0	4	0	0
56	0	0	0	2	2	4H	0	0	0	0	4	4H	4H
58	3H	0	3H	0	0	3	0	3	0	0	0	0	0
62	0	0	0	0	0	3	0	0	0	0	0	0	0
74	0	0	0	0	1	2H	0	0	0	0	3	2H	2H
89	4	2	4	4	5H	2	3	5	0	0	1H	2H	1H
95	1	4	2	2	3	2	1	2	0	0	0	0	0
108	0	0	0	0	0	0	0	0	0	0	0	0	0
116	0	0	0	2	2	0	0	0	0	0	0	0	0
129	0	0	0	0	1	0	0	0	0	0	0	0	0
144	0	0	0	3H	0	3H	0	2H	0	0	0	0	0
161	1	1	1	3	1	4	2	1	0	0	5	1H	0
166	0	0	0	3	0	2	1	1	0	0	3	0	0
173	0	0	0	2	1	2	1	1	0	0	0	0	0
Sum of zone widths (SZW)	9	9	10	25	16	46	8	15	0	0	20	9	7

Table 3.3 (Cont'd)

IS	18	19	20	21	22	23	25	27	28	29	30	31	32	33	34	35	36
15	0	0	0	0	2	0	0	1H	4H	2H	2H	0	2H	0	3H	0	2H
48	2	0	0	0	2	0	0	0	2H	0	0	0	2H	0	1	0	0
56	0	0	0	0	0	0	0	0	2H	0	0	0	0	0	3	0	0
58	0	0	2	0	0	0	0	0	2H	0	2H	2H	2H	2H	0	2H	2H
62	0	0	0	0	0	0	0	0	2H	0	25H	0	14H	0	0	0	1H
74	0	0	0	0	0	0	0	0	5H	0	0	0	0	2	0	0	0
89	0	0	0	2	0	0	0	0	3	0	0	0	0	2	1	1	1
95	0	0	0	0	0	0	0	0	8H	8H	8H	6H	6H	5H	4H	9H	6H
108	0	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
116	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	0	0	2	0	1	0	0	0	1H	0	0
144	1	0	0	0	2	0	0	0	0	0	0	0	0	0	0	0	0
161	3	0	0	0	3	0	0	1	3	1	0	0	0	0	0	3	0
166	0	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	0
173	0	0	0	0	0	0	0	0	2	1	1H	2H	2	0	0	1	0
SZW	6	0	2	2	11	0	0	2	39	12	39	10	28	11	13	16	12

Table 3.3 (Cont'd)

IS	38	39	40	42	43	46	48	50	51	52	53	54	55	56	58	59
15	1	0	0	0	0	0	0	0	0	0	0	3H	2	0	0	0
48	0	0	1H	1H	2H	1	0	2	2H	1H	1H	1H	0	0	1H	0
56	0	0	0	0	0	2	0	0	0	1	3H	2	4H	0	0	0
58	2	1	1	2H	2H	0	0	0	0	0	0	0	0	0	0	0
62	0	9H	0	1H	2H	11H	8H	0	0	0	0	0	0	0	0	0
74	0	0	0	0	0	0	0	0	0	3	3	0	4	0	0	0
89	0	0	0	3	2	4	0	0	0	1	0	1	2H	0	2H	0
95	5H	5H	4H	4H	4H	5H	7H	0	4H	0	0	0	0	0	0	0
108	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2H	0
116	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
129	0	2	1	0	1H	0	0	0	0	0	3H	0	0	0	0	0
144	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
161	0	0	0	0	0	4	0	0	0	0	3	0	0	0	0	0
166	0	0	0	0	0	2	0	0	0	1	0	1	3	0	0	0
173	1H	1	0	0	0	0	0	0	0	0	0	0	5	0	0	0
SZW	9	18	9	11	13	29	15	2	6	7	13	8	20	0	5	0

Table 3.3 (Cont'd)

IS	62	63	65	67	68	69	70	72	73	74	75	76	77	78	79	80	81
15	0	2H	2H	1	0	0	2H	0	0	0	0	0	0	0	0	5	0
48	0	0	0	0	2H	0	0	0	2H	0	0	0	3	0	0	4	0
56	0	3H	5H	0	0	0	5H	0	0	0	0	0	4	0	0	4	0
58	0	0	0	0	0	4H	0	0	0	0	0	0	1H	0	0	4	0
62	0	0	0	0	0	0	1	0	1H	0	0	0	3	0	0	2	0
74	0	0	0	0	0	3	2H	0	0	0	0	0	3	0	0	4	0
89	1	1H	0	0	2	6	4H	0	0	0	0	0	3	0	0	2	0
95	0	0	0	0	0	0	0	1H	0	0	0	0	0	0	0	0	0
108	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
116	0	0	0	0	0	0	3	2H	0	0	0	0	0	0	0	0	0
129	0	0	0	0	0	0	2	0	2H	0	0	2H	0	0	0	0	0
144	0	0	0	0	0	0	2H	2	0	0	0	0	0	0	0	0	0
161	0	0	0	0	0	2H	3	0	0	0	4H	4	0	0	0	2	0
166	0	0	0	0	0	0	2	0	0	0	3	3	0	0	0	2	0
173	0	0	2	2	0	0	4H	0	0	0	0	2	2H	0	0	1H	0
SZW	1	6	9	3	4	15	30	5	5	0	7	11	19	0	0	30	0

Table 3.3 (Cont'd)

IS	82	83	85	86	87	88	90	91	92	93	94	95	96	97	98	99
15	3H	3	3	4	0	6	0	0	0	4	0	0	0	0	3	3
48	0	3	3	4	0	6	0	0	0	4	0	2H	0	0	4	4
56	4H	3	2	0	0	6	0	0	0	2	0	2H	0	0	2	3
58	0	2	2	3H	0	6	0	0	0	1	0	1H	1H	0	2	1
62	5	3	4	3	0	7	0	0	0	6	0	4	0	0	4	4
74	4	5	0	0	0	6	0	0	0	5	0	4H	0	0	4H	4H
89	4	4	4	3	0	4	0	0	0	6	0	3H	0	0	5H	5H
95	1H	3H	0	0	0	0	0	0	0	2	0	0	0	0	2H	2H
108	0	1	1H	2H	0	5	0	0	0	1	0	6H	5H	0	2	2
116	3H	2H	0	0	0	3H	0	0	0	3	0	0	0	0	3	2
129	1H	1H	1H	2H	0	0	2H	0	2H	2H	2H	1H	2H	0	2	2
144	5H	3	6H	5	0	6	0	0	0	2	0	4	0	0	3	2
161	5H	5	2	2	0	5	0	0	0	5	0	0	0	0	5	3
166	3	5	2	2	0	7	0	0	0	5	0	3	0	0	4	4
173	3H	2H	2	3	0	6	3H	0	0	2	0	2H	0	0	2H	1H
SZW	41	45	32	33	0	73	5	0	2	50	2	32	8	0	47	45

Legend to Table 3.3

Bacteriocin production by the RS strains of thermophilic bacilli was measured using 15 selected RS strains as described in Methods (2.5). The radius of the zone of growth inhibition (mm) was recorded after overnight incubation (60^{o}C). H, denotes a "hazy" zone.

3.2. Screening of Thermus aquaticus strains and thermophilic Bacilli species for plasmid DNA

At the start of this investigation no plasmids had been isolated from thermophilic bacteria and a screening programme for the detection of CCC plasmid DNA was initiated.

Three strains of T. aquaticus, 4 of B. stearothermophilus and 3 of the caldo-active Bacilli were initially screened using the procedure described in Chapter 2 (6.1). This relies on the separation of plasmid (covalently closed circular) DNA from chromosomal DNA in caesium chloride density gradients by the inclusion of ethidium bromide which alters the bouyant density of plasmid DNA (Radloff et. al., 1967). Two bands appear in the gradient when viewed under UV light; a main chromosomal DNA band and a lower, smaller plasmid DNA band. Incorporating ^{3}H-thymidine into the DNA (Guerry et. al., 1973) aids detection of low levels of plasmid DNA that can not be seen visually.

The ^{3}H profiles of the density gradients for each of the strains tested are shown in Fig 3.1 (a-j). T. aquaticus B and T. aquaticus X1 both have a plasmid as detected by a satellite band. The plasmid bands were also seen visually under UV light as very faint bands indicating a very low yield of plasmid DNA. The only bacillus species to show a satellite peak of ^{3}H activity was B. caldolyticus (Fig 3.1h) but visually only one band could be detected. Analysis of the fraction from the density gradient containing most of this 'pseudo-satellite' band (fraction 11) with restriction endonucleases EcoRI, HindIII did not confirm the presence of plasmid DNA. Only heterogeneous sized fragments were obtained more characteristic of chromosomal DNA.

The use of plasmids isolated from the Thermus strains, pTA-B (strain B) and pTA-X (strain X), as vectors for genetic manipulation in Bacilli was not explored. Only low levels of plasmid could be isolated and it is unlikely they would be able to replicate in Bacilli. No plasmid from a Gram-negative bacterium, eg. E. coli has been shown to replicate in Bacilli (Broda, 1979). At a later date 4 plasmids were isolated from other strains of Thermus (Hishinuma et. al., 1978), 3 from T. flavus (pTF1, pTF61, pTF62) and 1 from T. thermophilus (pTT8) but these were not investigated for the same reason.

A selection of the RS strains of thermophilic bacilli showing antibiotic resistance or bacteriocin production were screened for the presence of plasmid DNA using the same procedure (2.6.1). The results are shown in

Table 3.4. Only one strain, RS93 a strong bacteriocin producer, showed a satellite plasmid band (Fig 3.2). This band was not visualised under UV light.

The quantity of plasmid (pT93) isolated from RS93 appeared to vary with the degree of lysis of the cells as determined by the viscosity of the lysate and amount of chromosomal DNA seen in the density gradient. In comparison to the T. aquaticus strains tested using this lysis method the thermophilic Bacilli were very resistant to lysis. Therefore attempts were made to improve the lysis of thermophilic bacilli based on the lysozyme-EDTA and SDS lysis described in Chapter 2 (6.1).

Firstly, 50 ml cultures were used without altering the lysis conditions, so increasing the concentration of cells present. Secondly, the amount of lysozyme used was increased five times to 5 $mg.ml^{-1}$. However, no significant improvement in lysis was observed with these conditions whilst complete lysis, ie. no pellet obtained after clearing centrifugation, was obtained with T. aquaticus and E. coli.

A prolonged incubation of the cells with lysozyme at 20°C (30 min) or a shorter incubation at 37°C (15 min) did improve lysis slightly. A dramatic improvement was observed however when the culture medium was altered. Cells were produced in either BS broth, TSB, Nutrient broth (Oxoid No. 2) or TYS and lysed using the best conditions developed. Growth of cells in TSB or nutrient broth gave no improvement in lysis over those from BS broth used previously. However, lysis was so efficient with cells produced in TYS that the incubation time with lysozyme at 37°C had to be reduced to prevent lysis before the addition of SDS. This was essential to prevent complete lysis of cells where the chromosomal DNA cannot be precipitated by SDS in the presence of high NaCl (Guerry et. al., 1973). In addition, washing the cells with TES buffer before incubation with lysozyme improved lysis slightly.

Two further modifications were made to the lysis procedure. Firstly, the method was 'scaled-up' to allow lysis of cells from 200 ml of culture and secondly extraction of the cleared-lysate with chloroform/3-methyl-butan-1-ol (24.1) was introduced to remove both SDS and denatured protein. This latter step reduces the contamination in the density gradients, and improves the electrophoresis of the extracted DNA in agarose gels.

When screening thermophilic bacilli (TB strains) for plasmid DNA with the improved method, DNA was not labelled with ^{3}H-thymidine. Therefore satellite bands were detected visually by fluorescence under UV light and by scanning the gradients for absorbance at 260 nm with a density gradient scanner (model 2580) coupled to a Gilford spectropho-

tometer.

The modified procedure for plasmid screening, described in detail in Chapter 2 (6.2), proved very effective for isolating pT93 from strain RS93.

The antibiotic resistant thermophilic bacilli (TB strains) including the heavy metal ion tolerant strains were screened for the presence of plasmid DNA using the improved method (2.6.2) and the results are shown in Table 3.5. Five of the strains contained plasmid DNA as shown by agarose-gel electrophoresis of samples of the cleared lysates where 3 plasmid bands were detected. These corresponded to the 3 physical species of the plasmid, covalently closed circular (CCC), open circular (OC) and linear (L). In agarose gels under the conditions described in methods (2.13), the CCC species migrate most rapidly followed by linear species and then OC as the slowest moving band (Dr. P.W.J. Rigby, pers. comm.). Caesium chloride-ethidium bromide density gradients confirmed the presence of a plasmid by visualisation of a satellite band in all cases except strains TB124 and TB144 (Fig 3.3 a-e). In these 2 cases the buoyant density of the plasmid DNA did not appear to differ significantly from that of chromosomal DNA. However analysis of the single DNA band by agarose-gel electrophoresis confirmed the presence of plasmid DNA.

FIG.3.1a. B.stearothermophilus 1503

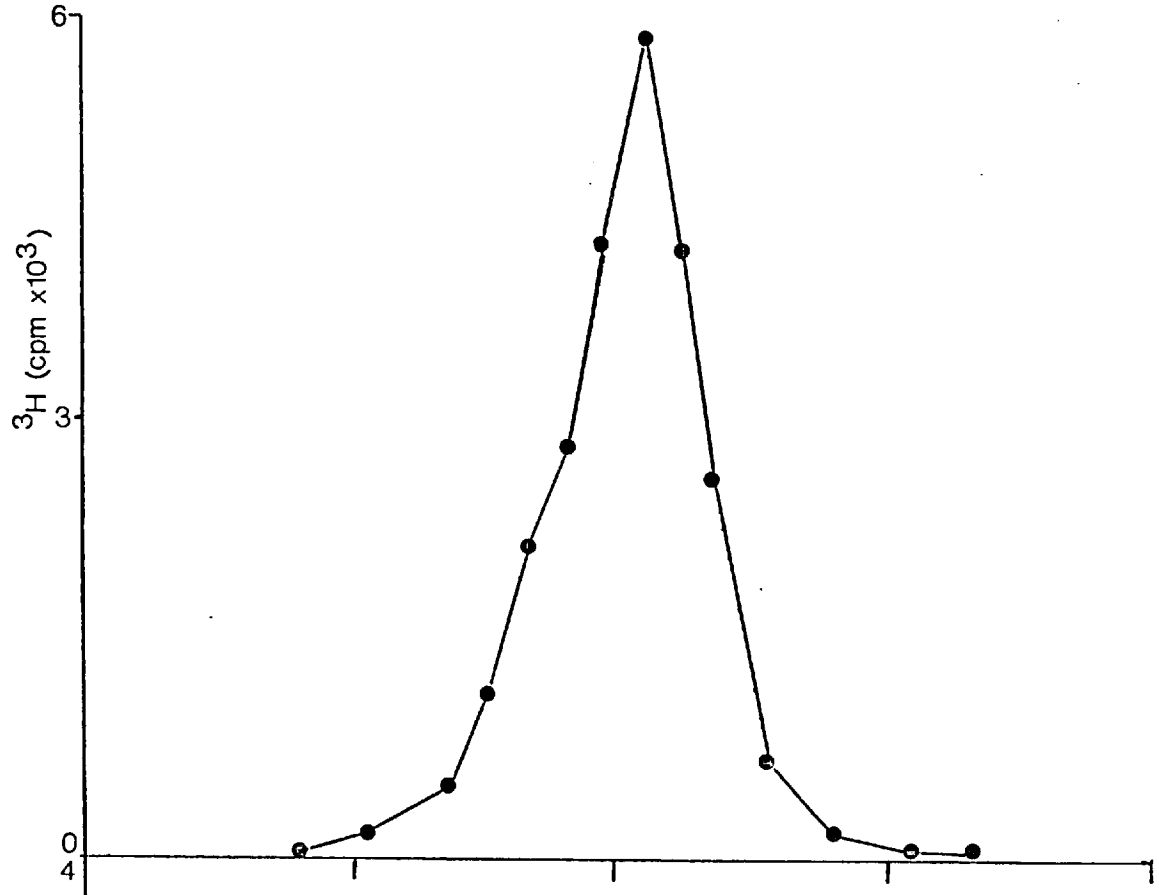
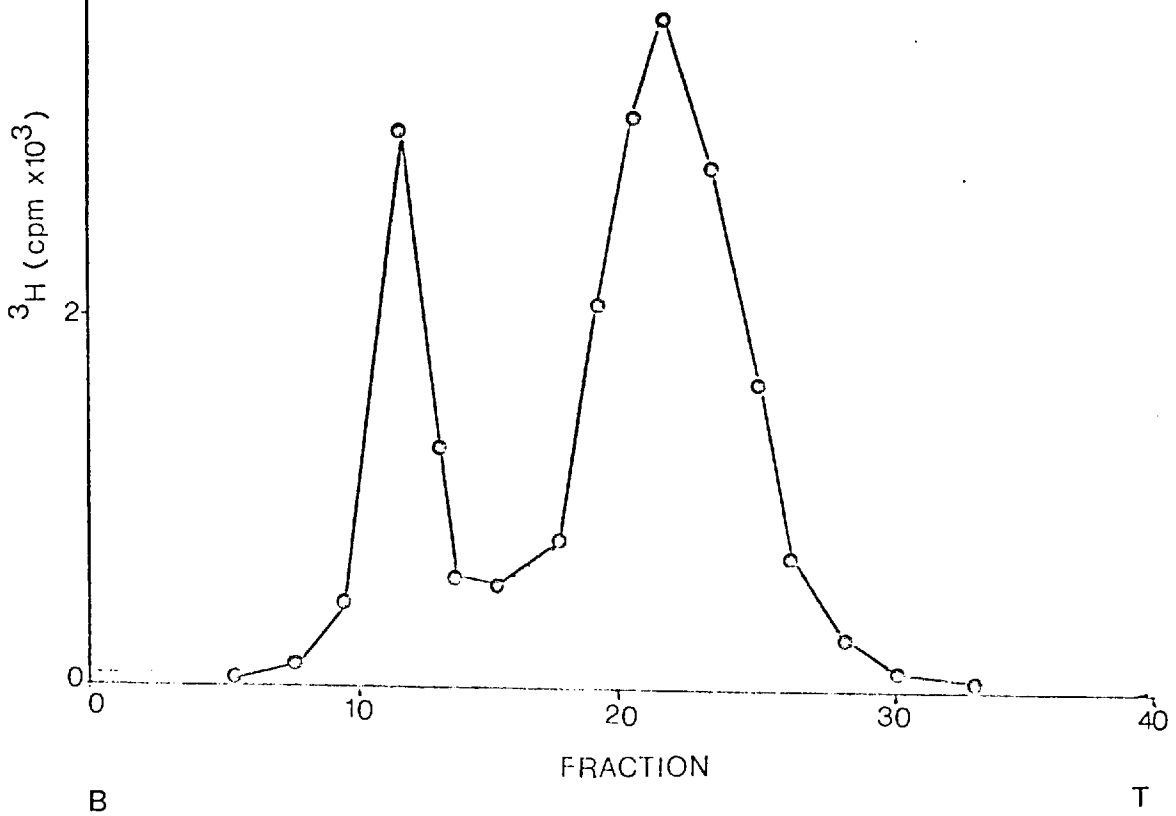


FIG.3.1b. T.aquaticus X



B T

FIG.31c. T.aquaticus YT1

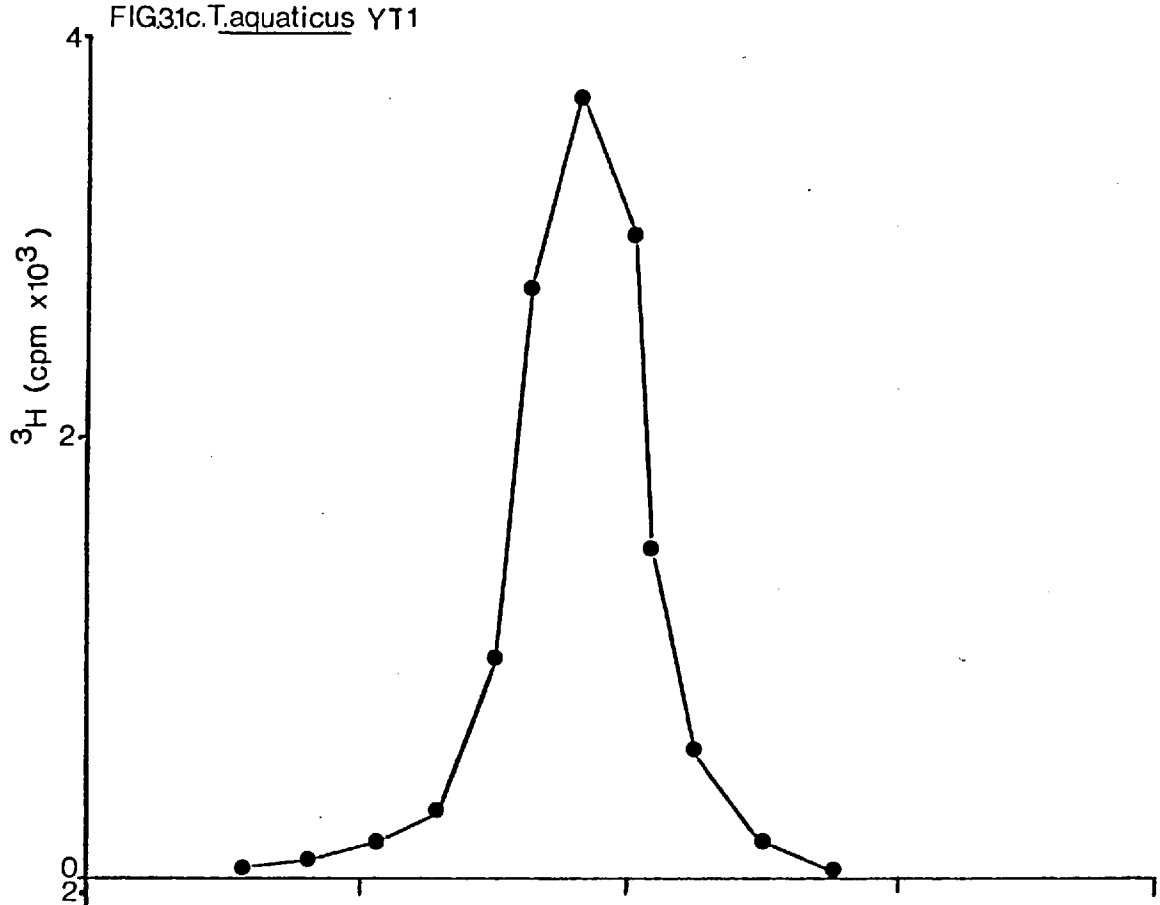
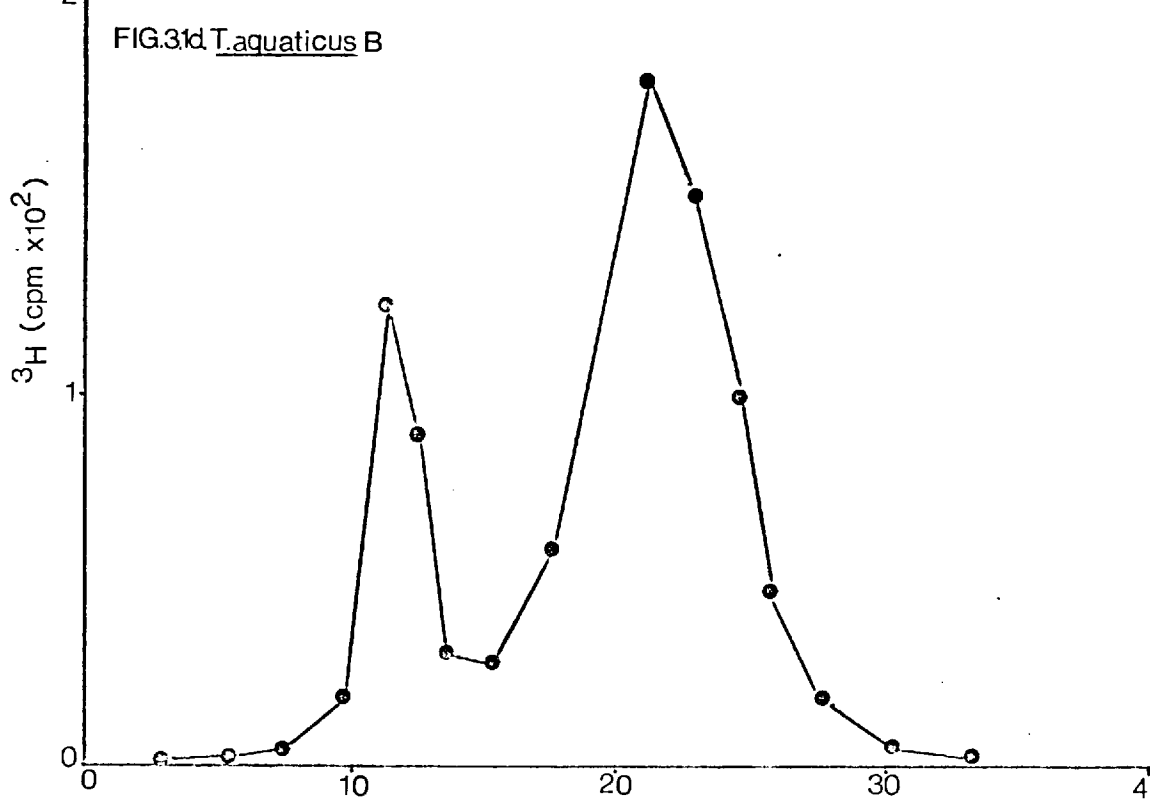


FIG.31d. T.aquaticus B



B

T

FIG.3.1e. B.stearothermophilus EP240

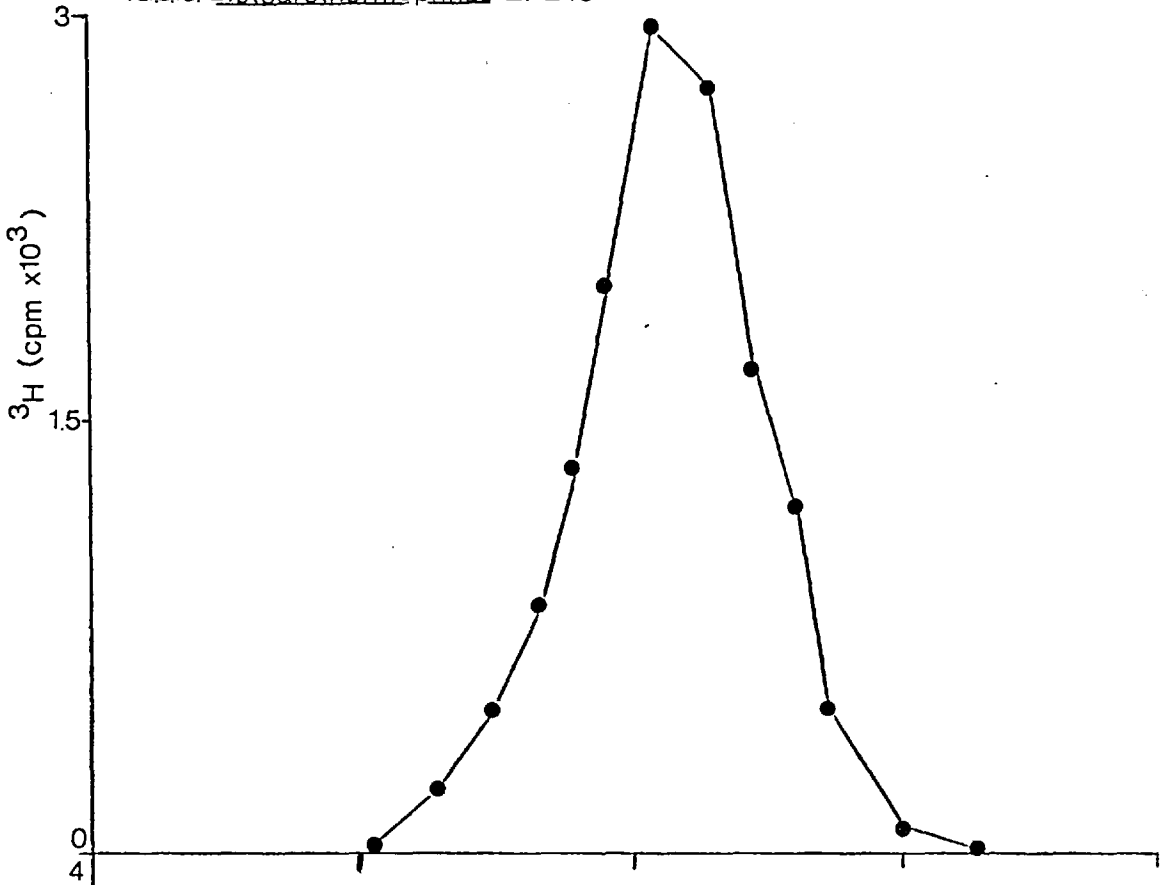


FIG.3.1f. B.stearothermophilus AY174

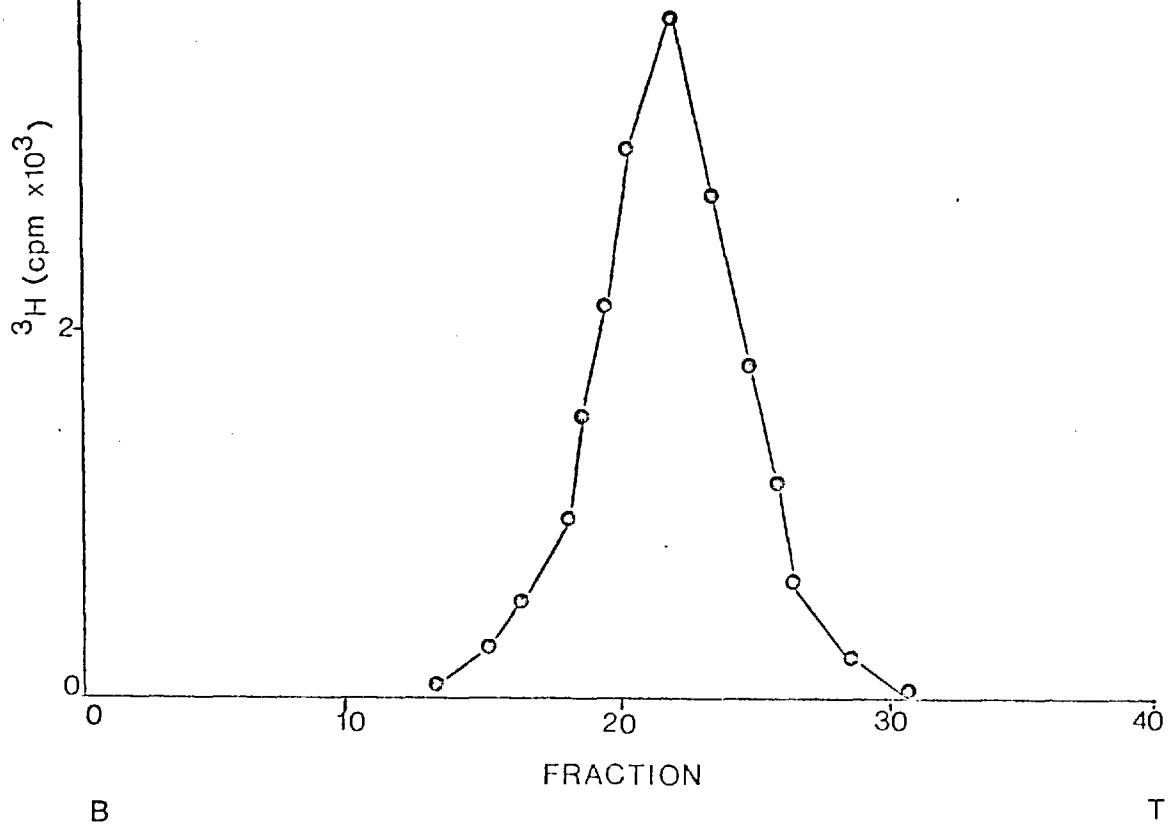


FIG3.1g. B.stearothermophilus 12016

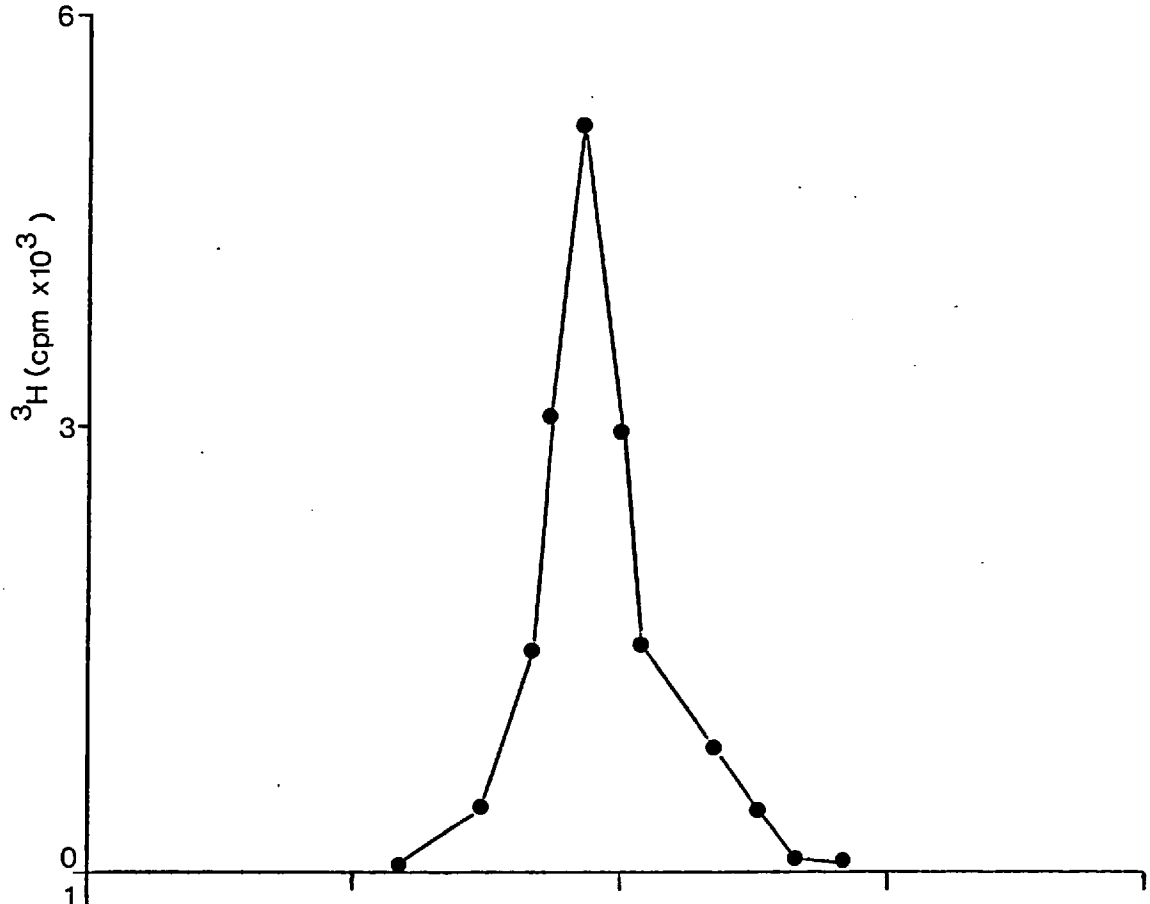
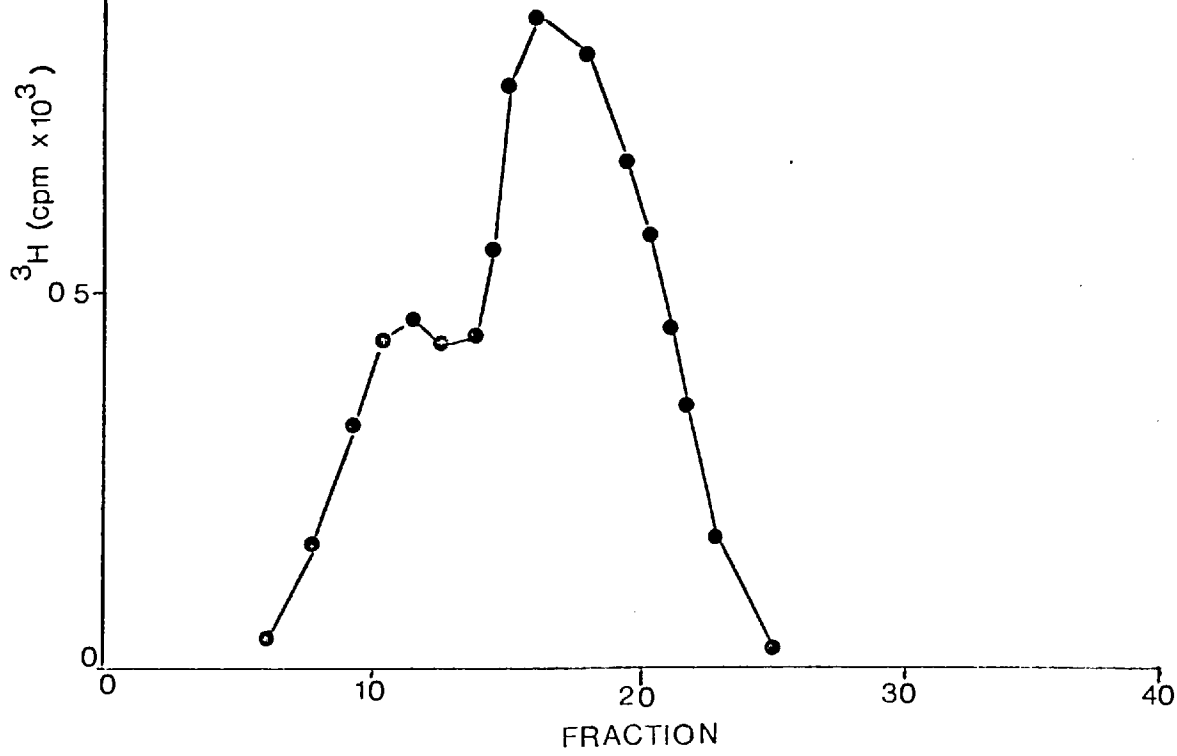


FIG3.1h. B.caldolyticus



B T

FIG31i. B.caldovelox

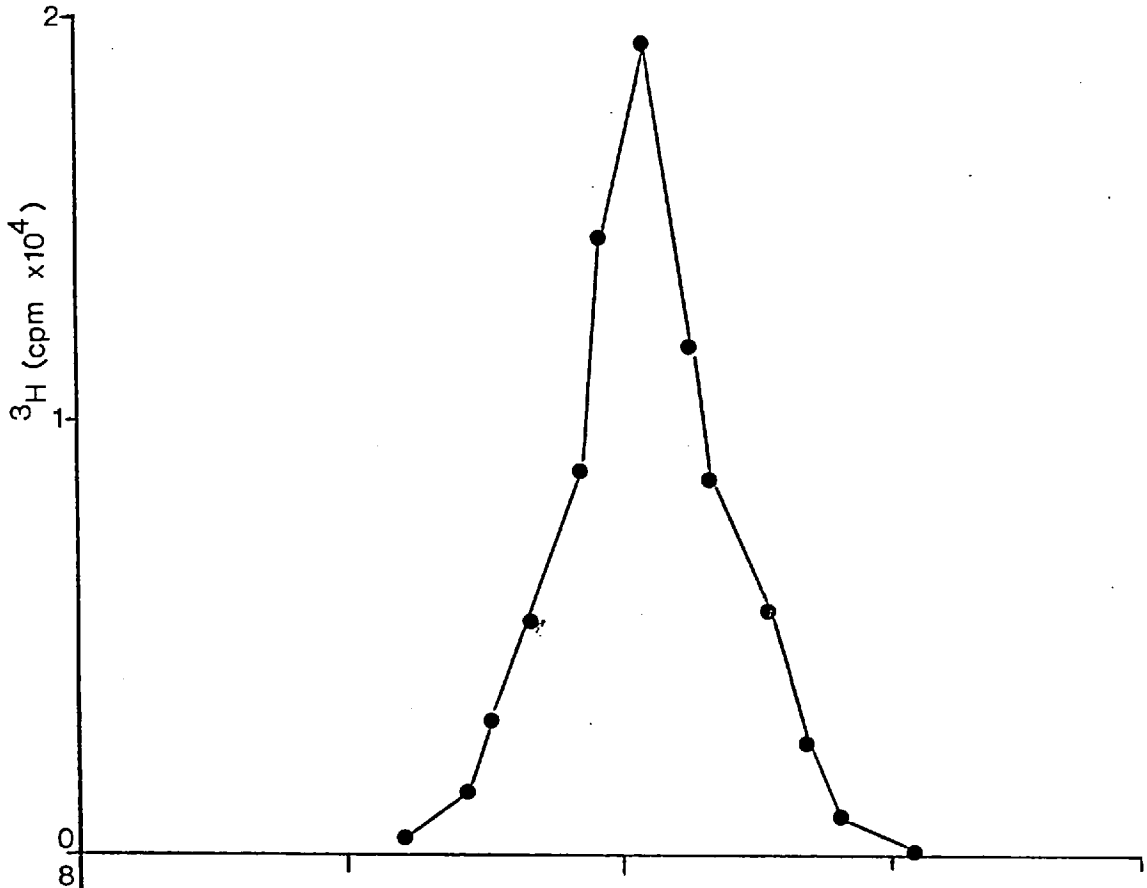


FIG31j. B.caldotenax

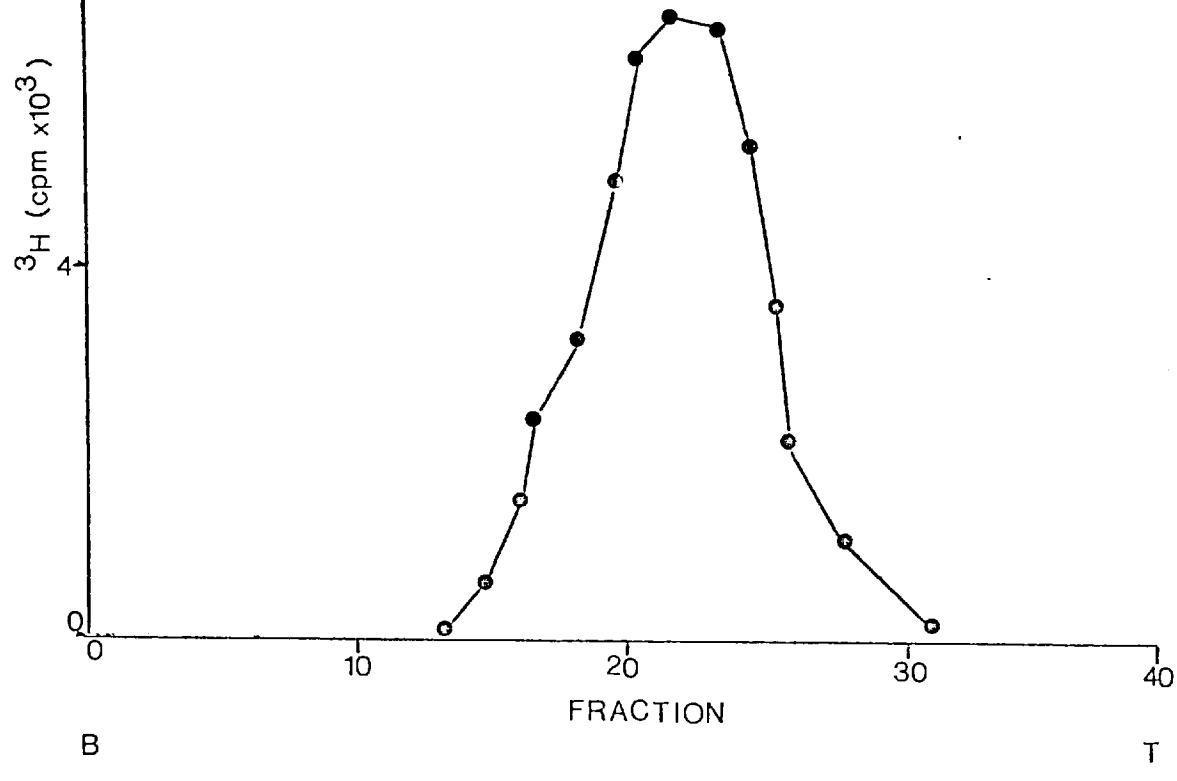


Figure 3.1. ^{3}H Profiles of Caesium Chloride Density Gradients

DNA was labelled with ^{3}H-thymine by incorporating ^{3}H-thymidine in the growth medium. Cleared lysates were prepared and subjected to ethidium bromide-caesium chloride density gradient centrifugation as described in Chapter 2 (6.1).

3.1.a. *B. stearothermophilus* NCA1503

3.1.b. *T. aquaticus* X

3.1.c. *T. aquaticus* YT1

3.1.d. *T. aquaticus* B

3.1.e. *B. stearothermophilus* EP240

3.1.f. *B. stearothermophilus* AY174

3.1.g. *B. stearothermophilus* ATCC12016

3.1.h. *B. caldolyticus*

3.1.i. *B. caldovelox*

3.1.j. *B. caldotenax*

Table 3.4. Screening of RS strains of thermophilic bacilli for plasmid DNA

Strain	Bacteriocin Production	Antibiotic Resistance	Plasmid
7	+	-	-
28	+	Cm^r	-
53	W	-	-
55	W	-	-
70	+	-	-
77	W	-	-
80	+	-	-
82	+	-	-
83	+	-	-
85	+	-	-
88	+	-	-
93	+	-	+ (pT93)*
95	+	-	-
98	+	-	-
99	+	-	-
10	W	Em^r Cm^r	-
29	W	Em^r Cm^r	-
30	+	Em^r	-
43	W	Em^r	-
54	W	Em^r	-

Legend to Table 3.4

RS strains showing bacteriocin production (Table 3.3) or antibiotic resistance (Table 3.2) or both; were screened for the presence of plasmid DNA as described in Methods (2.6.1).

W; weak bacteriocin production.

Em^r; Erythromycin resistant.

Cm^r; Chloramphenicol resistant

The plasmid isolated from strain RS93 was called pT93.

Figure 3.2. Caesium Chloride Density Gradient ^{3}H-Profile of an extract of B. stearothermophilus RS93

DNA was labelled with ^{3}H-thymine by incorporating ^{3}H-thymidine in the growth medium. Cleared lysates were prepared and subjected to ethidium bromide-caesium chloride density gradient centrifugation as described in Chapter 2 (6.1).

FIG. 3.2

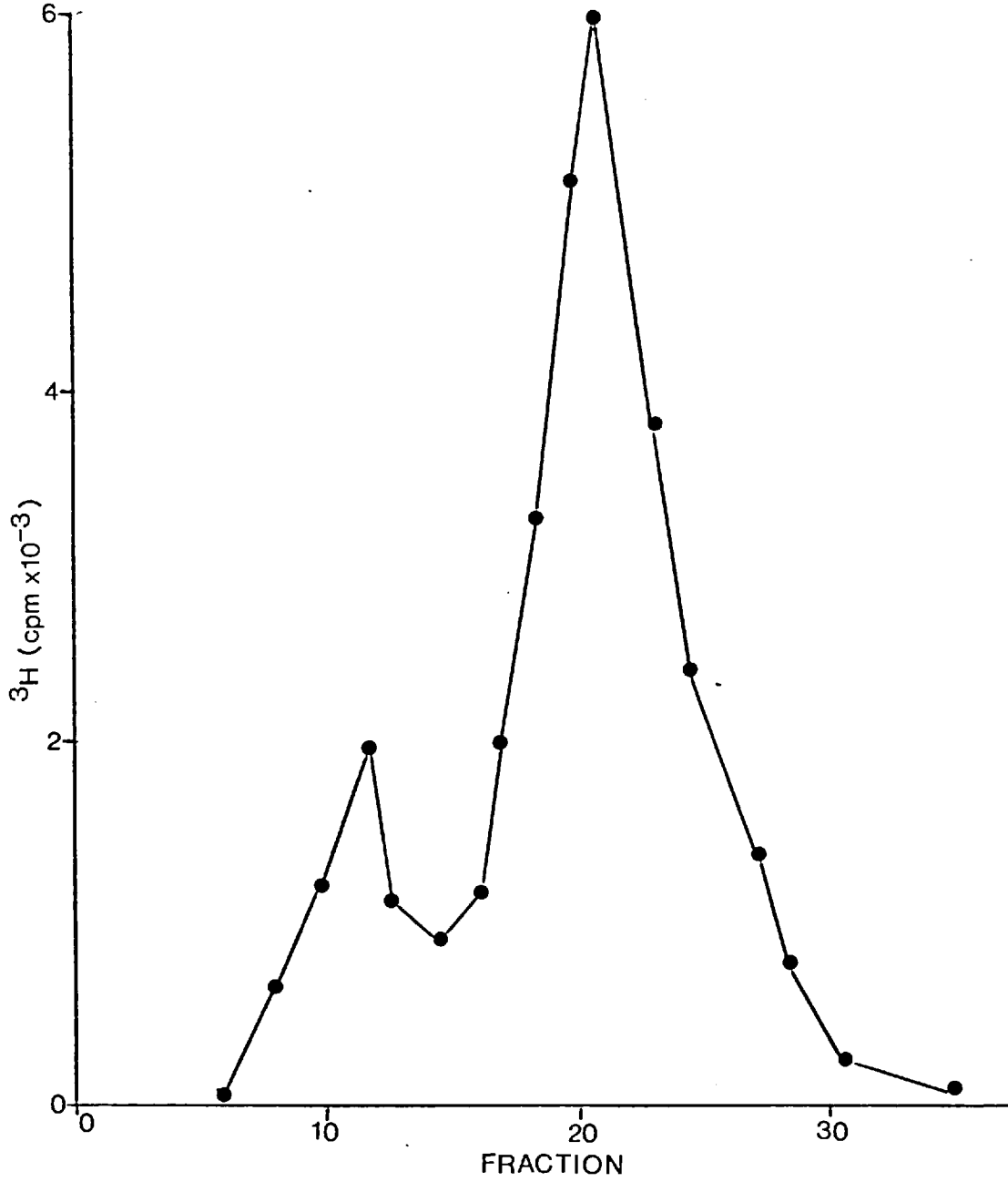


Table 3.5. Screening of TB strains of thermophilic bacilli for plasmid DNA

Strain no.	Selection (Antibiotic or heavy metal ion)	Detection of plasmid DNA
TB113 to 117	Streptomycin	-
TB118	"	+
TB119	"	-
TB150	"	+
TB120 to 122	Ampicillin	-
TB123	Tetracycline	-
TB124	"	+
TB125 to 127	"	-
TB128	"	+
TB144	"	+
TB129 to 134	Chloramphenicol	-
TB139 to 134	Kanamycin	-
TB135	Cadmium	-
TB136 to 138	Arsenate	-
TB143	Mercury	-

Legend to Table 3.5

The maximum growth temperature was 65°C for strains TB119 and TB123, and 60°C for all other strains. DNA was extracted as described in Methods (2.6.2) and cleared lysates were examined for the presence of plasmids by agarose-gel electrophoresis and isopycnic centrifugation as described (2.6.2; 2.13).

FIG. 3.3a TB118

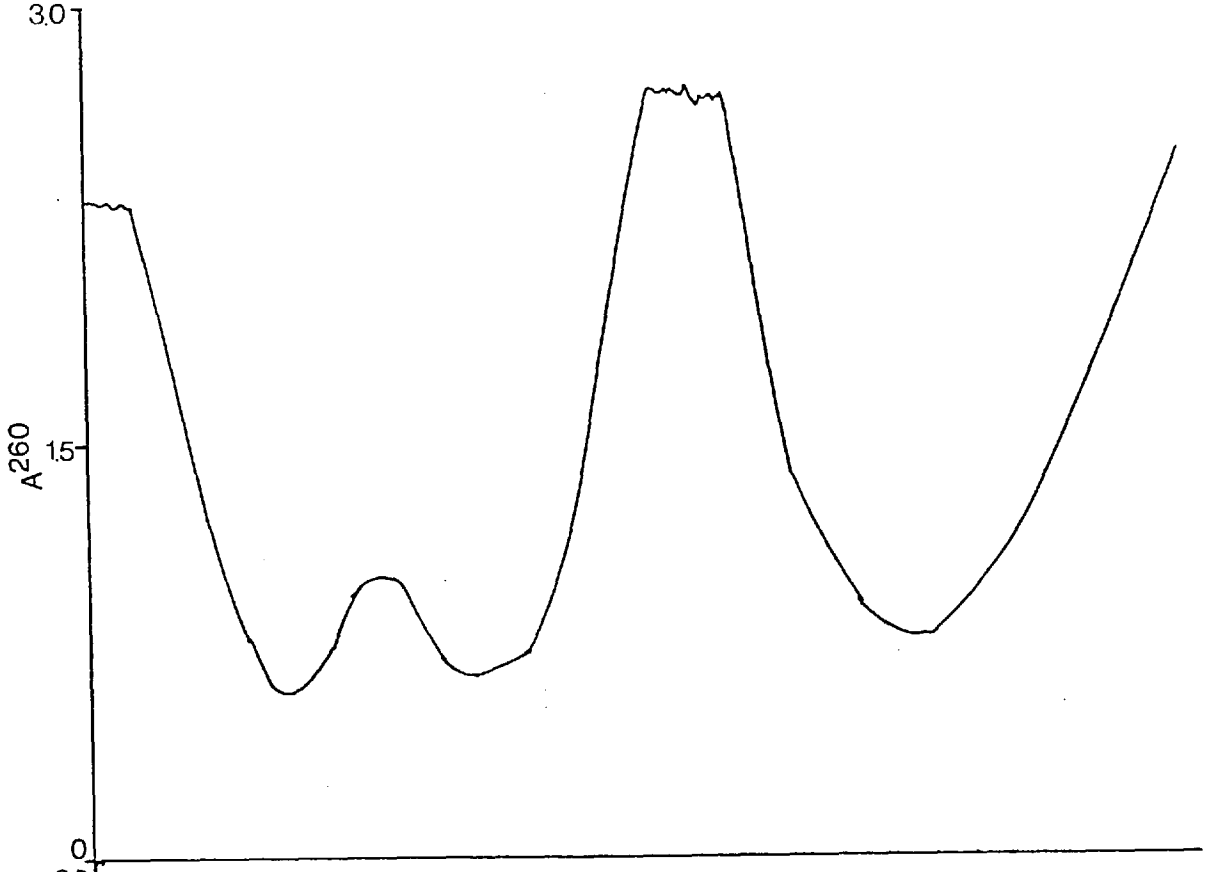


FIG. 3.3b TB150

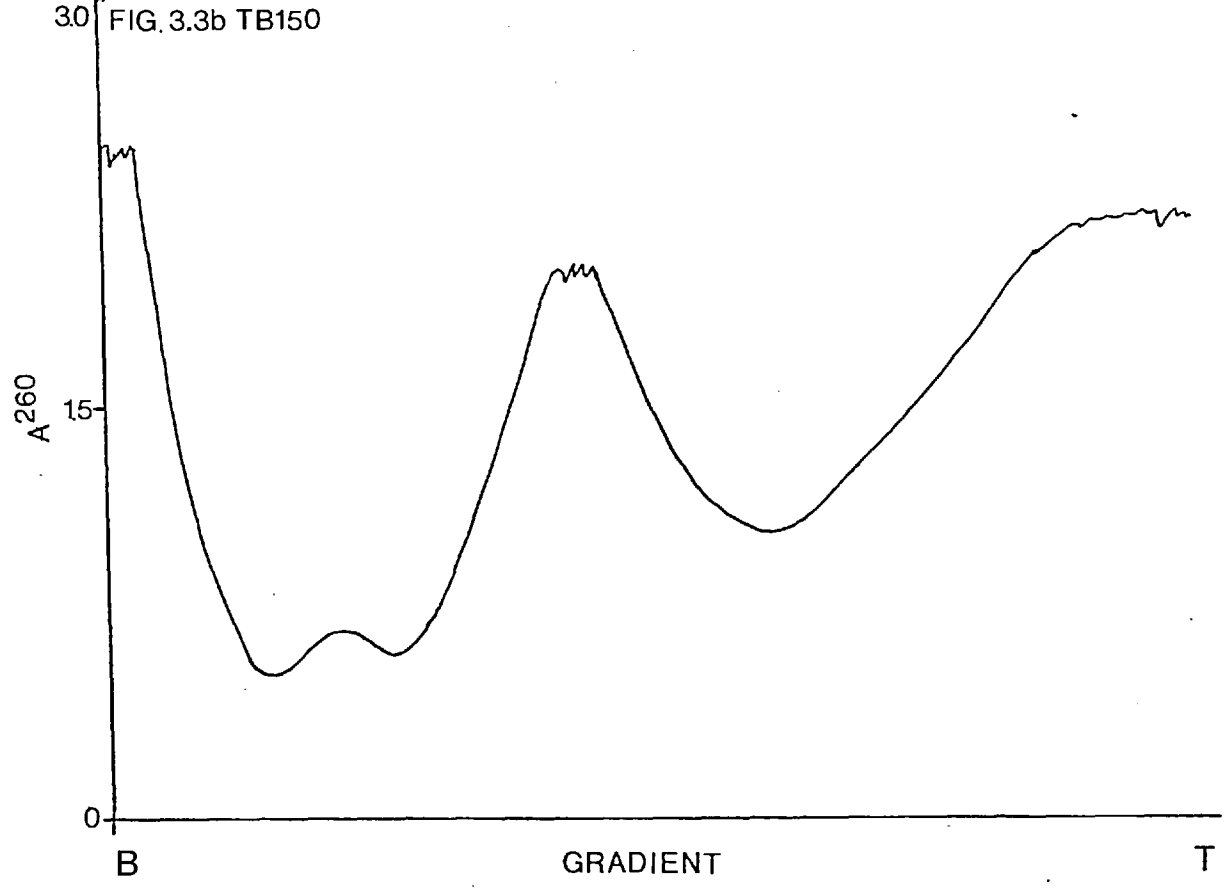


FIG. 3.3c TB124

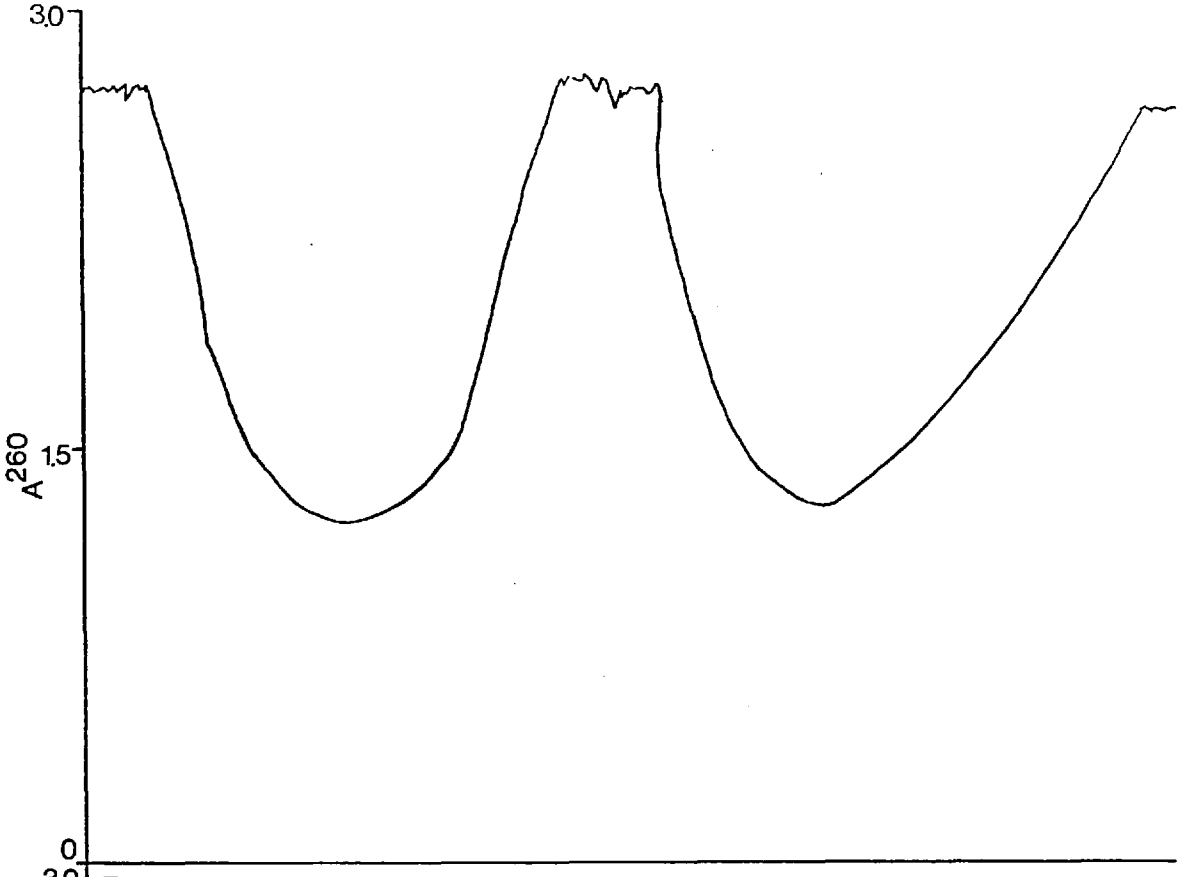


FIG. 3.3d TB144

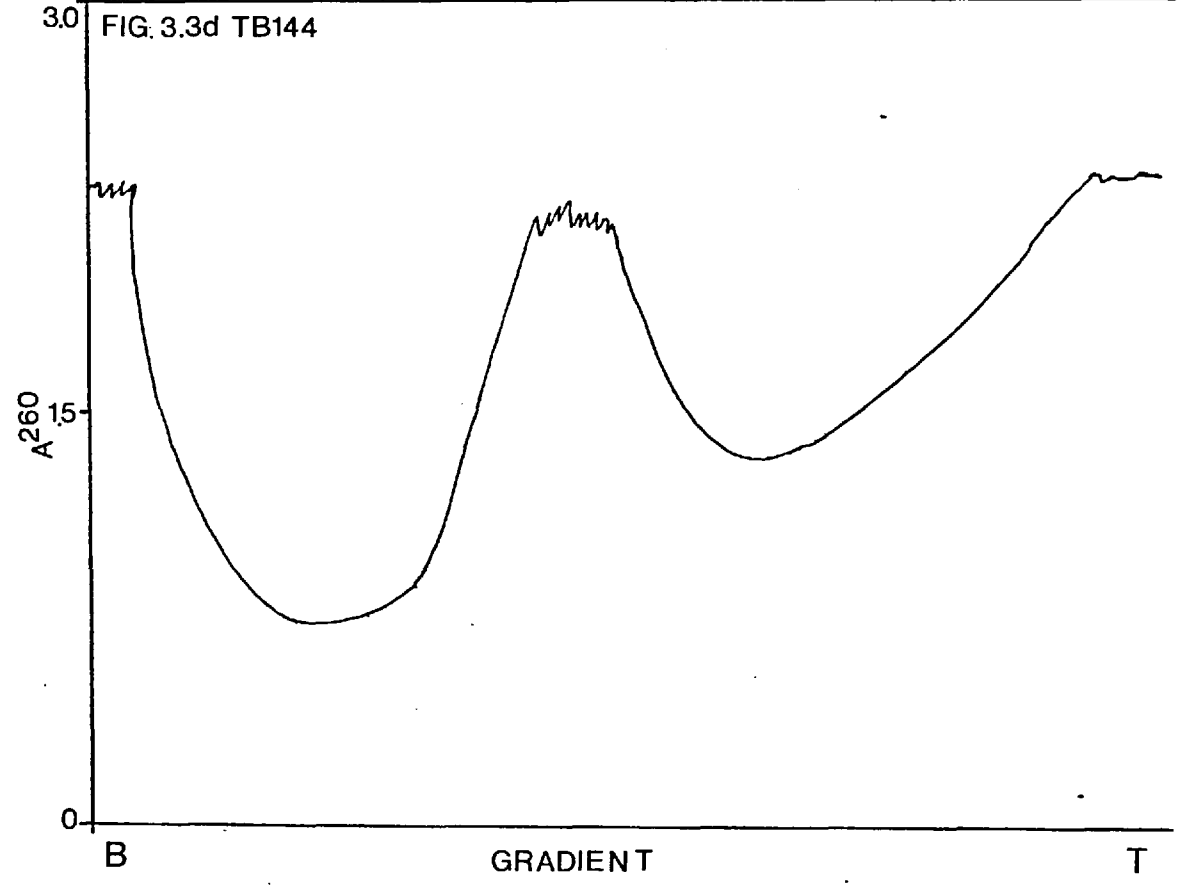


FIG. 3.3e TB128

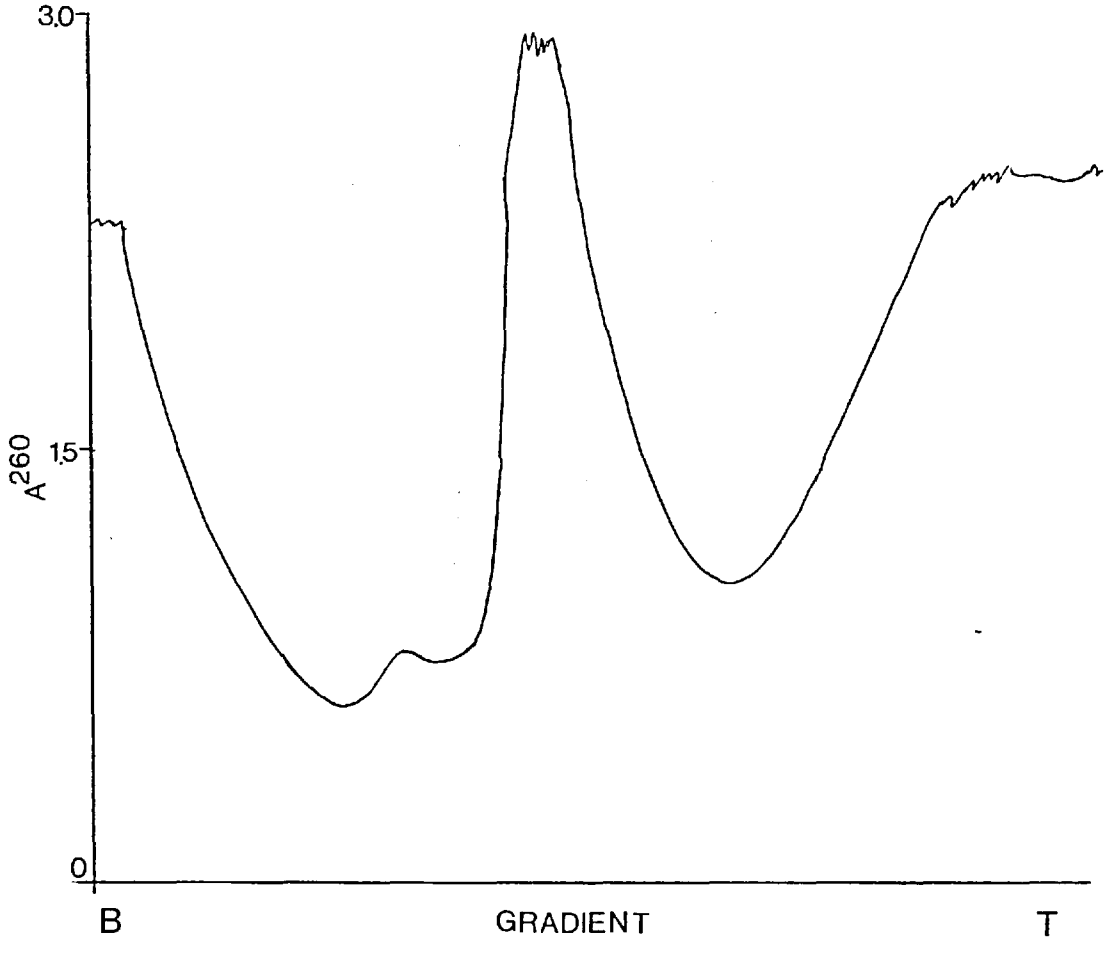


Figure 3.3. Caesium Chloride Density Gradient Absorbance Scans of extracts of TB strains of Thermophilic Bacilli

Cleared lysates were prepared and subjected to ethidium bromide-caesium chloride density gradient centrifugation as described in Chapter 2 (6.2). The gradients were collected via a density gradient scanner, whilst monitoring the absorbance at 260 nm with a Gilford 250 spectrophotometer. B, bottom of gradient. T, top of gradient.

3.3.a. TB118 (Sm^r)

3.3.b. TB150 (Sm^r)

3.3.c. TB124 (Tc^r)

3.3.d. TB144 (Tc^r)

3.3.e. TB128 (Tc^r)

3.3. Characterisation of the plasmid containing TB strains of thermophilic bacilli

Each of the 5 plasmid containing strains (Table 3.5) was characterised using several biochemical tests and growth characteristics. In addition *B. stearothermophilus* NCA1503 and *B. coagulans* ATCC8038 were subjected to the same tests as a comparison. The results are given in Table 3.6. TB strains 150 and 144 were isolated as contaminants from the original plates of TB118 (Sm^r) and TB124 (Tc^r) respectively (Table 2.1). Both TB118 and TB124 produced large, mucoid, budding colonies on TSBA plates while TB150 and TB144 appeared as small, round, non-mucoid colonies on the same plates and were assumed to be contaminants. However, both Sm^r strains (TB118, TB150) appeared virtually identical to each other when characterised as did the Tc^r strains TB124, and TB144, Table 3.6. It would appear that TB150 and TB144 were non-mucoid varients of TB118 and TB124 respectively. Analysis of the plasmids they contain (Chapter 4) confirmed this since TB118, TB150 contained the same two plasmids, and TB124, TB144 contained an identical plasmid.

Using a defined medium (AD) based on the medium of Rowe *et. al.*, (1975) the auxotrophic requirements of each plasmid-bearing strain were determined. Strains TB124, TB144 were prototrophic, TB118, TB150 required thiamine, while RS93 required arginine, histidine and proline, although these mutations were "leaky" since RS93 would grow slowly on a minimal medium. It was not possible to grow TB128 on AD medium even when all 20 naturally occurring amino acids, riboflavin, biotin, pantothenic acid, thiamine, thymine, adenine, cytidine and guanidine were included. However, inclusion of thymine in complex media greatly improved the growth of this strain indicating some degree of thymine requirement.

Strains TB124, TB128 and TB118 were tested for resistance to 20 different antibiotics using Oxoid multi-discs as described in Methods (2.4) and the results are shown in Table 3.7. As expected all the strains were resistant to the antibiotic that they were selected against (TB124, TB128 both Tc^r; TB118 Sm^r). However, TB128 showed a more specific tetracycline resistance than TB124. It was not resistant to oxytetracycline or chlorotetracycline unlike TB124. None of the strains were resistant to any other antibiotic.

Table 3.6. Characterisation of plasmid containing TB strains of thermophilic bacilli

	TB118	TB150	TB124	TB144	TB128	B. stearo-thermophilus	B. coag ulans
Growth at 25°C	+	+	+	+	–	–	+
" " 37°C	+	+	+	+	+	–	+
" " 60°C	+	+	+	+	+	+	–
" "	–	–	–	–	–	+	–
Growth in azide	–	–	W	W	–	–	+
" in 5% saline	+	+	+	+	W	–	–
Starch hydrolysis*	+	+	+	+	–	+	–
Caseine hydrolysis*	W	W	R	–	–	W	–
Gelatine hydrolysis	–	–	–	–	–	–	–
Citrate Utilization	+	+	+	+	–	–	–
Sabaraud agar	W	W	W	W	–	–	+
NO_3 redn	+	+	+	+	+	+	–
NO_2 redn	+	+	+	+	+	+	–
Catalase	+	+	+	+	+	–	+
Oxidase	+	+	+	+	+	–	–
Adonitol	–	–	–	–	–	–	–
Aesculin	–	–	–	–	–	–	–
Arabinose	+	+	+	W	–	–	–
Dextrin	+	+	W	W	–	+	+
Dextrose	+	+	+	W	+	+	+
Dulcitol	–	–	–	–	–	–	–
Erythritol	–	–	–	–	W	–	–
Galactose	–	–	–	+	W	W	+
Glycerol	+	W	W	W	–	+	+
Glycogen	W	W	W	W	–	+	W
Inositol	W	–	–	–	–	–	–
Inulin	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	+
Fructose	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	W
Manitol	+	+	+	+	W	–	+
Mannose	+	+	+	+	–	+	–
Raffinose	–	–	–	–	–	+	+

Table 3.6 (Cont'd)

	TB118	TB150	TB124	TB144	TB128	B. stearo-thermophilus	B. coag-ulans
Rhamnose	-	-	-	-	-	-	-
Saccharose	+	+	+	+	W	+	-
Salicin	+	+	+	+	-	-	+
Starch	-	-	-	-	-	+	W
Sorbitol	-	-	-	W	-	-	+
Trehalose	-	-	W	+	-	+	+
Xylose	+	+	+	+	W	-	W

All tests were carried out as described in Methods (2.3) and results recorded after 48h incubation; further incubation resulted in no additional activity. Weak reactions are denoted by W.

*Digestion recorded as + for a large zone of hydrolysis, and R for a restricted zone.

Table 3.7. Antibiotic resistance of plasmid-bearing TB strains of Thermophilic bacilli

Antibiotic	TB124 (Tc^r)	TB128 (Tc^r)	TB118 (Sm^r)
Chloramphenicol (C_{50})	17	12	12
Streptomycin (S_{25})	22	10	0
Gentamycin (CN_{10})	23	20	20
Erythromycin (E_{10})	24	20	23
Kanamycin (K_{50})	22	9	19
Neomycin (N_{10})	22	18	19
Ampicillin (PN_{25})	22	35	28
Penicillin G ($P_{1.5}$)	10	26	23
Carbenicillin (Py_{100})	25	30	31
Tetracycline (TE_{50})	0	0	14
Oxytetracycline (OT_{10})	0	10	10
Chlorotetracycline (CN_{10})	0	12	13
Claxacillin (OB_5)	15	20	23
Cephaloridine (CR_{25})	20	26	25
Colistin sulphate (CT_{10})	0	0	0
Nitrofurantoin (F_{200})	22	22	21
Furazolidone (FR_{50})	23	13	23
Sulphafurazole (SF_{500})	24	20	10
Sulfamethoxazole/ Trimethoprim (SXT_{25})	18	22	19

Legend to Table 3.7

Sensitivity of the TB strains of thermophilic bacilli to antibiotics was carried out as described in Methods (2.4). Results were recorded after overnight incubation as diameter of inhibitory zone, mm (includes 7 mm diameter of multodisc).

3.4. Species classification of plasmid containing thermophilic bacilli

As described previously (3.1) thermophilic bacilli can be classified as one of two species, B. stearothermophilus or B. coagulans (Buchanan & Gibbons, 1974). A summary of the different characteristics of these two species and the plasmid containing thermophilic bacilli is given in Table 3.8. Strain RS93 appeared very similar to B. stearothermophilus NCA1503 and can be classed as a previously unreported strain of this species. This classification was confirmed by Sharp et. al., (1980).

The TB strains, however, do not appear as closely related to B. stearothermophilus NCA1503 as RS93 mainly due to their more restricted growth temperature range. However, these strains show little similarity to B. coagulans (Table 3.8) and on balance should perhaps be regarded as new strains of B. stearothermophilus. It appears that TB128 is more closely related to B. stearothermophilus NCA1503 than the other TB strains since it is the only one to be sensitive to the bacteriocin from RS93. This bacteriocin is specific for thermophilic bacilli (Sharp et. al., 1979) and has no effect on B. coagulans or other mesophilic bacteria. The insensitivity of TB118 + TB150 and TB124 + TB144 to thermocin 93 is curious as the two strains show no real similarity to B. coagulans and are not mesophiles.

As mentioned previously (3.3) two morphologically distinct variants of strains TB118 and TB124 were isolated, TB150 and TB144 respectively. Examination of old agar plates failed to show the presence of spores in TB150 and TB144 while their mucoid variants clearly contained spores. It is possible that TB150 and TB144 may be asporogenic variants of TB118 and TB124 respectively.

SUMMARY

(1) A large number of strains of thermophilic bacilli were isolated and fully characterised.

(2) Strains showing antibiotic resistance or bacteriocin production were examined for the presence of covalently closed circular DNA molecules (plasmids).

(3) Six plasmid containing strains of thermophilic bacilli were identified and classified as previously unreported strains of Bacillus stearothermophilus.

Table 3.8. Differential characteristics of plasmid-bearing thermophilic bacilli species

	B. coagulans (ATCC 8083)	TB118	TB124	TB128	RS93	B. stearothermophilus NCA1503
Growth temperature (^{o}C) maximum	61	63	63	65	68	74
minimum	25	25	25	36	39	41
optimum	50-55	55-60	55-60	55-60	60-65	60-65
Growth in 0.02%(w/v) azide	+	W	W	W	-	-
Sabaraud agar	+	-	-	-	-	-
Catalase production	-	+	+	+	+	+
NO_3 reduction	-	+	+	+	+	+
NO_2 reduction	-	+	+	+	+	+
Position of spore	CT	T	T	T	T	T

Tests were carried out as described in Methods (2.3) and results recorded after 48h incubation. Weak reactions are denoted by W, Terminal spore by T and central spore by C.

CHAPTER FOUR

ISOLATION AND CHARACTERISATION OF PLASMIDS FROM THERMOPHILIC BACILLI

4.1. Isolation of plasmid DNA

The improved method of plasmid isolation described in Chapter 2 (6.2) was used as the basis of a large-scale method for plasmid preparation (Chapter 2.7).

Two additional steps were carried out that were not employed for the plasmid-screening procedure and were designed to remove as much chromosomal DNA present in extracts prior to density gradient centrifugation, as possible. An alkali denaturation of the cleared lysate (Currier & Nester, 1976) denatures DNA to single strands. Since the two strands of CCC plasmid DNA remain joined as interlocking rings, they renature much more efficiently than OC plasmid or chromosomal DNA where the complementary strands are lost to each other. The single strand DNA can be removed by phenol (Currier & Nester, 1976) or chloroform extraction (Methods 2.7.2). The results of such an experiment are shown in Plate 4.1: the removal of chromosomal DNA was virtually complete. Plate 4.1 further shows that the CCC species of plasmid appears to be at a much lower concentration than before alkali denaturation, due mainly to the dilution effect of adding alkali followed by Tris buffer. The CCC DNA was therefore concentrated by precipitation with polyethylene glycol (Bernhard et. al., 1978) and resuspended in a small volume of buffer (Methods 2.7.3) prior to further purification.

Alkali denaturation was essential for isolating plasmid DNA from TB124 and TB144 since the plasmid band was completely masked by chromosomal DNA present in the caesium chloride ethidium bromide density gradients when using the plasmid screening method (2.6.2), as illustrated by Fig 3.3. The improvement in isolation of plasmid facilitated by alkali denaturation is demonstrated by Fig 4.1, where the amount of the chromosomal DNA band is now so small as to allow efficient separation of the plasmid DNA band.

Another advantage of the alkali denaturation/polyethylene glycol steps is that, as so much chromosomal DNA is removed that the loading of DNA in a density gradient can be greatly increased, thus reducing the number of gradients needed for isolating plasmid from the cells of a 2L culture.

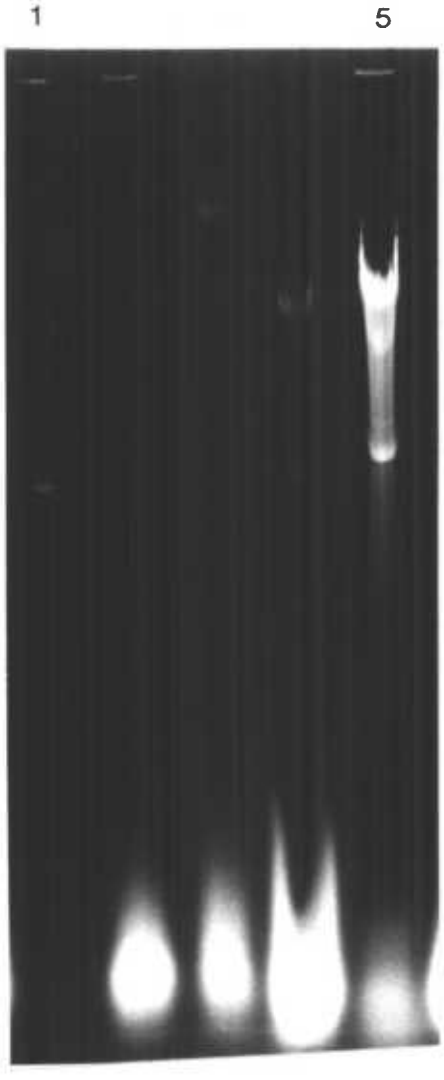
After removal of the plasmid band from the density gradient (Methods 2.7.5), protein and ethidium bromide was removed by phenol extraction as described (2.7.6).

The quantity of plasmid DNA isolated from each of the 6 plasmid containing strains of thermophilic bacilli varied considerably. Strains TB124, TB144, TB118 and TB150 yielded about 0.5 mg from cells of a 2L culture while only 0.12 mg was obtained from TB128 and only 50 µg from RS93. This variation could be due to degradation of substantial quantities of plasmid during isolation, or a reflection of the size and number of copies of the plasmid in the cell. The determination of plasmid copy number and size of plasmids is described below (4.2) and does show some variation. However, it is not sufficient to account for the very low amounts of plasmid isolated from RS93 and TB128.

It was found that if cleared lysates, in particular from RS93 or TB128, were left for any length of time at $4^{o}C$ extensive degradation of the DNA, particularly plasmid DNA, occurred. This was indicated in agarose gels by removal of high molecular weight chromosomal DNA and nicking of plasmid CCC species to the OC and linear form. Such nuclease activity was initially noted in extracts of TB128 and could be reduced by the inclusion of thymine (50 $\mu g.ml^{-1}$) in the medium. It can be inferred that TB128 is a thy^{-} strain which produces large amounts of nuclease in response to thymine limitation as a means of scavenging for indigenous thymine.

It was not possible to reduce nuclease activity in extracts of RS93 in a similar manner and nicking of the CCC plasmid species was a problem. Therefore all cleared lysates were treated with diethylpyrocarbonate (2.7.5) which inactivates most proteins by reacting with all amino and imidazole groups (Rosen & Fedorosak, 1966). A 25% improvement in the yield of pT93 was noted.

Plate 4.1. Removal of Chromosomal DNA from a Cleared-Lysate of TB124 by Alkali Denaturation



1. Cleared lysate of TB124 after alkali denaturation.

5. " " " " before " "

A cleared lysate of TB124 was prepared, and alkali denaturation carried out as described in Chapter 2 (2.7). Agarose-gel electrophoresis was carried out as described in 2.13.

FIG. 4.1a

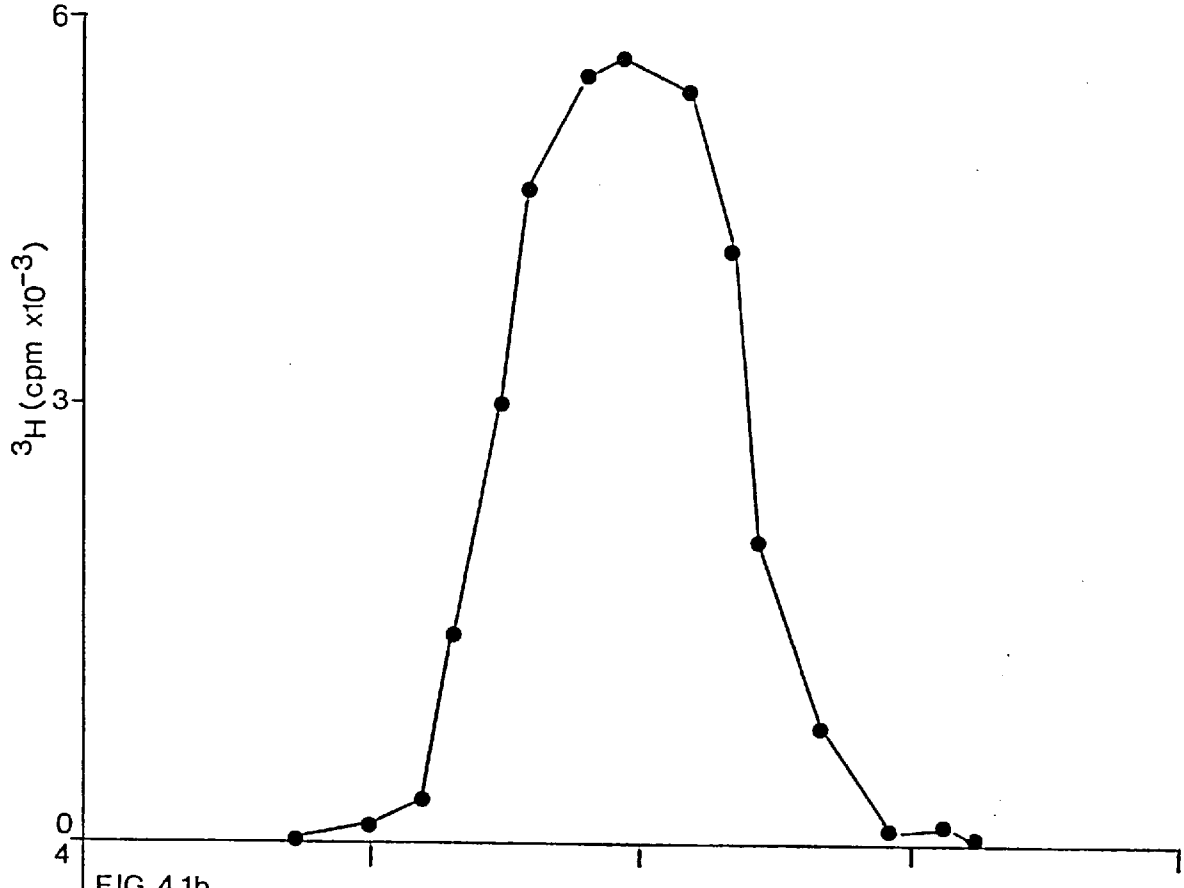
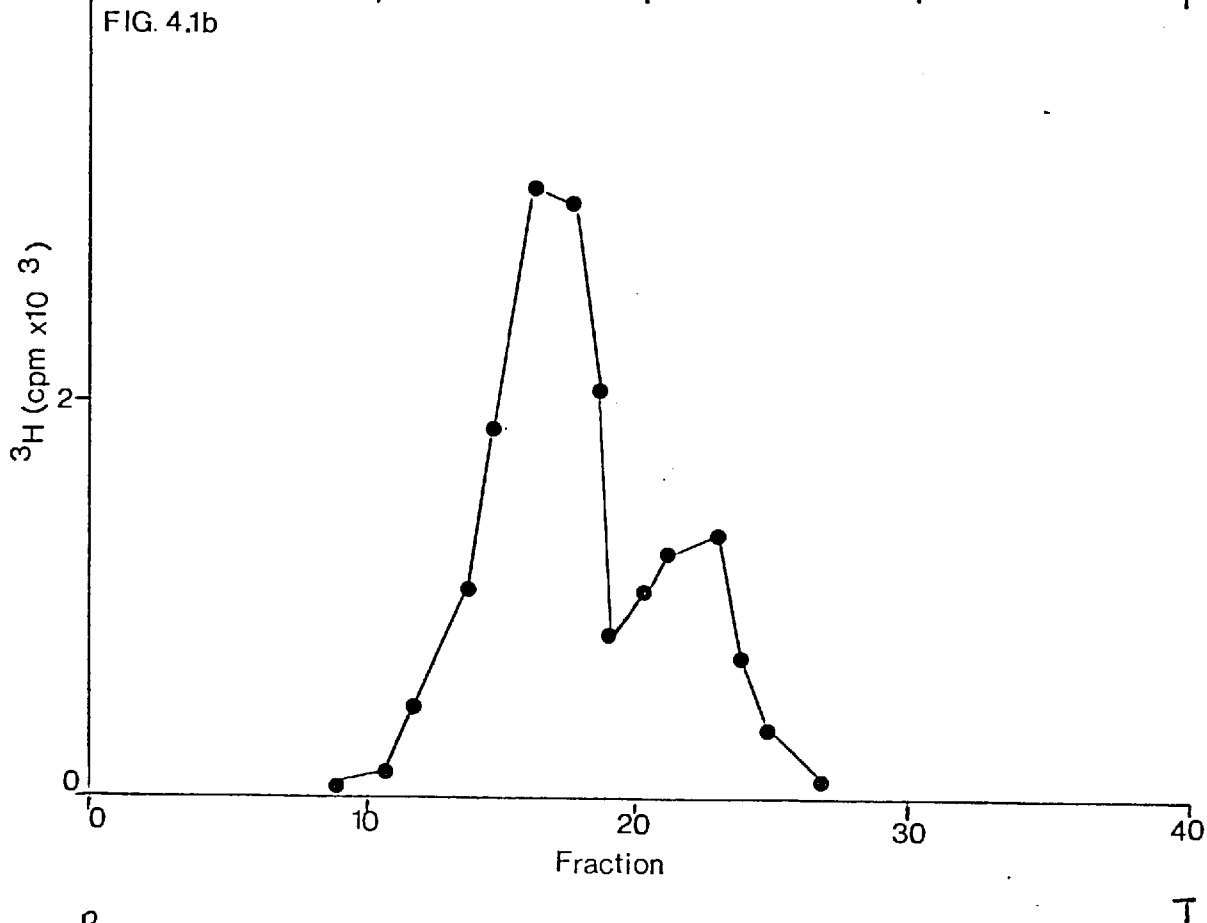


FIG. 4.1b



B

T

FIG. 4.1c

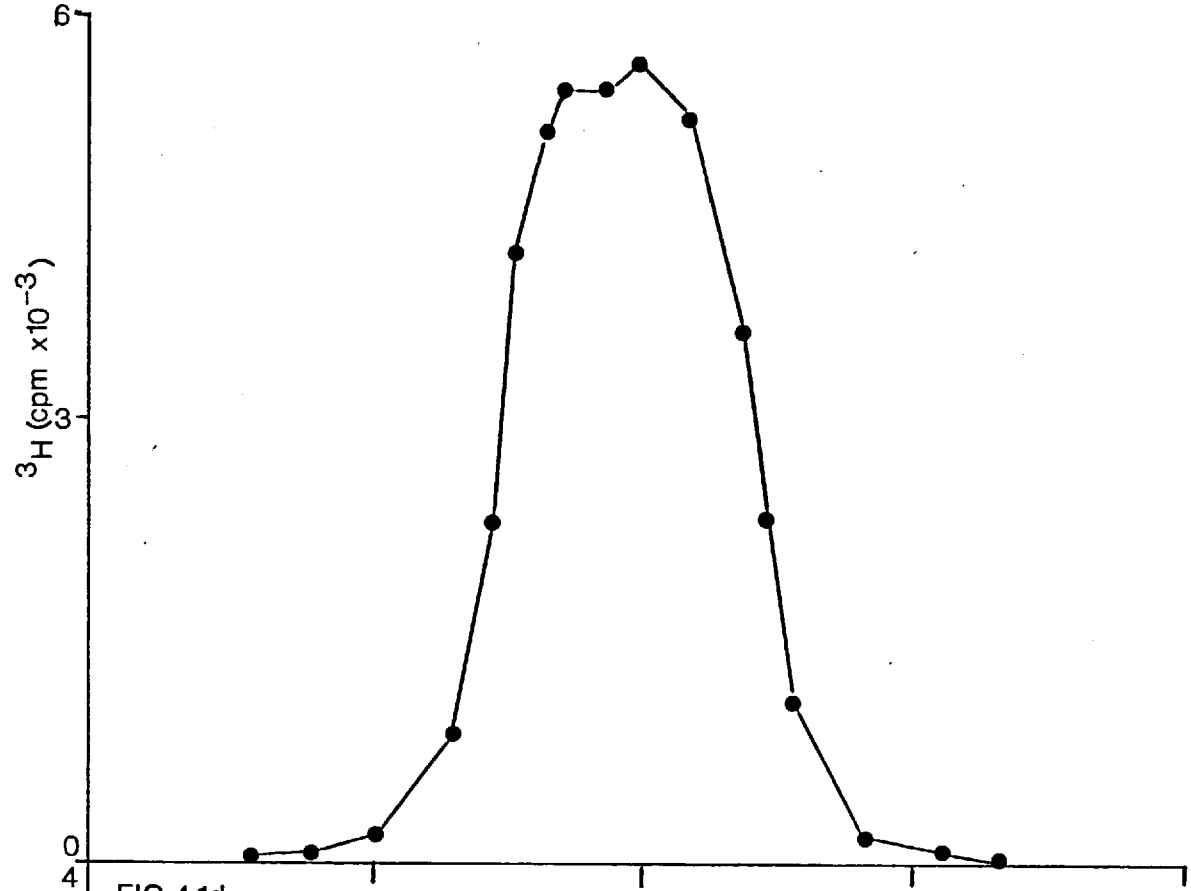
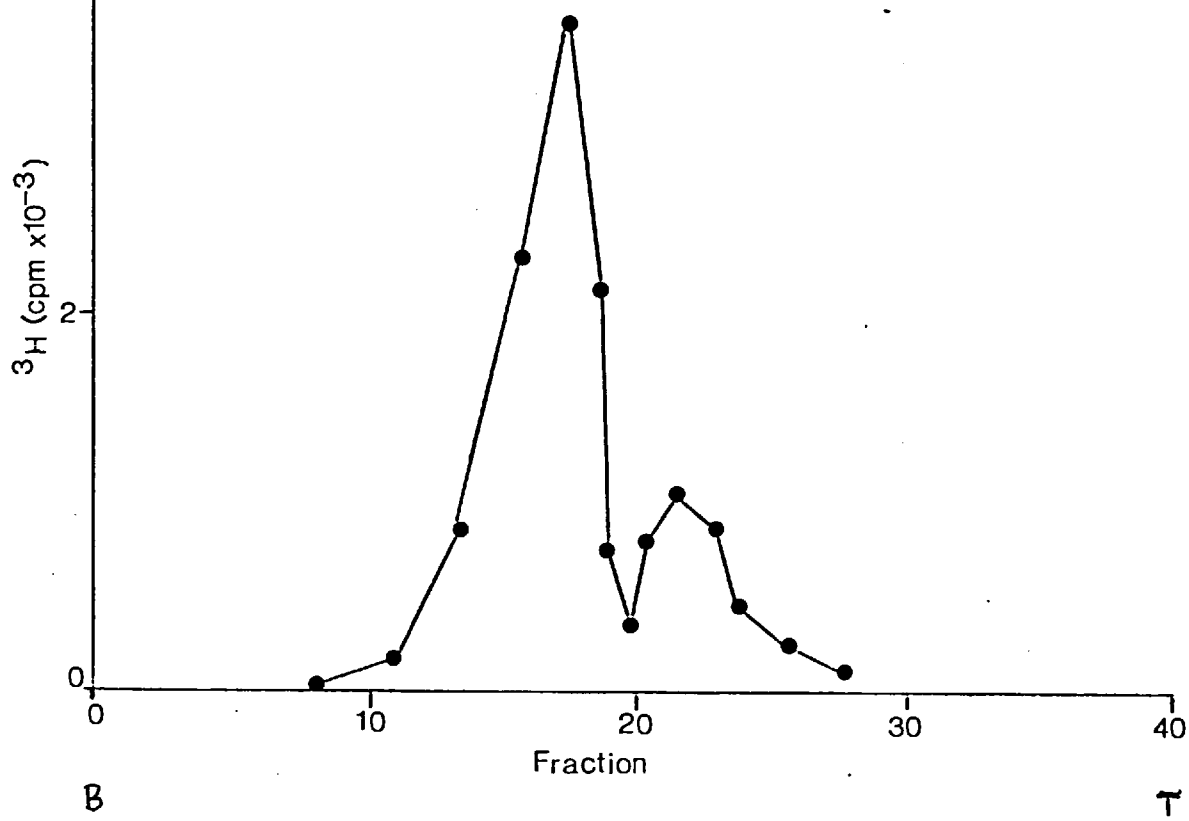


FIG. 4.1d



B

T

Figure 4.1. Ceasium Chloride Density Gradient ^{3}H-Profiles of an Extract of TB124 and TB144

DNA was labelled with ^{3}H-thymine by incorporating ^{3}H-thymidine in the growth medium (2.6.1). Cleared lysates were prepared and subjected to ethidium bromide-ceasium chloride density gradient centrifugation as described in Chapter 2 (.7).

4.1.a. TB124 without an alkali denaturation step

4.1.b. " with " " " "

4.1.c. TB144 without " " " "

4.1.d. " with " " " "

4.2. Characterisation of plasmids

4.2.1. Restriction endonuclease analysis

Each of the plasmid preparations from RS93, TB118, TB150, TB124, TB144, TB128, were digested with the following restriction endonucleases; BamHI, BclI, BglI&II CauI&II EcoRI, HaeIII, HhaI, HindII & III, HinfI, HpaI, KpnI, PstI, SalI, SstI, XbaI, XmaI (Methods 2.14) and the fragments separated in agarose gels as described in Chapter 2 (13).

A typical gel is shown in plate 4.2 where plasmid DNA from TB118 was digested with XbaI, SalI, PstI, and KpnI. The molecular weight of the fragments produced with the different enzymes was determined by constructing a calibration curve of Log molecular weight against distance travelled for the λ DNA standards run in the same gel (Methods 2.13.2). The sizes of the unknown fragments were determined using this curve (Fig 4.2) and are shown in Table 4.1. In the case of XbaI, SalI and KpnI one of the fragments produced was larger than the 3.35 Md fragment of the λ EcoRI + HindIII digested standards. Thus estimation of the molecular weight of the large fragment was by extrapalation and therefore inaccurate. A second set of standards (λ DNA digested with HindIII) was used where fragments of 6.23 Md and 4.18 Md were produced providing greater accuracy.

It was very surprising that 3 different enzymes with different cleavage sites (KpnI, SalI, XbaI) should all produce two fragments of identical size, 4.9 Md + 3.0 Md (Table 4.1). The implication is that the preparation contains two plasmids of 4.9 Md + 3.0 Md each with a single site for Xba, SalI and KpnI. Further evidence came from closer examination of the gel (plate 4.2), since the 4.9 Md fragment in each case was less intense than the smaller (3.0 Md) one; indicating that the two plasmids were not present in equimolar amounts. Also the undigested plasmid DNA had 4 bands. These bands must correspond to CCC and OC forms of the two plasmids. They can be identified since only the large plasmid contained an XmaI site and on digestion of the mixture 2 bands disappear to give an additional 4.9 Md fragment (Fig 4.3). The two remaining bands therefore correspond to CCC and OC forms of the small plasmid. Confirmation of their being 2 plasmids in TB118 was obtained by electron microscopy (Plate 4.3) and as expected the plasmid preparation from TB150 contained the same two plasmids. The large plasmid (4.9 Md) was called pAB118A and the small (3.0 Md) pAB118B.

The two plasmids from TB118 or TB150 had to be separated to determine

the number of restriction endonuclease cleavage sites each individual contained. A mixture of the two plasmids produces a meaningless fragment pattern. Separation of the plasmids was achieved by digesting 10 µg plasmid DNA from TB118 with KpnI and separating the two fragments (plasmids) in a preparative agarose gel (Methods 2.13). In earlier experiments the part of the gel containing each of the bands was cut out and the DNA recovered by the freeze-squeeze technique of Thuring et. al., (1975), with about a 50% yield. A more efficient way of recovering the DNA was simply to cut a well in the agar just in front of each band and allow it to migrate electrophoretically into the well filled with buffer (Methods 2.13.3): a 95% recovery of DNA was achieved with this method.

The purified plasmids (KpnI digested) were then digested with 18 restriction endonucleases and the number of fragments produced for pAB118A, pAB118B are given in Table 4.2.

Restriction endonuclease digests of plasmid DNA from TB124 and TB144 showed that they contained the same plasmid, named pAB124. The number of fragments produced by digestion with restriction endonucleases for pAB124, pAB128 (from strain TB128) and for pT93 (from strain RS93) are given in Table 4.2; and the size of the fragments shown in Table 4.3.

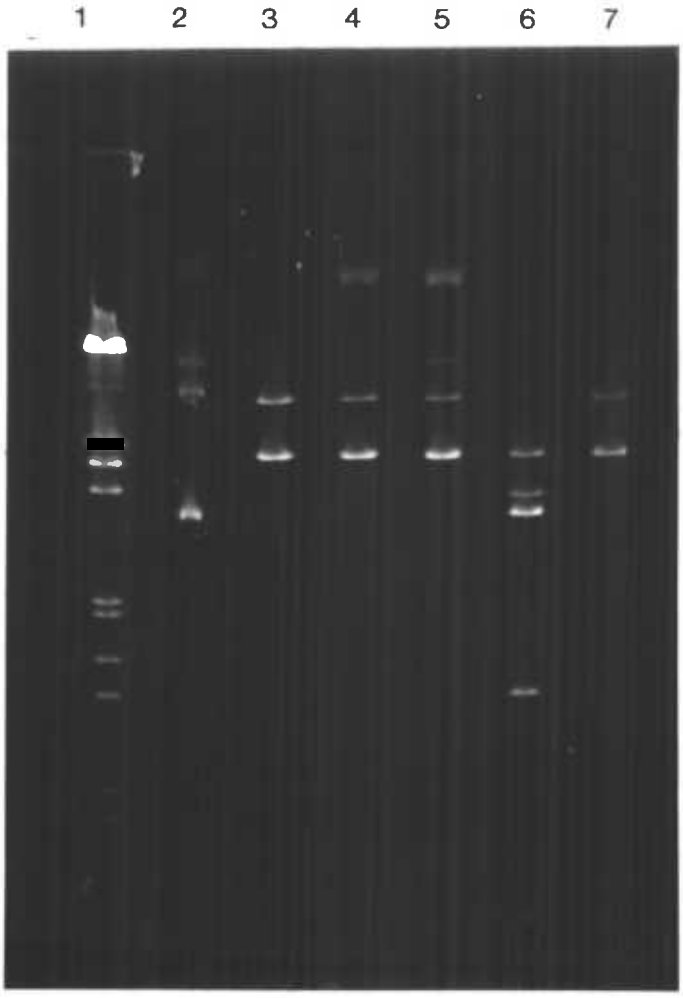
The 6 plasmid bearing strains of thermophilic bacilli were therefore shown to contain 5 different plasmids; two from a streptomycin resistant strain (pAB118A, pAB118B), two from two tetracycline resistant strains (pAB124, pAB128) and one from a strong bacteriocin producer (pT93). The size of the plasmids was determined from the sum of all the sizes of fragments produced by the restriction endonucleases given in Table 4.1, 4.3 and range from 4.9 Md (pAB118A) through 4.15 Md (pT93), 3.0 Md (pAB118B), 2.9 Md (pAB124) to 2.5 Md (pAB128).

All the plasmids are relatively small and could be developed as vectors for gene cloning in thermophilic bacilli.

Ideally a vector should contain one cleavage site for a particular restriction endonuclease which produces a cohesive terminus. The two plasmids from TB118 have several such sites. pAB118A has one BamHI, KpnI, SalI, XbaI, XmaI site and pAB118B contains one site for BamHI, KpnI, SalI and XbaI (Table 4.2). Unfortunately pAB124 is the only other plasmid with a suitable site (XbaI); the other enzymes with a single site in pAB124 (CauII, HpaI) and in pAB128 (BglI, CauII, HpaI) all produce blunt-ended

termini. Because of the difficulty of isolating pT93 in quantity this plasmid was not fully characterised.

Plate 4.2. Digestion of Plasmid DNA from TB118 with several Restriction Endonucleases



1: λ DNA digested with EcoRI and HindIII.

2. Plasmid DNA from TB118.

3. " " " " digested with SalI.

4. " " " " " " XbaI (1 unit).

5. " " " " " " XbaI (0.5 unit).

6. " " " " " " PstI.

7. " " " " " " KpnI.

Plasmid DNA was prepared from TB118 as described in Chapter 2 (2.7). Digestions and agarose-gel electrophoresis were carried out as described in 2.14 and 2.13.

Table 4.1. Molecular Weights of Fragments Produced by Digestion of Plasmid DNA from TB118 with Several Restriction Endonucleases

Enzyme (Fragment)[a]	Distance travelled[a] (cm)	Molecular weight[b] (Md)
SalI (A)	3.65	4.90
(B)	4.35	3.00
XbaI (A)	3.65	4.90
(B)	4.35	3.00
PstI (A)	4.35	3.00
(B)	4.90	2.32
(C)	5.10	2.21
(D)	7.60	0.80
KpnI (A)	3.65	4.90
(B)	4.35	3.00
λ DNA standards[c]		
(A)	2.80	13.40
(B)	4.25	3.20
(C)	4.50	2.80
(D)	4.90	2.32
(E)	6.40	1.33
(F)	6.55	1.27
(G)	7.30	1.05
(H)	7.75	0.89
(I)	8.80	0.59
(J)	9.40	0.47

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14), and the results shown in Plate 4.2.

a, See Plate 4.2. b, Determined from Fig 4.2.

c, See Chapter 2 (2.13.2).

Figure 4.2. Calibration Curve for the Determination of the Molecular Weights of DNA Fragments in Agarose-gels

An EcoRI + HindIII digest of lambda DNA was subjected to agarose-gel electrophoresis (Plate 4.2) as described in Chapter 2 (2.13.1, 2.13.2). A graph was constructed of Log molecular weight against distance travelled for each fragment;
a, 3.20; b, 2.80; c, 2.32; d, 1.33; e, 1.27; f, 1.05; g, 0.89; h,0.59; i, 0.47 (megadaltons) Murray & Murray, 1975.

FIG. 4.2

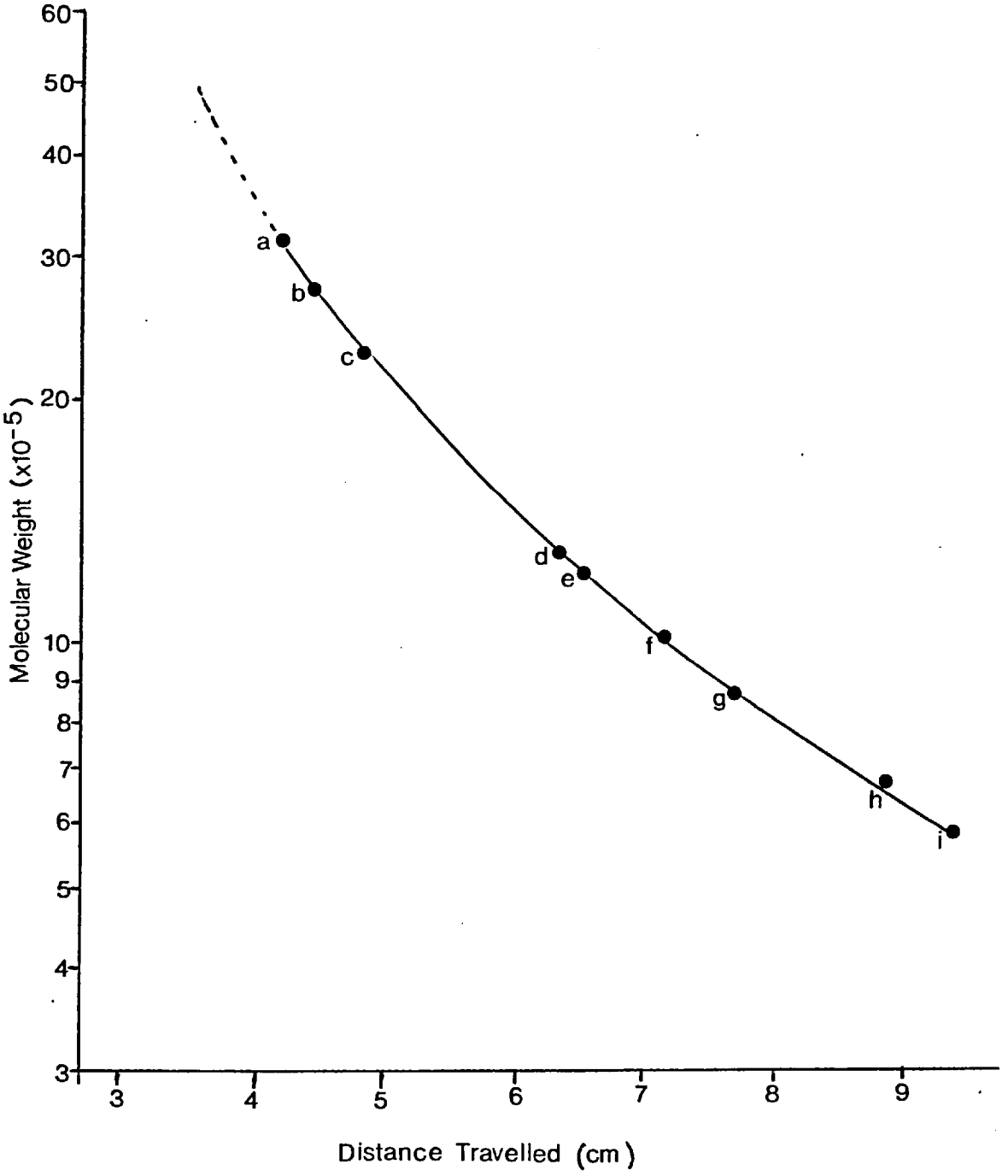

60
50
40
30
20
10
9
8
7
6
5
4
3
Molecular Weight (x10⁻⁵)
a
b
c
d
e
f
g
h
i
3
4
5
6
7
8
9
Distance Travelled (cm)

Figure 4.3. Digestion of Plasmid DNA Isolated from TB118 with Restriction Endonucleases KpnI and XmaI

Plasmid DNA was isolated from strain TB118 as described in Methods (2.7) and digested with either KpnI or XmaI as described in Chapter 2 (2.14).

1, λ DNA digest with EcoRI + HindIII producing fragments of molecular weight (Md); 13.4, 3.35, 3.20, 2.80, 2.32, 1.33, 1.27, 1.05, 0.89, 0.59, 0.47, 0.31 (Murray & Murray, 1975).

2, pAB118A/pAB118B digested with KpnI.

3, pAB118A/pAB118B digested with XmaI.

4, pAB118A/pAB118B undigested.

FIG. 4.3

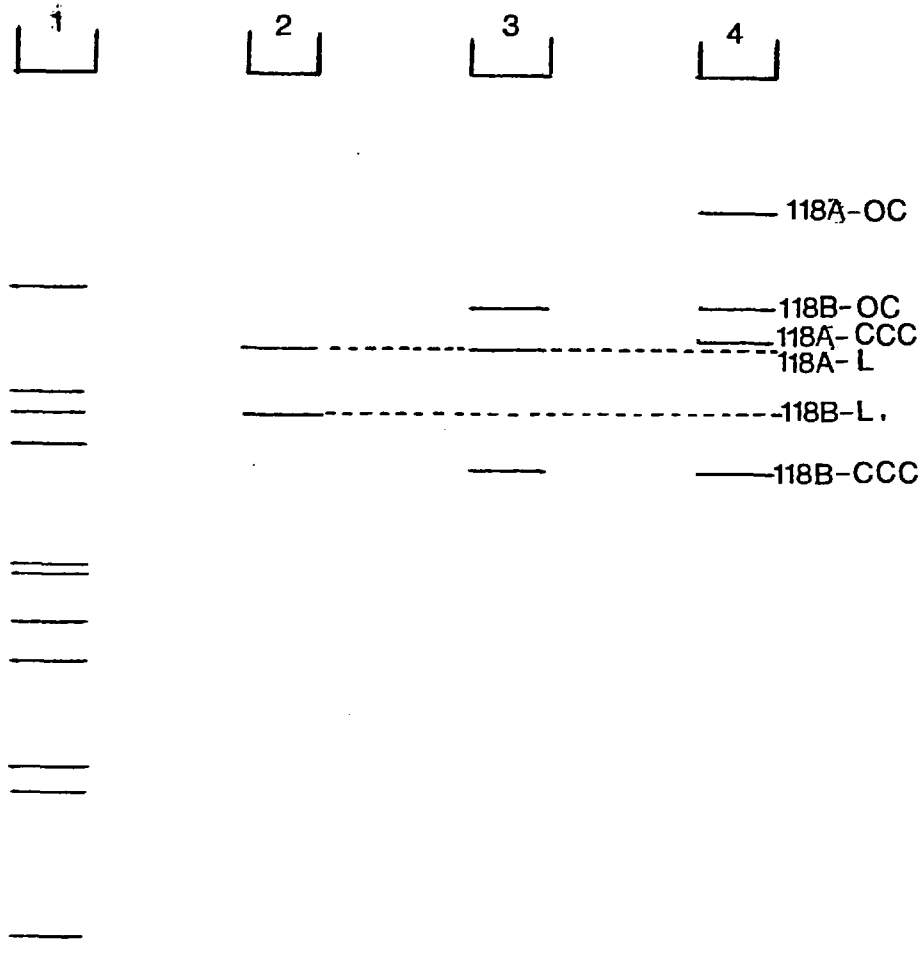


Table 4.2. Restriction endonuclease cleavage sites in plasmid DNA

Enzyme (sequence)	No. of fragments detected				
	pAB118A	pAB118B	pAB124	pAB128	pT93
BamHI (G/GATCC)	1	1	0	0	NT
BclI (T/GATCA)	4	2	3[a]	4	NT
BglI ($GCCN_5GGC$)	3	0	0	1	NT
BglII (A/GATCT)	0	0	0	0	0
CauI (GG^A_TCC)	7	4	4	5	NT
CauII (CC^C_GGG)	3	2	1	1	NT
EcoRI (G/AATTC)	2	0	3	3	2
HaeIII (GG/CC)	6	3	3	3	NT
HhaI (GCG/C)	6	5	2	4	2
HindII (GTPy/PuAC)	4	2	2	2	NT
HindIII (A/AGCTT)	3	0	0	0	NT
HinfI (G/ANTC)	10	6	6	6	NT
HpaI (GTT/AAC)	2	0	1	1	NT
KpnI (GGTAC/C)	1	1	0	0	NT
PstI (CTGCA/G)	4	2	0	0	NT
SalI (G/TCGAC)	1	1	0	0	NT
SstI (GAGCT/C)	0	0	0	0	NT
XbaI (T/CTAGA)	1	1	1[a]	0	0
XmaI (C/CCGGG)	1	0	0	0	NT

Legend to Table 4.2

Plasmid DNA was purified and digested with restriction endonucleases as described in Methods (2.7, 2.14) and the resulting fragments separated by agarose-gel electrophoresis (2.13).

a, pAB124 DNA isolated from *B. stearothermophilus* is partially modified against *Bcl*I and *Xba*I.

NT denotes not tested.

Table 4.3a. Size of pAB124 fragments produced by digestion with restriction endonucleases

Enzyme	Size of fragments (Md)				Sum of fragment sizes (Md)
	A	B	C	D	
CauII	2.90				2.90
HpaI	2.90				2.90
XbaI	2.90				2.90
HhaI	2.23	0.70			2.93
HindII	2.30	0.62			2.92
BclI	1.63	0.68	0.58		2.89
EcoRI	1.95	0.61	0.33		2.89
HaeIII	1.50	1.18	0.25		2.93
CauI	1.27	0.77	0.60	0.28	2.92
HinfI	0.86	0.55	(0.45)[a]	(0.31)[a]	2.93

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using DNA isolated from TB124(2.7) a, this fragment was "over intense" and assumed to be a "double".

Table 4.3b. Size of pAB128, pT93 fragments produced by digestion with Restriction Endonucleases

Enzyme	Size of fragments (Md)					Sum of fragment sizes (Md)
	A	B	C	D	E	
pAB128						
BglI	2.50					2.50
CauII	2.50					2.50
HpaI	2.50					2.50
HindII	1.95	0.56				2.51
EcoRI	1.70	0.55	0.25			2.50
HaeIII	1.50	0.68	0.30			2.48
HhaI	1.05	(0.50)[c]				2.55
BclI	1.40	0.44	0.37	0.30		2.51
CauI	1.00	0.55	(0.41)[a]	(0.20)[b]		2.57
HinfI	0.67	0.60	(0.40)[a]	0.35	(0.07?)[d]	2.43[d]
pT93						
EcoRI	2.30	1.85				4.15
HhaI	2.35	1.80				4.15

Legend to Table 4.3b

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using DNA isolated from TB128 and RS93 (2.7).

a, this fragment was over intense and assumed to be a "double".

b, an accurate estimation of the molecular weight of this fragment is not possible.

c, this fragment was very intense and assumed to be a "triplet".

d, this fragment was not detected, but can be predicted from the sum of the fragment sizes.

4.2.2. ElectronMicroscopy and plasmid copy number

All the plasmid preparations were shown by agarose gels to contain about 10% open circles which can be easily seen under the electron microscope. Samples were spread on grids as described in Methods (2.19) and electron micrographs of each plasmid are shown in Plate 4.3, confirming that TB118 contained two plasmids of differing sizes.

The number of copies of a plasmid per cell can be determined from the ratio, or percentage of plasmid DNA to chromosomal DNA isolated in caesium chloride-ethidium bromide density gradients (Bernhard et. al., 1978). This requires labelling of the DNA with ^{3}H-thymidine to allow estimation of the amount of DNA in each species and is described fully in Chapter 2 (2.17). This method however cannot be used for pAB124 nor pAB118A/pAB118B, since pAB124 does not separate well from the chromosomal DNA band in density gradients (Chapter 3) and the two TB118 plasmids have the same buoyant densities. Therefore an additional method was employed where ^{3}H-labelled plasmid DNA was separated from the chromosomal DNA in agarose gels. The plasmid band was removed from the gel by dissolving the agarose with Potassium iodide (Blin et. al., 1975) and then counted for ^{3}H activity as described in Methods (2.17.4). The two CCC species of pAB118A and pAB118B separated quite well using agarose gels (Plate 4.2) and the ^{3}H-activity attributed to each was determined. The amount of background ^{3}H-activity in the gel due to chromosomal DNA contamination was estimated by removing an equal sized piece of gel just in front of the CCC plasmid band and counting for ^{3}H-activity as before.

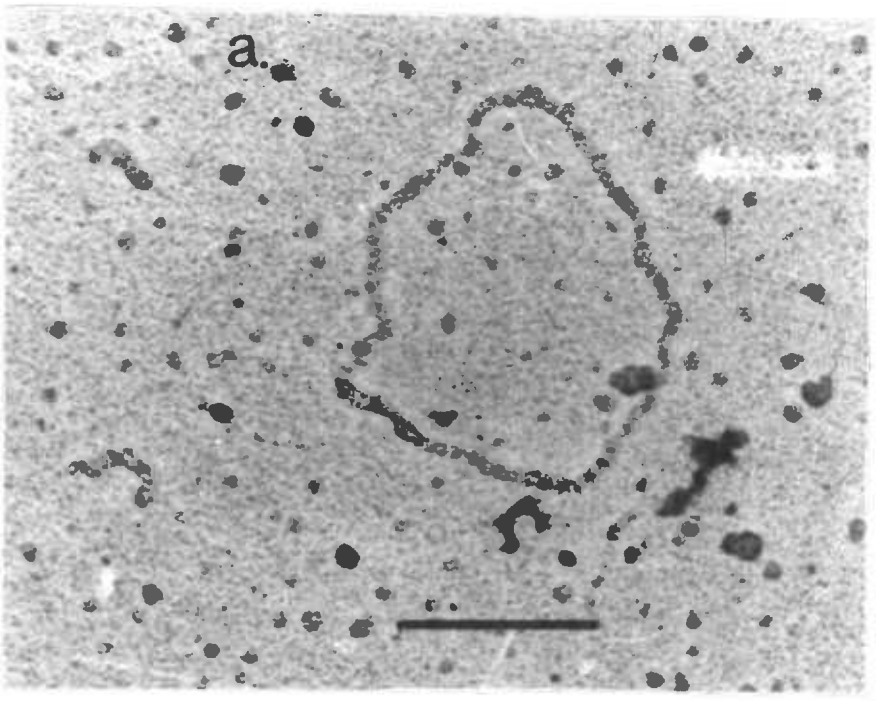
The copy numbers of each plasmid are shown in Table 4.4 and an example of the calculation is given below.

The percentage of pAB128 (CCC) DNA was estimated as 0.3% of the chromosomal by agarose-gel electrophoresis (Table 4.4). Assuming the molecular weight of the B. stearothermophilus chromosome is approximately the same as that of B. subtilis 3.0×10^{9} (Bernhard et. al., 1978) then the amount of plasmid DNA must be 0.3% of this value ie, 9×10^{6}. Since the molecular weight of pAB128 is 2.5×10^{6} there must be 3-5 copies present. It was estimated from agarose gels that about 40-50% of plasmid DNA in extracts were as open circles or linear, therefore the maximum copy number shown in Table 4.4 takes this loss into account.

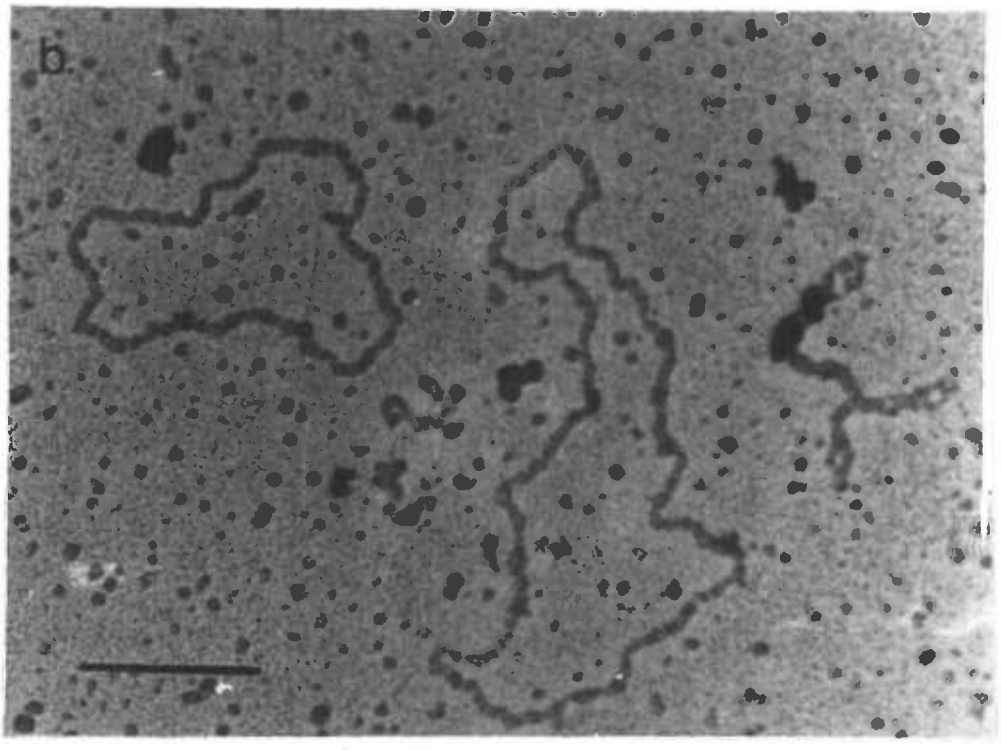
The low copy number of pT93 (Table 4.4) explains in part the problem of isolating sufficient quantity of plasmid for characterisation. The highest copy number was shown by pAB124 (6-9) which can be isolated in high yields from TB124/TB144.

Plate 4.3. Electron Micrographs of Plasmid DNA

4.3.a., pT93 (4.1 Md)



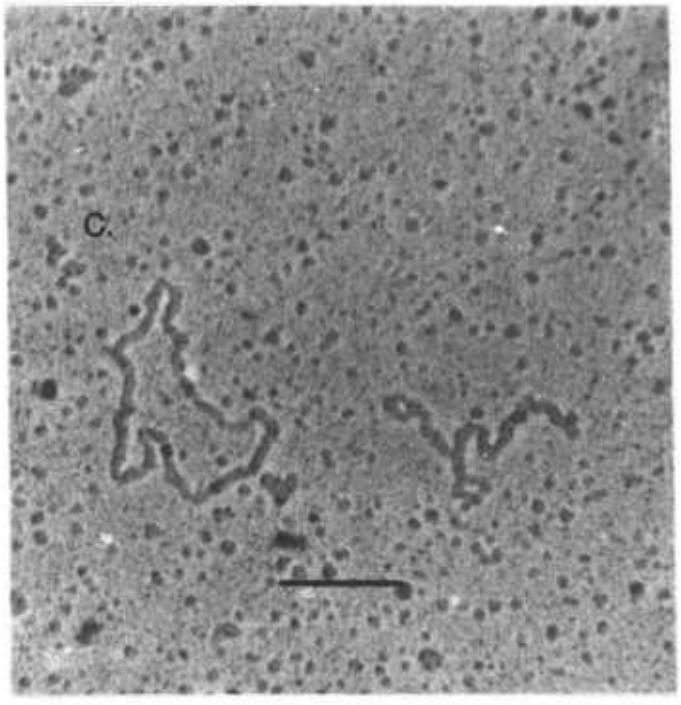
4.3.b., pAB118A (4.9 Md), pAB118B (3.0 Md)



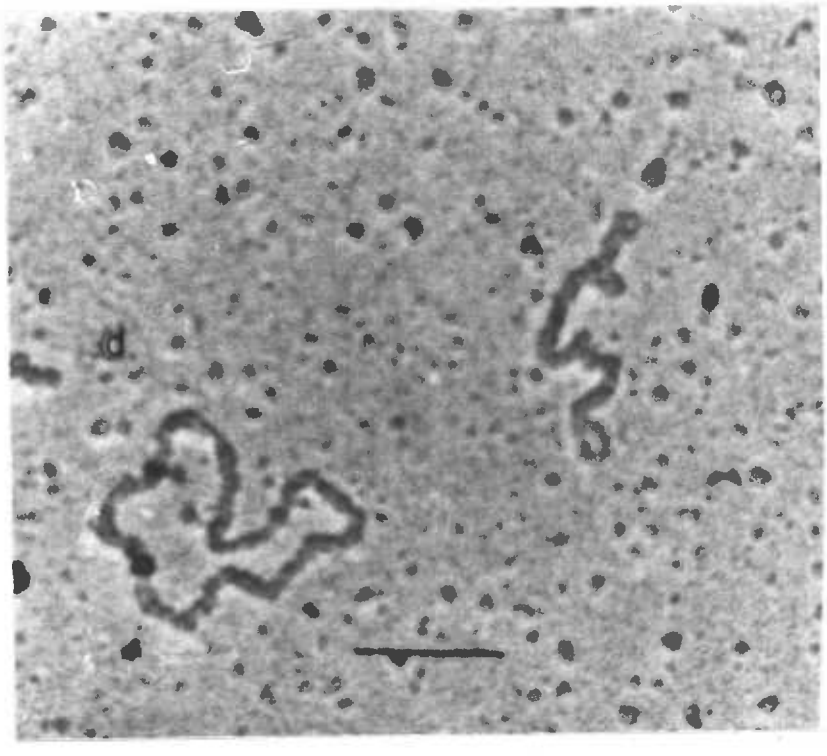
Bar represents 0.2 µm. Electron microscopy was carried out as described in Chapter 2 (2.19).

Plate 4.3. (Cont'd)

4.3.c., pAB124 (2.9 Md)



4.3.d., pAB128 (2.5 Md)



Bar represents 0.2 μm. Electron microscopy was carried out as described in Chapter 2 (2.19).

Table 4.4. Plasmid copy numbers

	Agarose gel method				Isopycnic centrifugation method			
	^{3}H Counts				^{3}H Counts			
	Chromosomal DNA	Plasmid DNA	Plasmid Content (%)	Copy No. (min-max)	Chromosomal DNA	Plasmid DNA	Plasmid Content (%)	Copy No. (min-max)
pAB128	8,700	27	0.30	3-6	31,200	97	0.31	3-6
pT93	9,500	20	0.20	1-2	40,510	90	0.22	2-3
pAB118A	8,900	49	0.55	3-5				
pAB118B	8,900	37	0.41	4-6				
pAB124	5,250	32	0.60	6-9				

Cell extracts were prepared as described in Methods (2.17) and the amount of CCC plasmid (%) determined. Plasmid copy number was calculated using the following molecular weights; B. stearothermophilus chromosome, 3.0×10^3 Md; pAB118A, 4.9 Md; pAB118B, 3.0 Md; pAB128, 2.5 Md; pT93, 4.1 Md; pAB124, 2.9 Md.

4.2.3. Transformation of Bacillus subtilis with plasmid DNA

Although the plasmids isolated from the TB strains were from antibiotic resistant thermophilic bacilli there is no evidence to indicate whether the plasmid carries the antibiotic resistance genes. The majority of plasmids isolated from Bacilli have, in fact, no known genetic function. This point is well illustrated by Bernhard et. al., (1978); of the 23 plasmids identified in strains of B. subtilis and B. cereus only two conferred an identifiable genetic trait, pBC7 (bacteriocin) and pBC16 (Tc^r) to strains of B. subtilis. Plasmids from Staphylococcus aureus can also express well in B. subtilis (Gryczan et. al., 1978), and the expression of the plasmids from the thermophilic bacilli in B. subtilis was examined.

As a positive control in the transformation experiments with thermophilic bacilli plasmid DNA, the Staphylococcal plasmid pUB110 (Neo^r) was used which transforms B. subtilis to Neo^r at high frequency (Keggins et. al., 1978). The results of the transformation experiments with B. subtilis IG20 (trpC2, $r.^-m.^-$) are shown in Table 4.5.

Only pAB124 was shown to confer tetracycline resistance on B. subtilis and several transformants were examined for the presence of plasmid DNA using the small-scale extraction procedure described in Methods (2.8). The results (Plate 4.4) confirm the presence of plasmid DNA in the transformants, and a large-scale preparation of one transformant was carried out. The plasmid DNA obtained was digested with several restriction endonucleases and generated identical fragments to pAB124 confirming that the plasmid present in the transformant was indeed pAB124.

The transformation was repeated several times but no transformants were obtained with pAB118A/B (Sm^r) or pAB128 (Tc^r). The high level of spontaneous mutation to streptomycin resistance by B. subtilis (Table 4.5) makes identification of Sm^r pAB118A/B transformants difficult. The level of streptomycin in the selection plates in later experiments was increased to 500 $\mu g.ml^{-1}$ but resistance to this level was shown by at least 10^3 cells from a population of 10^8 (1×10^{-5} spontaneous mutation frequency). If pAB118A/B was conferring streptomycin resistance then the number of Sm^r cells should increase substantially from 10^3. In a few experiments 2×10^4 Sm^r cells

were obtained. A random selection of these were screened for the presence of plasmid DNA (Methods 2.8), however none was detected. It is possible that the transformation frequency of cells to Sm^r is so low that it reduces the chance of identifying transformed cells in a background of spontaneous mutants.

This method of transformation gave very variable results the level of competence of cells, as determined by the number of transformants with a given amount of pUB110, varied from 2×10^2 to 1×10^4. During the course of a typical preparation of competent cells the level of competence was measured using uptake of pAB124 at 30 min intervals and the results shown in Fig 4.4 indicated a decrease in competence on subculture in starvation medium. It was noticed that B. subtilis IG20 produced a linear response with increase in optical density against time when tryptophan was not limiting and the results in Fig 4.5 show that 60-90 min after growth became non-linear (tryptophan limitation) the cells were at maximum competence. This is a much easier and reproducible method of preparing competent cells and was also found to be applicable for several B. subtilis strains (Chapter 6).

Using this modified procedure, transformations with pAB124 were more reproducible. Frequencies of up to 7×10^3 per µg DNA were regularly obtained. However, when pAB124 DNA isolated from B. subtilis was used, transformation frequencies increased to 5×10^4 per µg DNA. This difference could not be explained by a simple restriction and modification system since the strain used was r^-m^-. Work by Canosi et. al., (1978) explains this, because oligomeric forms of plasmids are produced in B. subtilis and these transform at a much higher frequency than the monomeric species.

Repeated transformations with pAB118A/B using the modified method failed to provide conclusive evidence that these plasmids conferred streptomycin resistance. However, one preparation of pAB128 gave 2.8×10^3 Tc^r transformants per µg DNA (12.5 $\mu g.ml^{-1}$ tetracycline) while only 10 were obtained with the original preparation. This would indicate that the latter preparation contained some inhibiton of transformation, possibly trace amounts of phenol or ethidium bromide. Further examination of the original preparation revealed a very high percentage of open circular form of plasmid which cannot transform competent cells (Canosi et. al., 1978; Contente & Dubnau, 1979a) explaining the lower transformation frequency. The Tc^r transformants were

examined for the presence of plasmid DNA (Methods 2.9.2)which was detected in all cases. A large-scale plasmid preparation of one transformant was carried out (Methods 2.9.1). The DNA was digested with several restriction endonucleases and generated indentical fragments to pAB128 isolated from TB128 (Table 4.3).

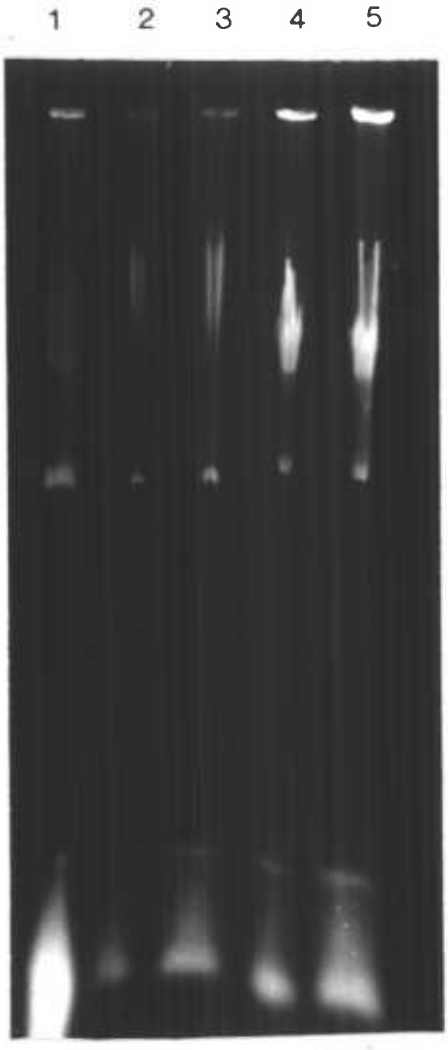
The two tetracycline resistance plasmids pAB124 and pAB128 produced similar restriction endonuclease fragment patterns when digested with several enzymes. HindII and HhaI generated one large and one small fragment for both plasmids. The EcoRI and HaeIII fragment patterns were very similar and each plasmid contained 1 site for CauII and HpaI. The presence of any sequence homology between the two plasmids was examined by DNA-DNA hybridisation using the method of Southern (1975) described in Methods (2.23). Plasmid pAB128 (1 μg) was digested with HpaI subjected to agarose-gel electrophoresis (Methods 2.13, 2.14), denatured with alkali and transferred to a nitrocellulose filter. 'Nick-translated pAB324 (^{32}P) DNA (this plasmid contains the cloned tetracycline genes of pAB124; see Chapter 6) was added to the filter and any hybridisation was detected by autoradiography. The results shown in Plate 4.5 indicated some homology between the plasmids.

Table 4.5. Transformation of B. subtilis IG20 with plasmid DNA

Plasmid (Source)	No. Spontaneous mutants	No. Transformants. ($\mu g\ DNA^{-1}$)	Transformation frequency ($\times 10^{-5}$)	Antibiotic selection ($\mu g.ml^{-1}$)
pAB124 (from mucoid strain TB124)	0	1000	3.6	Tetracycline (25)
	0	1950	6.9	Tetracycline (12.5)
pAB124 (from non-mucoid strain TB144)	0	1050	3.8	Tetracycline (25)
	0	1800	6.4	Tetracycline (12.5)
pAB128 (from strain TB128 thy^-)	0	0	0	Tetracycline (25)
	0	0	0	Tetracycline (12.5)
pAB118A/B (from mucoid strain TB118)	3500	0	0	Streptomycin (100)
	1950	0	0	Streptomycin (200)
pAB118A/B (from non-mucoid strain TB150)	3480	0	0	Streptomycin (100)
	1705	0	0	Streptomycin (200)
pUB110 (from B. subtilis)	0	3000	11	Neomycin (25)
	0	3550	13	Neomycin (12.5)

Transformations were carried out as described in Methods (2.20) using 2.8×10^8 bacteria in minimal medium (SMS).

Plate 4.4. Agarose-gel of Plasmid DNA Isolated from B. subtilis Transformed with pAB124 from TB124



1: pAB124 plasmid DNA isolated from TB124.

2: " " " " " B. subtilis Tc^r (transformed with pAB124), clone 1.

3: Plasmid DNA isolated from B. subtilis Tc^r (transformed with pAB124), clone 8.

4: " " " " " " " " clone 21.

5: " " " " " " " " clone 40.

Plasmid DNA was extracted from Tc^r transformants of B. subtilis and agarose-gel electrophoresis was carried out as described in Chapter 2 (2.9; 2.13).

Figure 4.4. Transformation of B. subtilis IG20 with pAB124

Plasmid DNA was isolated from strain TB124 as described in Methods (2.7) and added to cells prepared by the method of Stacey (1968).

●———● Optical Density (550 nm) of culture.

o- - - -o Transformants per μg pAB124 DNA.

FIG. 4.4

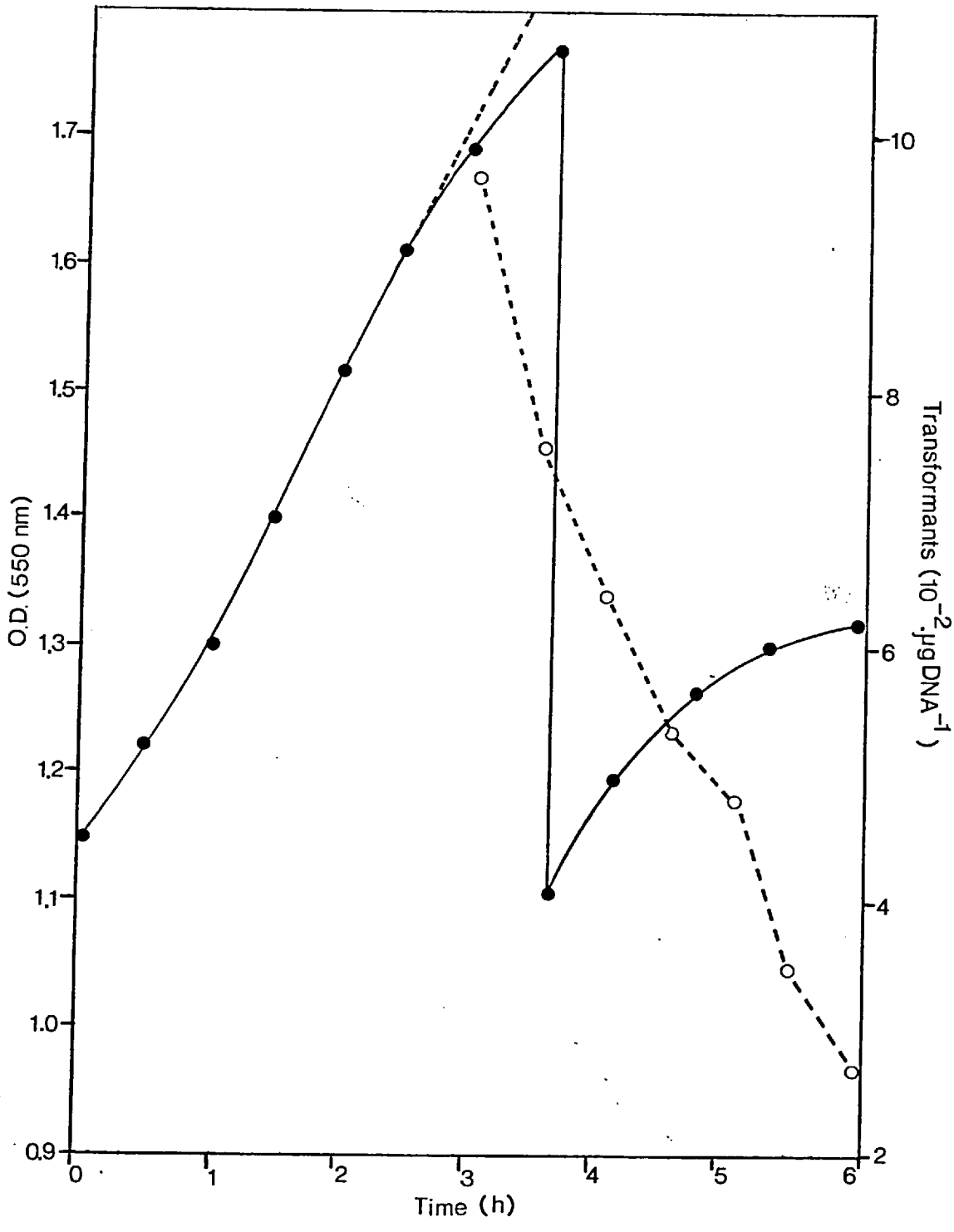

O.D. (550 nm)
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
Transformants (10^{-2}.µg DNA^{-1})
10
8
6
4
2
0
1
2
3
4
5
6
Time (h)

Figure 4.5. Transformation with pAB124 During the Growth Cycle of *B. subtilis* IG20

Plasmid DNA was isolated from strain TB124 as described in Methods (2.7) and added to cells as described in Chapter 4 (4.22).

●———● Optical Density (550 nm) of culture.

o- - - -o Transformants per µg pAB124 DNA.

FIG. 4.5

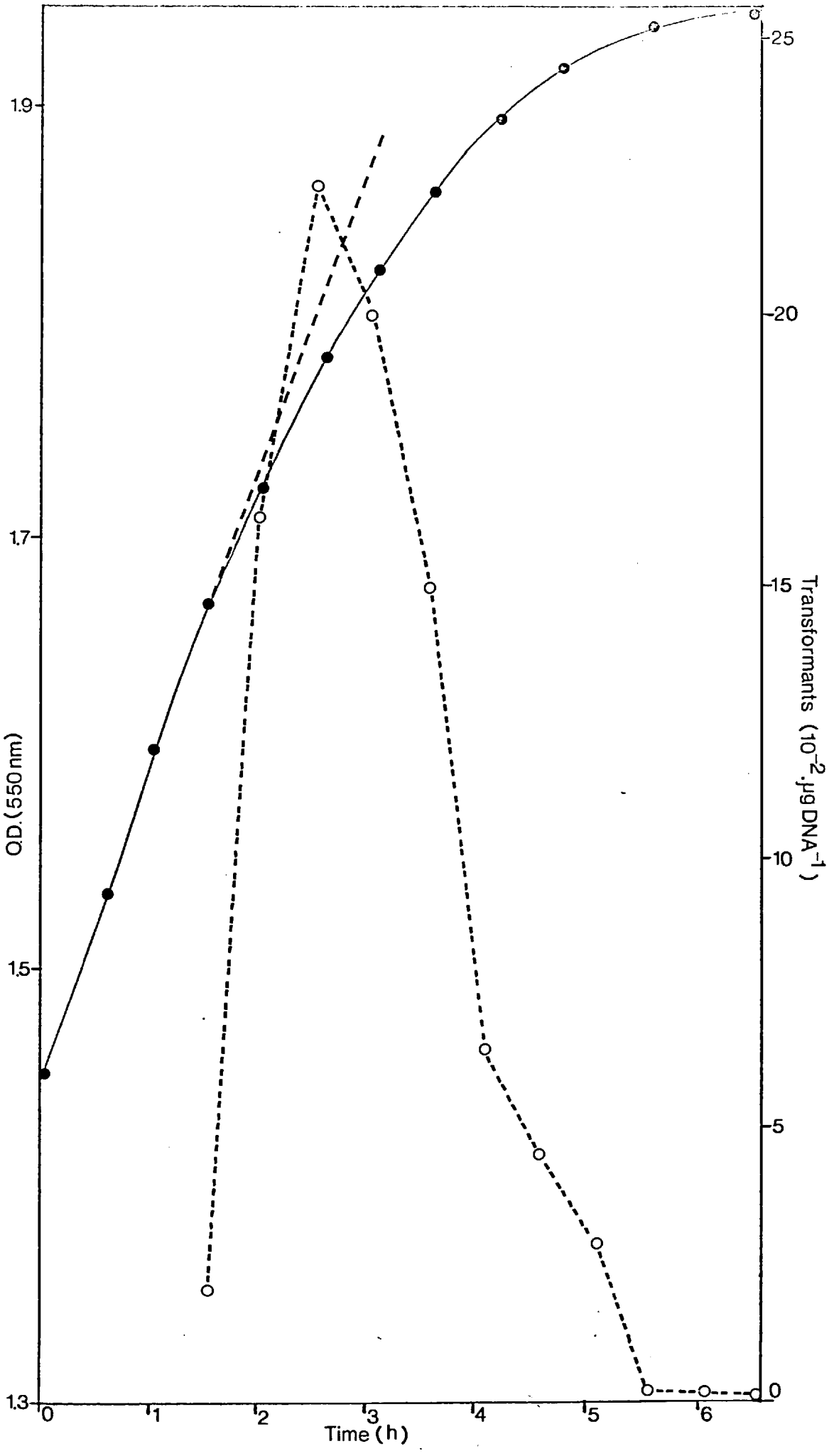
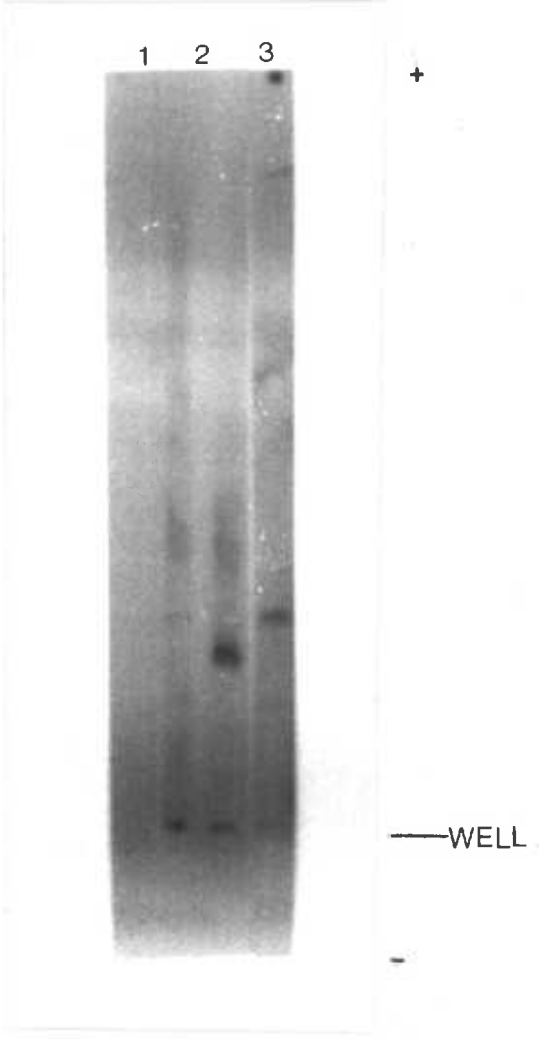

1.9
1.7
1.5
1.3
O.D. (550 nm)
0
1
2
3
4
5
6
Time (h)
25
20
15
10
5
0
Transformants (10^{-2}, µg DNA^{-1})

Plate 4.5. X-ray plate of a Nitrocellulose filter where pAB324 ^{32}P DNA was hybridised to pAB124 and pAB128



1: pAB128 digested with HpaI (1 day exposure at -70°C).
2: pAB124 " " " (2 days " " ").
3: pAB128 " " " (" " " ").

Plasmid DNA was isolated from TB124, TB128; digested with restriction endonuclease HpaI and subjected to agarose-gel electrophoresis as described in Chapter 2 (2.7., 2.14., 2.13.). The DNA was transferred to a nitrocellulose filter and hybridised to ^{32}P pAB324 as described in 2.23.

4.2.4. Stability and curing of plasmids

The plasmids pAB124 and pAB128 were shown to confer Tc^r; the position concerning Sm^r associated with pAB118A/B remains unclear. The stability of these plasmids without any selective pressure was examined and attempts were made to cure the plasmids from the strains by heat shock or chemical treatment. This approach has two advantages: firstly it will confirm if the plasmids do confer antibiotic resistance in their original host as when the plasmid is lost the strain should revert to antibiotic sensitivity, and secondly such a strain could then be used as a host, and experiments to transform the plasmid back into the cell could then be carried out.

Each strain was inoculated into several flasks containing TYS medium (100 ml) and incubated overnight at either 30°C, 37°C, 50°C or 60°C. Each culture was used to inoculate (a 0.1 ml inoculum) 100 ml of fresh medium and incubation continued at the same temperature. This subculturing was carried out twice more after which the percentage of the population retaining antibiotic resistance was determined by plating on TSBA and TSBA+ antibiotic.

All the strains, TB124/TB144, TB118/TB150, TB128 retained antibiotic resistance in TYS and when they were also subcultured using minimal medium (AD). It was noted that none of the strains grew well at 63°C and the effect of heat shock (70°C for 30 min or 60 min) was examined. TB124/TB144 and TB118/TB150 retained antibiotic resistance while 10% of the TB128 population were now Tc^s. Examination of several of the Tc^s colonies revealed that they still contained the plasmid. Therefore it would seem that the heat shock caused a mutation in the tetracycline resistance genes or promotor functions, not plasmid expulsion.

The evidence from the above experiments strongly indicated that the plasmids were very stable in their host strains. Therefore, chemicals that are known to promote plasmid expulsion were used in an effort to cure the plasmids. (Hirota, 1956; Hirota, 1960; Gale et. al., 1972). These were acridine orange, ethidium bromide and mitomycin C. The first two were used, as described in Methods (2.18) without success. However, after 2 subcultures of TB144 in TYS medium containing 0.01 $\mu g.ml^{-1}$ mitomycin C, 0.1% of the population appeared Tc^s. Twenty of these colonies were examined for the presence of plasmid DNA as described in Methods (2.8). The results showed that 50% Tc^s

strains had lost the plasmid (strains termed LO2) while the remainder still had a plasmid. This indicated that a mutation in the tetracycline resistance genes or promotor function had occurred in the latter group without plasmid loss.

It was not possible to isolate strains that had lost the plasmids pAB118A/B on pAB128 by chemical treatment.

SUMMARY

(1) A method for the large-scale purification of the plasmids from antibiotic resistant thermophilic bacilli was developed.

(2) Two plasmids, of differing molecular weight, were identified in Sm^r strains TB118 and TB150.

(3) The number of restriction endonuclease cleavage sites present in pAB118A, pAB118B, pAB124, pAB128 was determined for 18 enzymes, and the number of copies of each plasmid in the host cell determined.

(4) Plasmids pAB124 and pAB128 were shown to confer Tc^r to B. subtilis. Some sequence homology between these two plasmids was demonstrated.

(5) Strain TB144 was cured of the plasmid pAB124 with mitomycin C. The host then reverted to Tc^s.

CHAPTER FIVE

CHARACTERISATION OF PLASMID pAB124 AND CONSTRUCTION OF DELETION VARIANTS

Of the five plasmids isolated and characterised (Chapter 4) pAB124 appears to have most potential as a vector for gene-cloning, it is small (2.9 Md), present in fairly high copy number (6-9) contains a suitable cloning site, XbaI and expresses its tetracycline resistance genes well in B. subtilis. This plasmid was therefore further characterised.

5.1. Restriction endonuclease cleavage site map of pAB124

As shown in the previous chapter (Table 4.2), pAB124 contained one cleavage site for CauII, HpaI, XbaI, two cleavage sites for HhaI, HindII and three cleavage sites for HaeIII, EcoRI, BclI and a map of all these sites was constructed. As more restriction endonucleases became available, they were also tested against pAB124 and the results are shown in Table 5.1. It was found that pAB124 had one cleavage site for BalI, BstEII, HgaI, HgiAI, PvuII and three sites for HpaII, HphI, ThaI; and these were also included in the construction of a restriction endonuclease map.

The double-digest method of restriction endonuclease site mapping (Nathans & Smith, 1975; Roberts, 1976) is described in Methods (2.15).

Firstly, the relative positions of the enzymes having just one site in pAB124 (BalI, BstEII, HgaI, HgiAI, HpaI, CauII, PvuII, XbaI) was determined where the CauII site was taken as reference point. The fragment sizes produced in the double digest experiments are shown in Table 5.2.

A HpaI + CauII, and a BstEII + CauII digest both generated two fragments of 2.55 and 0.35 Md, it can be assumed therefore both BstEII and HpaI are the same distance from the CauII site, either on the same side, ie very close together, or on opposite sides 0.7 Md apart. The latter position was confirmed by a BstEII + HpaI digest where 2.2 + 0.7 Md fragments were generated. A CauII + XbaI digest generated 1.85 + 1.05 Md fragments, and it was assumed that the XbaI site was 1.85 Md to the right of CauII and the HpaI and BstEII sites were positioned using this orientation (Fig 5.1). If the HpaI site lies 0.35 Md to the right of the CauII site then a HpaI + XbaI digest would generate 1.48 +

1.40 Md fragments, whereas, if it lies to the left, 0.8 + 2.1 Md fragments would result. Since the former sized fragments were obtained, the HpaI site lies to the right of the CauII site. Therefore the BstEII site will be 0.35 Md to the left as they are 0.7 Md apart. This orientation of the four sites was consistent with all the double digests.

A BstEII + HgiAI digest generated 0.3 + 2.6 Md fragments. The HgiAI site must be 0.3 Md to the left of the BstEII site (Fig 5.2) since a fragment of 0.4 Md was generated by a HgiAI + XbaI digest: a 1.0 Md fragment would be produced for the other position. All the other double digests confirmed the position of the HgiAI site.

The HgaI site was positioned in a similar manner. A BstEII + HgaI digest generated 0.52 + 2.35 Md fragments, therefore the HgaI site lies to the left of the BstEII site (Fig 5.2) since the opposite orientation would bring it very close to the HpaI site: 1.20 + 1.72 Md fragments were generated by a HpaI + HgaI digest. This position of the HgaI site brings it close to the XbaI site (0.18 Md) and a XbaI + HgaI digest confirmed this (Table 5.2).

Double digests of HpaI + PvuII, HpaI + BalI indicated that the PvuII and BalI sites were very close to the HpaI site (Table 5.2) and their precise position (Fig 5.2) was determined from the result of a double digest with CauII (Table 5.2).

The position of the BalI, HgaI, HgiAI and PvuII sites (Fig 5.2) was consistent with all the double digests carried out.

A diagramatic representation of the position of the fragments of pAB124 produced by HhaI, HindII, HpaII, HaeIII, ThaI, BclI and EcoRI is given in Fig 5.3.

Double digests with HhaI indicated that the large HhaI fragment (A) contained the HpaI and XbaI sites where the HpaI site was quite close (0.1 Md) to a HhaI site. Since the small HhaI fragment B (0.7 Md) was shown to contain the BstEII and CauII cleavage sites, (Table 5.3) only one orientation of the HhaI fragments was possible. The large fragment produced by a HhaI + HpaI digest lies to the right of the HpaI site with the small fragment (0.1 Md) to the left, otherwise HhaI (A) would contain the BstEII and CauII sites which was not the case. HhaI (B) was therefore positioned in the gap over the BstEII and CauII sites.

The HindII fragments were positioned in a similar manner,

only one orientation was again possible. One HindII site lies very close to the HpaI site since a HindII + HpaI digest showed no observable alteration in the size of HindII fragments (Table 5.3). The large HindII fragment (A) runs to the right from the HpaI site since it contained the XbaI site but not the CauII site. Thus HindII (B) lies over the CauII site in the position shown (Fig 5.3). The position of the HindII and HhaI fragments was consistent with all the double digests carried out.

The four restriction endonucleases, BstEII, CauII, HpaI, XbaI were used to map the three fragments produced by HpaII, HaeIII, ThaI, EcoRI and BclI and the results of the double digest experiments are shown in Table 5.3.

The double digests involving HpaII indicated that the large HpaII fragment (A) contained the HpaI site, while HpaII (B) the BstEII site and HpaII (C) the XbaI site. A CauII + HpaII digest showed no observable alteration in the size of HpaII fragments, thus these two sites are close together. To be consistent with this only one orientation of HpaII (A) is possible, where it lies to the right of the CauII site so containing the HpaI site. (Fig 5.3.) Therefore only one orientation of HpaII (C) is possible to fit it in the gap over the XbaI site. This resulted in HpaII (B) lying over the BstEII site and as expected running up to the CauII site.

Since it was shown that HaeIII (A) did not contain the HpaI site the larger fragment (1.24 Md) produced by a CauII + HaeIII digest lies to the left of the CauII site and so containing the BstEII and XbaI sites as confirmed by double digests. The HaeIII (C) fragment was shown to contain the HpaI site, although there is some inaccuracy in determining the molecular weights of the small fragments produced by a HaeIII + HpaI digest (0.1, 0.15 Md), the estimated values fit very well when locating HaeIII (C) next to HaeIII (A). Therefore HaeIII (B), which does not contain any of the single sites, lies in the gap to the right of HaeIII (C) Fig 5.3.

A double digest of ThaI and BstEII gave a slight (0.05 Md) reduction in the size of ThaI (B) indicating the two sites were close together. ThaI (B) also contains the CauII site and a double digest gave 0.42 and 0.23 Md fragments. Only one orientation is possible, the 0.42 Md fragment must lie to the left of the CauII site bringing it close (0.07 Md) to the BstEII site, since the other orientation would mean ThaI (B) contained the HpaI site which is not the case. The ThaI (A) fragment can now be positioned

as only one orientation is consistent with it containing the HpaI and XbaI sites. The small ThaI (C) therefore lies between ThaI (A) and ThaI (B) as shown in Fig 5.3.

The EcoRI fragments were positioned in a similar manner as only one orientation of EcoRI (A) is possible to be consistent with it containing the BstEII, CauII and HpaI sites but not the XbaI site which EcoRI (B) contains. A CauII + EcoRI digest generated 1.55 and 0.4 Md fragments from EcoRI (A) therefore the 1.55 Md fragment can only lie to the right of CauII site with the 0.4 Md fragment to the left. The EcoRI (B) fragment lies to the right of EcoRI (A) over the XbaI site in the orientation shown as an EcoRI + XbaI digest yields 0.33 and 0.29 Md fragments from EcoRI (B) and the distance from EcoRI (A) to the XbaI site is 0.35 Md. The remaining fragment EcoRI (C) therefore lies to the right of EcoRI (B) (Fig 5.3).

Only one orientation of the large BclI fragment (A) is possible since it was shown to contain the XbaI site but not the CauII, HpaI or BstEII sites. The location of this fragment places one BclI site very close to the HpaI site (Fig 5.3) a position that was confirmed by a BclI + HpaI digest which showed no alteration in the size of BclI fragments. The results in Table 5.3 show that the 0.68 Md Bcll fragment (B) contained the CauII site and 0.38 + 0.28 Md fragments were produced from a CauII + BclI digest. Only one orientation of fragment B was possible to bring a BclI site very close to the HpaI site. Therefore the third BclI fragment lies to the right of BclI (A) overlapping the BstEII site by 0.08 Md (Fig 5.3) as confirmed by a double digest of BstEII + BclI.

The results of digests with HphI (Table 5.3) clearly showed that the sum of all the fragments did not add up to 2.9 Md, indicating that there were probably additional fragments produced by HphI that were not detected in the gels. This was confirmed when mapping the 3 observed HphI sites as described below, and illustrated in Fig 5.4.

The large HphI fragment (A) was shown to contain the XbaI site and only one orientation of this fragment is possible (Fig 5.4) since it does not contain the BstEII, CauII or HpaI sites. The 0.65 Md HphI fragment contains the BstEII site and this fragment must lie to the right of HphI fragment A in the orientation shown as it does not contain the CauII site. Therefore 3 HphI sites have been located, however, the distance

between HphI fragment B and fragment A is 0.53 Md and a HphI + CauII digest showed that the 0.38 Md fragment must lie in this gap. The discrepancy in size of the gap and the fragment (0.15 Md) indicated that this region must contain additional undetected fragments. The 0.38 Md HphI fragment could be positioned in this gap since it was shown also to contain the PvuII site. A CauII + HphI digest indicated that one HphI site was quite close (0.06 Md) to the CauII site. Only one orientation of the 0.38 Md fragment was possible to be consistent with this and for it to contain the PvuII site. (Fig 5.4). This means there are at least 5 HphI sites, resulting in two new fragments (0.06 + 0.10 Md) that were not detected in gels.

The relative positions of all the mapped restriction endonuclease cleavage sites are shown in Fig 5.5.

Table 5.1. Restriction endonuclease cleavage sites in pABI24

Enzyme (Sequence)	No.	Fragments Size (Md)	Sum of fragment Sizes (Md)
AccI (GT/$^{AG}_{CT}$AC)	0		-
AluI (AG/CT)	>5	1.70, (0.25)[a], (0.20)[a], ( )[ac]	(2.60)
AvaI (G/G$^{A}_{T}$CC)	0		-
BalI (TGG/CCA)	1	2.90	2.90
BstEII (G/GTNACC)	1	2.90	2.90
EcoRII (/CC$^{A}_{T}$GG)	0		-
HaeII (PuGCGC/Py)	0		-
HgaI (GACGC)	1	2.90	2.90
HgiAI (G$^{A}_{T}$GC$^{A}_{T}$/C)	1	2.90	2.90
HpaII (C/CGG)	3	1.80, 0.70, 0.42	2.92
HphI (GGTGA)	3	1.70, 0.65, 0.40	2.75
MboII (GAAGA)	>6	0.90, 0.41, 0.45, 0.31, 0.20, ( )[c]	(2.27)
MnlI (CCTC)	>6	0.53, 0.27, 0.22, ( )[c]	(1.02)
PvuI (CGATCG)	0		-
PvuII (CAG/CTG)	1	2.90	2.90
RspI (?)	0		-
Sau3A (/GATC)	>6	0.90, 0.59, 0.48, (0.43)[a], ( )[c]	(2.83)
Sau96I (G/GNCC)	>6	0.80, 0.76, 0.54, 0.30, ( )[c]	(2.40)
SstII (CCGC/GG)	0		-
TacI/ThaI (CG/CG)	3	1.84, 0.65, 0.40	2.89
TaqI (T/CGA)	>8	1.05, (0.53)[a], (0.15)[ab], (0.09)[ab], (0.07)[ab], ( )[c]	(2.73)
XhoI (C/TCGAG)	0		-

Legend to Table 5.1

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using DNA isolate from B. subtilis (pAB124).

a, this fragment was over intense and assumed to be a "double".

b, this fragment was too small for an accurate molecular weight determination.

c, one or more fragments were detected but were too small for an estimation of molecular weight.

Table 5.2. Fragments produced by digestion of pAB124 with two Restriction Endonucleases having one cleavage site

Enzyme pair	Fragment size (Md) A	B	Sum of fragment sizes (Md)
BstEII + CauII	2.55	0.35	2.90
BstEII + HpaI	2.23	0.70	2.93
BstEII + XbaI	2.20	0.70	2.90
CauII + HpaI	2.57	0.35	2.92
CauII + XbaI	1.82	1.05	2.90
HpaI + XbaI	1.48	1.40	2.88
HgiAI + BstEII	2.60	0.31	2.91
HgiAI + CauII	2.23	0.65	2.88
HgiAI + HpaI	1.90	1.02	2.92
HgiAI + XbaI	2.50	0.40	2.90
HgaI + BstEII	2.35	0.52	2.87
HgaI + CauII	2.00	0.88	2.88
HgaI + HpaI	1.72	1.20	2.92
HgaI + XbaI	2.72	(0.20)[a]	2.92
PvuII + BstEII	2.25	0.66	2.91
PvuII + CauII	2.60	0.31	2.91
PvuII + HpaI	2.90	-	2.90
PvuII + XbaI	1.55	1.35	2.90
BalI + BstEII	2.25	0.65	2.90
BalI + CauII	2.60	0.30	2.90
BalI + HpaI	2.90	-	2.90
BalI + XbaI	1.57	1.32	2.89

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using pAB124 DNA isolated from B. subtilis.

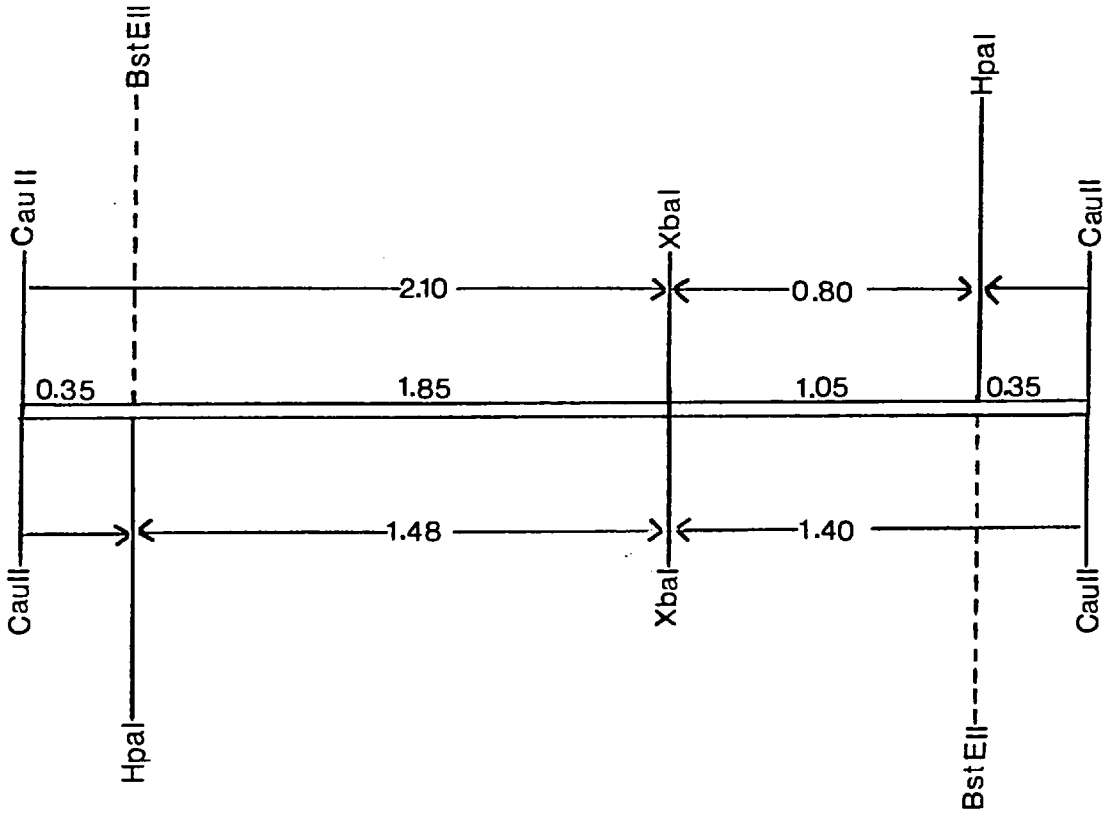
a, This fragment was too small for an accurate molecular weight estimation.

Figure 5.1. Orientation of CauII, BstEII, HpaI and XbaI Restriction Endonuclease cleavage sites in pAB124

Plasmid DNA was isolated from B. subtilis as described in Methods (2.7). Double digests involving BstEII, CauII, HpaI and XbaI and agarose-gel electrophoresis were carried out as described in Chapter 2 (2.15, 2.13) and the results shown in Table 5.2.

FIG.5.1

Orientation A



Orientation B (Actual)

Figure 5.2. Relative Position of BalI, BstEII, CauII, HgaI, HgiAI, HpaI, PvuII and XbaI Restriction Endonuclease Cleavage Sites in pAB124

The relative positions of the restriction endonuclease cleavage sites were determined from the results of double-digest experiments (Table 5.2) as described in Chapter 5 (5.1).

FIG 5.2

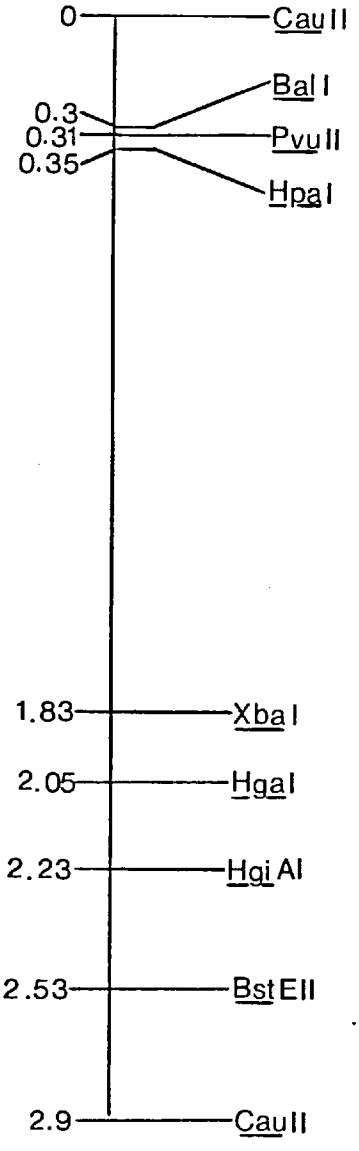

0
CauII
BalI
0.3
0.31
PvuII
0.35
HpaI
1.83
XbaI
2.05
HgaI
2.23
HgiAI
2.53
BstEII
2.9
CauII

Table 5.3. Fragments produced by digestion of pAB124 with two Restriction Endonucleases, one of which has more than one cleavage site in pAB124

Enzyme pair	Fragment sizes (Md) A	B	C	D	Sum of fragment sizes (Md)
HhaI	2.20	0.70			2.90
HhaI + BstEII	2.20	0.59	(0.12)[a]		2.91
HhaI + CauII	2.20	0.44	0.25		2.89
HhaI + XbaI	1.59	0.71	0.60		2.90
HhaI + HpaI	2.10	0.70	(0.10)[a]		2.90
HindII	2.30	0.62			2.92
HindII + BstEII	2.30	0.55	(0.05)[b]		2.90
HindII + CauII	2.30	0.33	0.28		2.91
HindII + XbaI	1.50	0.78	0.62		2.90
HindII + HpaI	2.30	0.61	-		2.91
HaeIII	1.50	1.18	0.25		2.93
HaeIII + BstEII	1.18	0.89	0.59	0.25	2.91
HaeIII + CauII	1.24	1.18	0.25	0.24	2.91
HaeIII + XbaI	1.30	1.18	0.25	(0.18)[a]	2.91
HaeIII + HpaI	1.51	1.19	(0.16)[a]	(0.10)[a]	2.96
HpaII	1.80	0.70	0.42		2.92
HpaII + BstEII	1.80	0.42	0.35	0.32	2.89
HpaII + CauII	1.80	0.70	0.42	-	2.92
HpaII + XbaI	1.80	0.70	0.38	(0.05)[b]	2.93
HpaII + HpaI	1.45	0.72	0.42	0.35	2.94
ThaI	1.84	0.65	0.40		2.90
ThaI + BstEII	1.83	0.62	0.40	(0.05)[b]	2.90
ThaI + CauII	1.80	0.42	0.40	0.23	2.88
ThaI + XbaI	1.60	0.65	0.40	0.26	2.91
ThaI + HpaI	1.75	0.65	0.40	(0.10)[a]	2.90
EcoRI	1.95	0.61	0.33		2.89
EcoRI + BstEII	1.90	0.60	0.33	(0.05)[b]	2.90
EcoRI + CauII	1.55	0.60	0.40	0.33	2.88
EcoRI + XbaI	1.95	(0.33)[c]	0.29	-	2.88
EcoRI + HpaI	1.20	0.75	0.60	0.32	2.87

Table 5.3 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	Sum of fragment sizes of (Md)
BclI	1.63	0.68	0.58		2.89
BclI + BstEII	1.63	0.68	0.50	(0.08)[b]	2.89
BclI + CauII	1.63	0.58	0.38	0.28	2.87
BclI + XbaI	1.44	0.68	0.58	0.21	2.91
BclI + HpaI	1.63	0.68	0.58	-	2.89
BclI + HgiAI	1.63	0.68	0.38	0.21	2.90
HphI	1.70	0.65	0.40		2.75
HphI + BstEII	1.70	0.45	0.40	0.20	2.76
HphI + CauII	1.70	0.65	0.34	(0.06)[b]	2.75
HphI + XbaI	1.45	0.65	0.40	0.25	2.75
HphI + HpaI	1.70	0.65	0.40	-	2.75
HphI + PvuII	1.70	0.65	0.35	(0.05)[b]	2.75

Double digests and electrophoresis were carried out as described in Methods (2.13, 2.14) using pAB124 DNA isolated from B. subtilis.

a, this fragment was detected but was too small for an accurate molecular weight determination.

b, this fragment was not detected but its presence can be predicted.

c, this fragment was "over intense" and assumed to be a "double".

Figure 5.3. Relative Positions of Fragments Produced by Digestion of pAB124 with Several Restriction Endonucleases

The relative position of the fragments was determined from the results of double-digest experiments (Table 5.3) as described in Chapter 5 (5.1).

FIG. 5.3

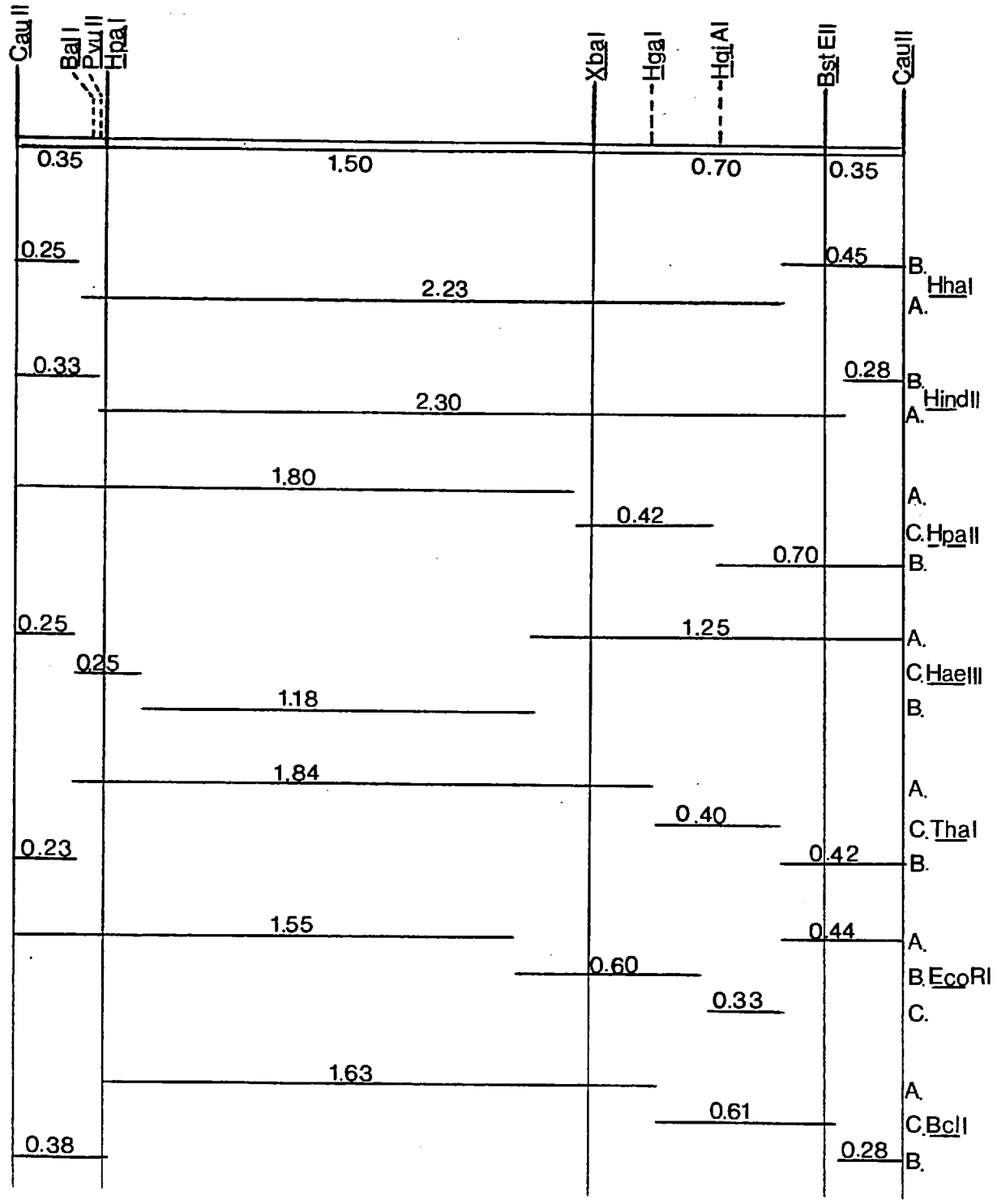

CauII
BalI
PvuII
HpaI
XbaI
HgaI
HgiAI
BstEII
CauII
0.35
1.50
0.70
0.35
0.25
0.45
B.
2.23
HhaI
A.
0.33
0.28
B.
2.30
HindII
A.
1.80
A.
0.42
C.HpaII
0.70
B.
0.25
1.25
A.
0.25
C.HaeIII
1.18
B.
1.84
A.
0.40
C.TaqI
0.23
0.42
B.
1.55
0.44
A.
0.60
B.EcoRI
0.33
C.
1.63
A.
0.61
C.BclI
0.38
0.28
B.

Figure 5.4. Relative Position of Fragments Produced by Digestion of pAB124 with HphI Restriction Endonuclease

The relative position of the HphI fragments was determined from the results of double-digest experiments (Table 5.3) as described in Chapter 5 (5.1).

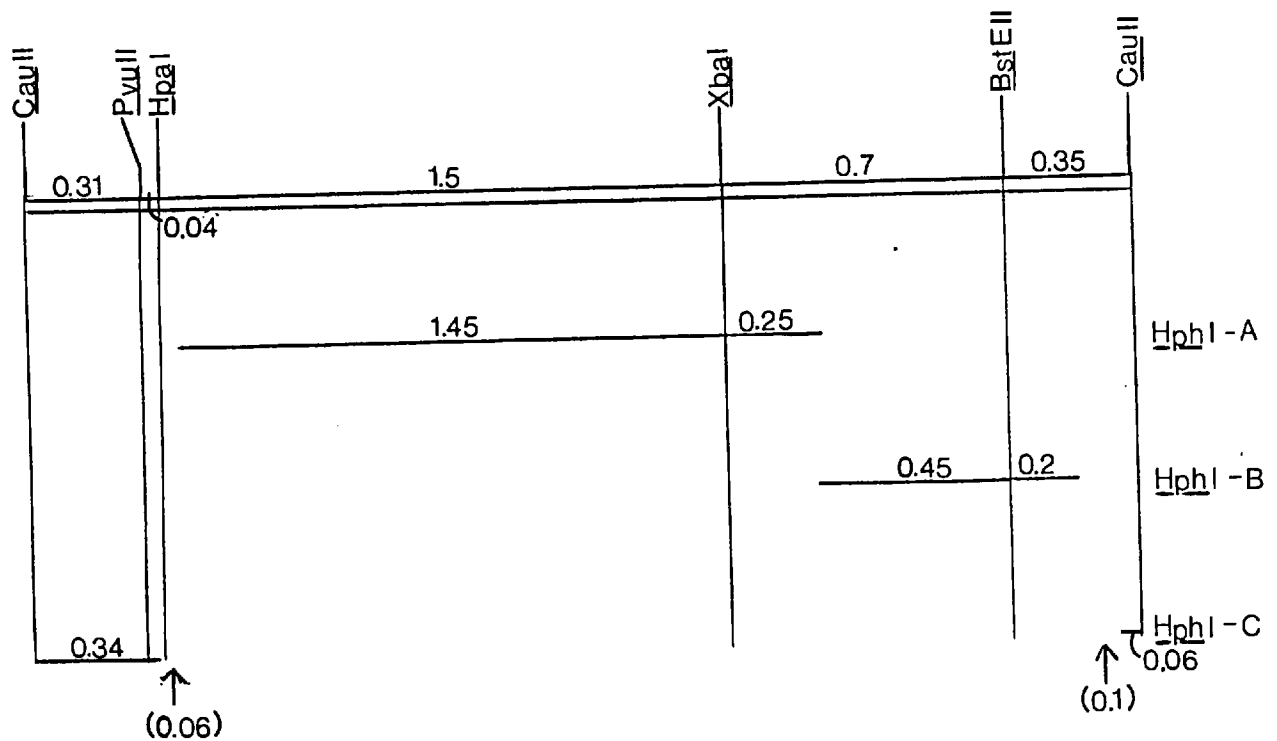

CauII
PvuII
HpaI
XbaI
BstEII
CauII
0.31
1.5
0.7
0.35
0.04
1.45
0.25
HphI-A
0.45
0.2
HphI-B
HphI-C
0.34
0.06
(0.06)
(0.1)

FIG 5.4

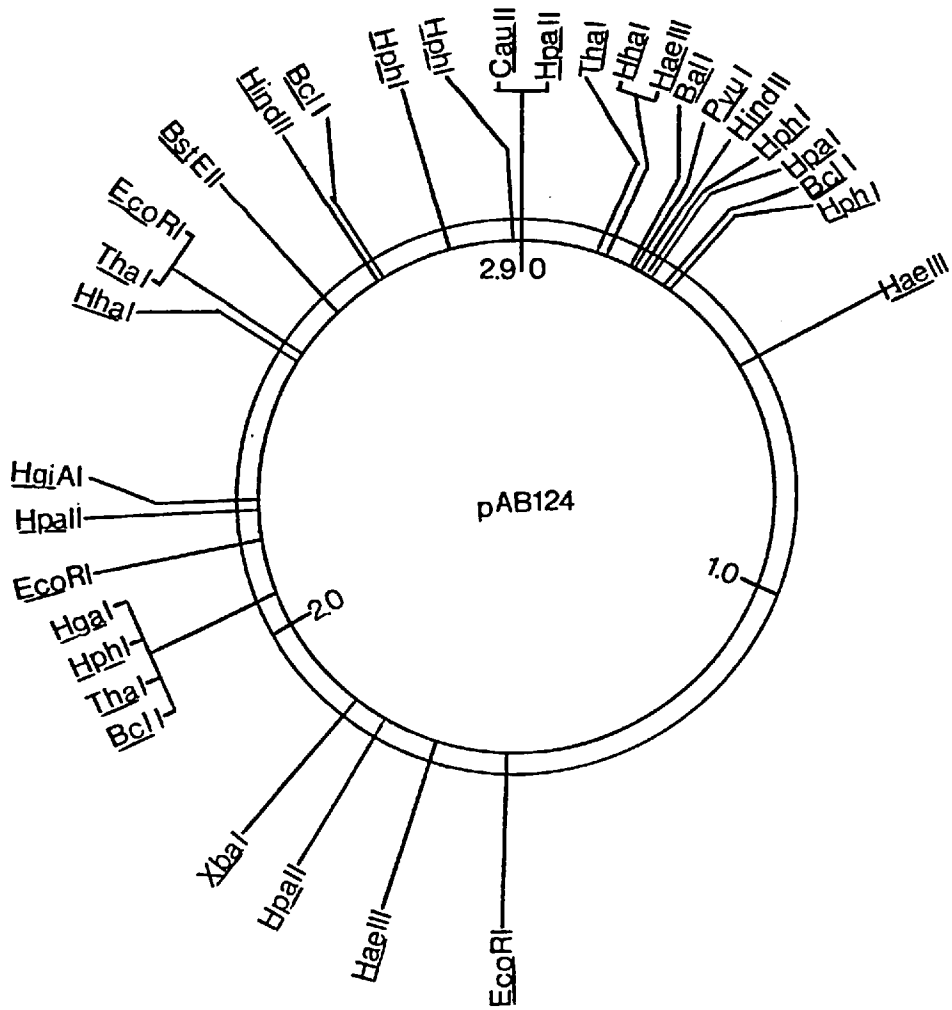
Figure 5.5. Restriction Endonuclease Cleavage Site map of pAB124

The restriction endonuclease cleavage site map was constructed as described in Chapter 5 (5.1) from data shown in Tables 5.1, 5.2, 5.3.

Map distances

CauII & HpaII, 0/2.90; ThaI, 0.23; HhaI & HaeIII, 0.25; BalI, 0.30; PvuII, 0.31; HindII, 0.33; HphI, 0.34; HpaI, 0.35; BclI, 0.38; HphI, 0.40; HaeIII, 0.53; EcoRI, 1.55; HaeIII, 1.68; HpaII, 1.80; XbaI, 1.85; HgaI, BclI, ThaI & HphI, 2.05; EcoRI, 2.15; HpaII, 2.20; HgiAI, 2.23; HhaI, 2.45; ThaI & EcoRI, 2.47; BstEII, 2.55; HindII, 2.62; BclI, 2.63; HphI, 2.74; HphI, 2.84.

FIG. 5.5


pAB124
2.9/0
1.0
2.0
HgiAI
HpaII
EcoRI
HgaI
HphI
ThaI
BclI
XbaI
HpaII
HaeIII
EcoRI
HaeIII
HphI
HphI
CauII
HpaII
ThaI
HhaI
HaeIII
BalI
PvuI
HindIII
HphI
HpaI
BclI
HphI
BclI
HindIII
BstEII
EcoRI
ThaI
HhaI

5.2. Cloning of EcoRI fragments of pAB124

The restriction endonuclease EcoRI produced three fragments in pAB124 (Table 5.3), of 1.95 Md, 0.61 Md and 0.33 Md, and if these fragments are cloned in another plasmid vector it would be possible to identify which of the fragments are required for tetracycline resistance.

A Staphylococcal plasmid pUB1654 (Fig 5.6) conferring streptomycin and neomycin resistance was used, where a single EcoRI cleavage site in the plasmid lies within the streptomycin resistance gene (A. Docherty, Pers. Comm.). It would therefore be anticipated that insertion of pAB124 EcoRI fragments at this site would destroy Sm^r, resulting in a Neo^r Sm^s phenotype of pUB1654.

Both plasmids (1 µg) were digested with EcoRI and mixed. After heat inactivation they were ligated with T4-DNA ligase as described in Methods (2.14, 2.16). The recombinant molecules were then transformed into competent cells of B. subtilis IG20 (trpC2, r^-m^-) and Tc^r Neo^r clones selected on TSBA plates containing neomycin and tetracycline at 12 $\mu g.ml^{-1}$; 3×10^2 transformants per µg DNA were obtained. Clones were then transferred to streptomycin plates (100 $\mu g.ml^{-1}$) by replica plating (Collins & Lyne, 1970) and Sm^s clones identified. Approximately 35% of transformants were $Tc^r.Neo^r.Sm^s$ and plasmid DNA was extracted from several of these clones (Methods 2.9.2) and analysed with EcoRI (Plate 5.1). The results of the cloning experiment are summarised in Table 5.4 and show that the minimum requirement for tetracycline resistance was the EcoRI fragment A of pAB124. Although this fragment (1.95 Md) confers a Tc^r phenotype when cloned in pUB1654 it may not contain the replicon of pAB124. The presence of the replicon in this fragment was tested by removing it (with EcoRI) from the recombinant molecule (pHI) and attempting to construct a viable Tc^r plasmid by circularisation with T_4-DNA ligase.

Plasmid DNA (1 µg) of pHI, pHII, pHIII, pHIV, representing all combinations of pAB124 EcoRI fragments cloned in pUB1654 (Table 5.4) was digested to completion with EcoRI and after heat inactivation of EcoRI ligated with T_4-DNA ligase (Chapter 2; 2.14, 2.16). The DNA was then transformed into competent

B. subtilis IG20 cells and Tc^r transformants selected on TSBA containing tetracycline (12 $\mu g.ml^{-1}$). Clones were then replica plated (Collins & Lyne, 1970) on to TSBA containing neomycin (12 $\mu g.ml^{-1}$) and streptomycin (100 $\mu g.ml^{-1}$); and $Tc^r.Sm^s.Neo^s$. clones identified. These clones could not therefore be pUB1654 Tc^r recombinants. Plasmid DNA was isolated from these clones (Methods 2.9.2) and digested with EcoRI (Methods 2.14). The results are summarised in Table 5.5 and show that two types of plasmids were obtained on the basis of EcoRI digestion. A small plasmid (1.95 Md) termed pAB224, containing 1 EcoRI site and a 2.3 Md plasmid (pAB524) containing 2 EcoRI sites producing fragments of 1.95 Md + 0.33 Md. On the basis of size and EcoRI digestion, fragment A of an EcoRI digest of pAB124 was circularised to produce a viable Tc^r plasmid (pAB224) and fragments of A + C of a similar digest were also circularised to produce a viable Tc^r plasmid (pAB524), Fig 5.7. Both plasmids were characterised with restriction endonucleases to confirm they were deletion variants of pAB124.

Figure 5.6. Restriction Endonuclease Cleavage Site Map of pUB1654
(A. Docherty, unpublished)

Map distances

EcoRI, 0/3.75; HindIII, 0.04; HpaII, 0.22; HindII, 1.30; HpaII, 1.31; BglII, 1.51; HindII, 1.56; HpaII, 1.75; HpaII, 2.25; BamHI, 2.55; XbaI, 2.75; HpaII, 2.83.

FIG. 5.6

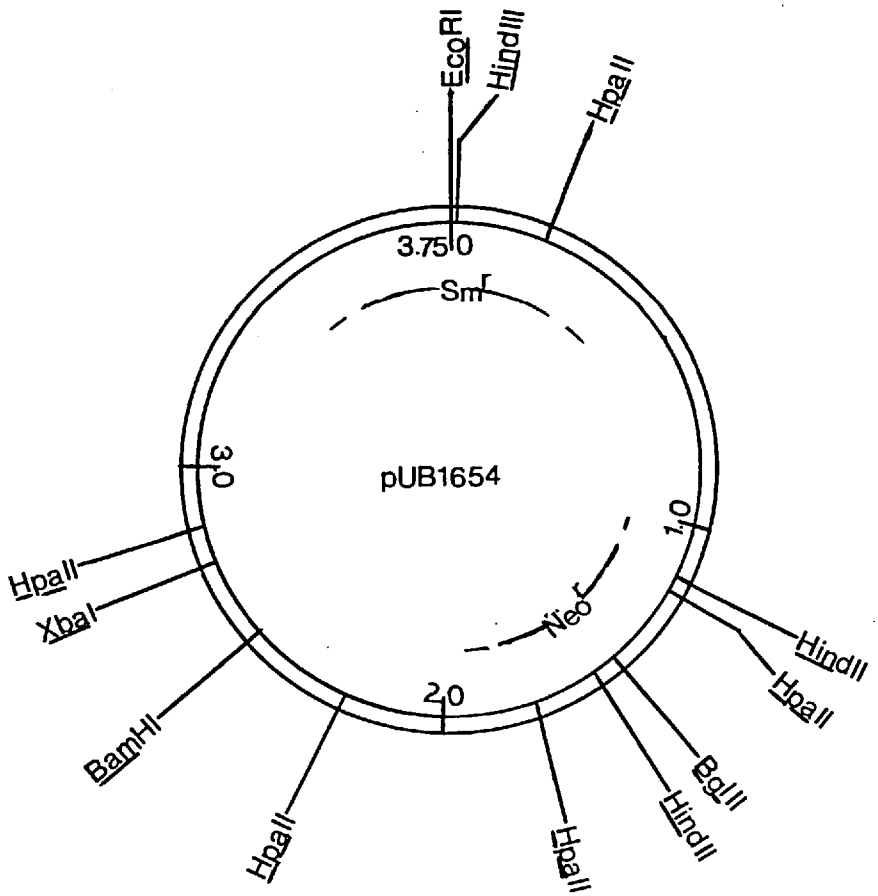
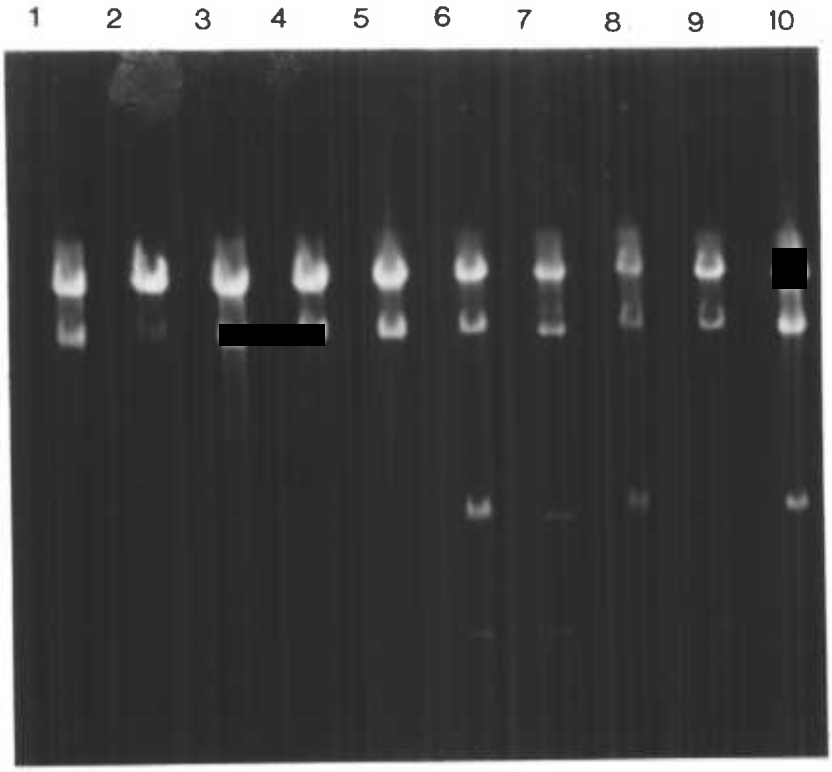

EcoRI
HindIII
HpaII
3.75 0
Sm^r
3,0
pUB1654
1,0
HpaII
XbaI
Neo^r
HindII
HpaII
2,0
BamHI
HpaII
HpaII
BglII
HindII

Plate 5.1. Agarose-gel of Plasmid DNA from Cleared Lysates of *B. subtilis* $Neo^r Tc^r Sm^S$ clones (5.2)



1: Clone 1 (pUB1654 + pAB124 EcoRI fragment A).
2: pUB1654 in *B. subtilis* ($Neo^r Sm^r$).
3: Clone 5 (pUB1654 + pAB124 EcoRI fragment A).
4: Clone 8 (" " " " A).
5: Clone 9 (" " " " A).
6: Clone 11 (" " " " A+B+C).
7: Clone 13 (" " " " A+B+C).
8: Clone 20 (" " " " A+B+C).
9: Clone 25 (" " " " A+C).
10: Clone 30 (" " " " A+B).

Plasmid DNA was extracted from $Neo^r Tc^r Sm^s$ transformants of *B. subtilis*, digested with EcoRI and subjected to agarose-gel electrophoresis as described in Chapter 2 (2.9., 2.14., 2.13.).

Table 5.4. Restriction Endonuclease analysis of recombinant plasmids of pAB124 EcoRI fragments and pUB1654

Clones identified No.	Plasmid size (Md)	EcoRI digestion Fragment size (Md)	pAB124 fragment inserted
10	6.0, pHI	(4.00)[a]	
		1.95	A
3	6.55, pHII	(4.00)[a]	
		1.95	A
		0.61	B
2	6.30, pHIII	(4.00)[a]	
		1.95	A
		0.33	C
4	6.90, pHIV	(4.00)[a]	
		1.95	A
		0.61	B
		0.33	C

Plasmid DNA was extracted from $Neo^{r}Tc^{r}Sm^{s}$ clones and digested with EcoRI as described in Methods (2.9.2., 2.14).

a, This fragment corresponds to pUB1654, with an estimated molecular weight of 4.0 Md.

Table 5.5. EcoRI analysis of tetracycline resistance plasmids derived from pAB124: pUB1654 recombinant molecules

DNA source[a]		Tetracycline resistance plasmids			
Recombinant	pAB124 EcoRI fragment present	No. Clones[b]	Size (Md)	EcoRI fragments (Md)	pAB124 EcoRI fragments present
pHI	A	10	1.95	1.95	A (pAB224)
pHII	A+B	10	1.95	1.95	A (pAB224)
pHIII	A+C	7	1.95	1.95	A (pAB224)
		3	2.30	1.95) 0.33)	A+C (pAB524)
pHIV	A+B+C	8	1.95	1.95	A (pAB224)
		2	2.30	1.95) 0.33)	A+C (pAB524)

Plasmid DNA was extracted from $Tc^r.Neo^s.Sm^s$. clones and digested with EcoRI as in Methods (2.9.2, 2.14).

a, Recombinant plasmids of EcoRI fragments of pAB124 and pUB1654 (Table 5.4)

b, 10 clones of each plasmid type was examined.

5.3. Characterisation of pAB224

The restriction endonuclease cleavage sites present in pAB224 (Table 5.6 and Table 5.8) were consistent with that expected from the circularisation of EcoRI fragment A of pAB124 (Fig 5.7). Confirmation of this was obtained by double-digests using restriction endonucleases having one site in pAB224 (Table 5.7). The results were entirely consistent with the calculated values based on circularisation of EcoRI (A) of pAB124 (Fig 5.7), and a circular map of pAB224 is shown in Fig 5.8.

This small plasmid is potentially a useful vector for genetic manipulations since there are 9 restriction endonucleases having just one site in pAB224, the single EcoRI being particularly useful.

Only the CauI fragments can be easily mapped using double-digests (Nathans & Smith, 1975; Roberts, 1976) since the other enzymes produced some very small fragments (Table 5.8). The results of these double-digest experiments are shown in Table 5.9.

The large CauI fragment (A) contained the HhaI and HpaI cleavage sites only. Thus only one orientation is possible, where the 1.0 Md fragment from a CauI + HpaI digest lies to the right of the HpaI site (Fig 5.9) and the 0.2 Md fragment to the left. The CauI B fragment was positioned to the right of CauI A since a BstEII digest produced two 0.25 Md fragments. This leaves CauI C positioned over the CauII site as shown (Fig 5.9). The position of these fragments was consistent with all the double digests.

The location of the tetracycline resistance genes in pAB224 can be determined by cloning another plasmid eg, pUB110, at the various single sites for restriction endonucleases and identifying Neo^r Tc^s or Neo^r Tc^r transformants.

Insertion of DNA into the EcoRI site of pUB110 does not inactivate the neomycin resistance genes (Gryczan & Dubnau, 1978) and this site was used for cloning. pUB110 DNA (20 µg) was digested with EcoRI (Methods 2.14), extracted with phenol followed by three diethyl ether extractions and precipitated with ethanol (Methods 2.7.6). The DNA was resuspended in 10 mM Tris-HCl pH 7.5 at a concentration of 500 $\mu g.ml^{-1}$ and poly-dC

tails attached with terminal transferase (Uniscience Ltd., Cambridge) based on the method of Lobban & Kaiser (1973).

Six samples of pAB224 (2 µg) were digested with one of the following enzymes; EcoRI, HpaI, HpaII, HhaI, HaeIII, ThaI (Methods 2.14) extracted with phenol followed by three diethyl ether extractions and precipitated with ethanol (Methods 2.7.6). The DNA was resuspended in buffer as above and poly-dG tails attached with terminal transferase (Lobban & Kaiser, 1973).

The tailed pUB110 DNA was divided equally into 6 tubes and one of the tailed pAB224 samples added to one tube, for all six samples. The DNA was ligated overnight with T_4-DNA ligase as described in Methods (2.16) and transformed into competent B. subtilis IG20 cells. No transformants (Neo^r) were obtained with any of the samples however 1.2×10^4 Tc^r transformants $\mu g\ DNA^{-1}$ were obtained with undigested pAB224 transformed using the same competent cells.

The reason for this failure to obtain any transformants with the 'tailed' recombinant molecules was later explained by the work of Canosi, et. al., (1978), who found that oligomers were required for transformation in competent cells. The monomeric species of DNA had one thousandth the transforming activity of a dimer of the same molecule. The production of recombinant molecules by 'tailing' with terminal transferase so each species has a complementary tail excludes the formation of dimers. Each individual species of DNA used will have the same tail on each 5' terminus, ie non-complementary. This problem can be overcome by using transformation of B. subtilis protoplasts which have no requirement for plasmid oligomers (Chang & Cohen, 1979). However, time did not allow for repeating this experiment and using protoplast transformation.

Figure 5.7. Location of pAB224 and pAB524 within the Restriction Endonuclease Cleavage Site Map of pAB124

Deletion variants of pAB124 (pAB224 and pAB524) were constructed as described in Chapter 5 (5.2) and restriction endonuclease cleavage site maps constructed (5.3; 5.4). Map distances are shown in Fig 5.5.

FIG. 5.7

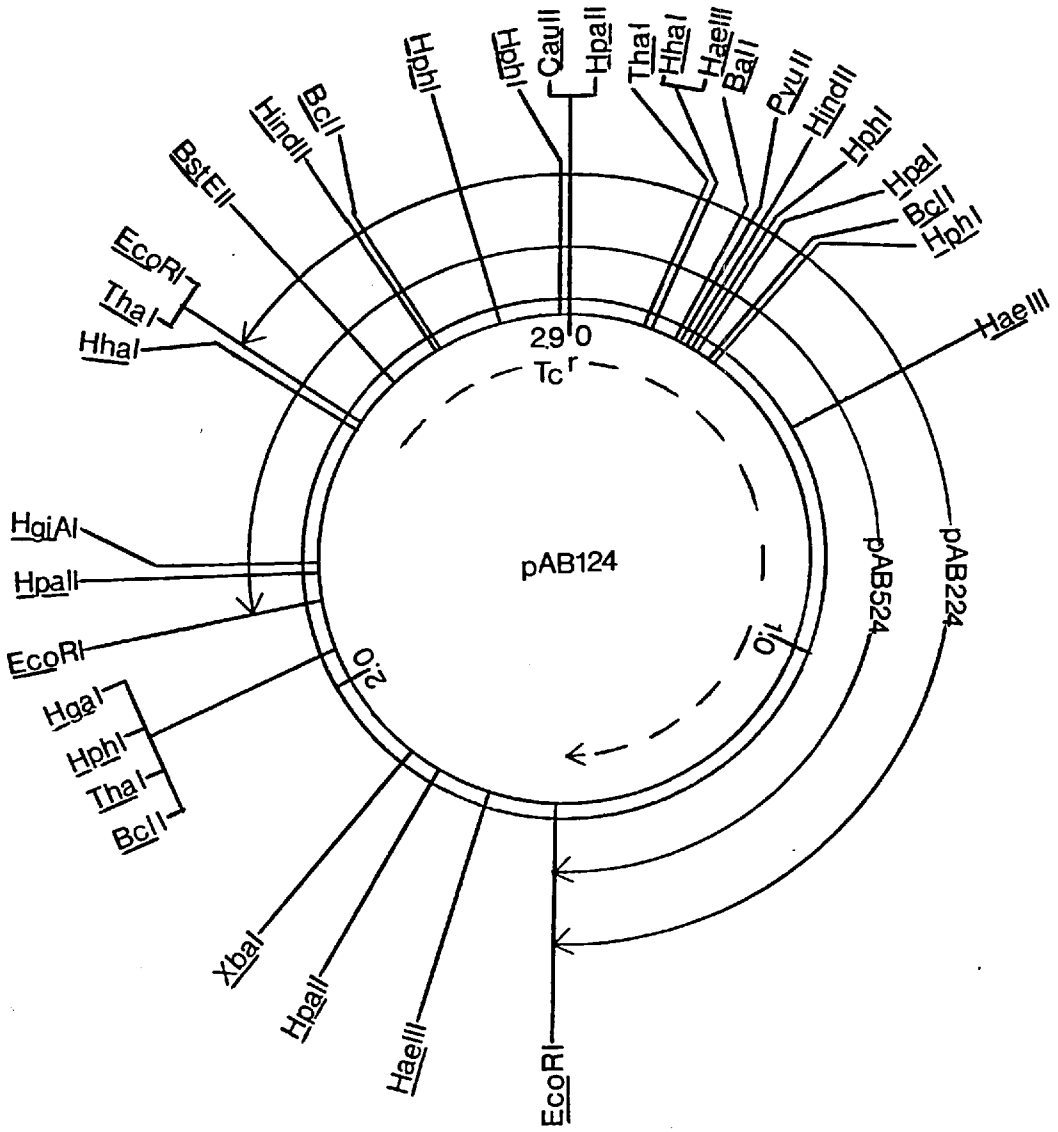

pAB124
2.9
0
Tc^r
ori
rio
HgiAI
HpaII
EcoRI
HgaI
HphI
ThaI
BclI
XbaI
HpaII
HaeIII
EcoRI
HaeIII
pAB224
pAB524
HaeIII
HpaI
BclI
HphI
HphI
PvuII
HindIII
BalI
HaeIII
ThaI
HhaI
CauII
HpaII
HphI
HphI
BclI
HindII
BstEII
EcoRI
ThaI
HhaI

Table 5.6. Restriction Endonuclease cleavage sites in pAB124 and deletion derivatives pAB224, pAB524

Enzyme (sequence)	No. fragments		
	pAB224	pAB524	pAB124
BalI (TGG/CCA)	1	1	1
BclI (T/GATCA)	2	2	3
BstEII (G/GTNACC)	1	1	1
CauII ($CC^{C}_{G}GG$)	1	1	1
EcoRI (G/AATTC)	1	2	3
HaeIII (GG/CC)	2	2	3
HhaI (GCG/C)	1	2	2
HindII (GTPy/PuAC)	2	2	2
HpaI (GTT/AAC)	1	1	1
HpaII (C/CGG)	1	2	3
ThaI (CG/CG)	1	2	3
PvuII (CAG/CTG)	1	1	1
HgiAI ($G^{A}_{T}GC^{A}_{T}$/C)	0	1	1
HgaI (GACGC)	0	0	1
XbaI (T/TCGAG)	0	0	1

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14).

Table 5.7. Fragments produced by digestion of pAB224 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md)			
	Actual		Calculated*	
	A	B	A	B
BstEII + CauII	1.60	0.35	1.60	0.35
BstEII + EcoRI	1.90	(0.07)[a]	1.88	0.07
BstEII + HhaI	1.35	0.60	1.35	0.60
BstEII + HpaI	1.25	0.70	1.25	0.70
BstEII + HpaII	1.60	0.35	1.60	0.35
BstEII + ThaI	1.25	0.70	1.25	0.70
CauII + EcoRI	1.52	0.42	1.53	0.42
CauII + HhaI	1.71	0.25	1.70	0.25
CauII + HpaI	1.60	0.35	1.60	0.35
CauII + HpaII	1.95	-	1.95	-
CauII + ThaI	1.58	0.35	1.60	0.35
EcoRI + HhaI	1.28	0.66	1.28	0.67
EcoRI + HpaI	1.16	0.77	1.18	0.77
EcoRI + HpaII	1.53	0.42	1.53	0.42
EcoRI + ThaI	1.26	0.67	1.28	0.67
HhaI + HpaI	1.86	(0.10)[b]	1.85	(0.10)[b]
HhaI + HpaII	1.70	0.25	1.70	0.25
HhaI + ThaI	1.95	-	1.95	-
HpaI + HpaII	1.60	0.35	1.60	0.35
HpaI + ThaI	1.85	(0.10)[b]	1.85	(0.10)[b]
HpaII + ThaI	1.70	0.25	1.70	0.25

Double digests and agarose-gel electrophoresis was carried out as described in Methods (2.13, 2.14) using pAB224 DNA isolated as described in Chapter 5 (5.2).

a, This fragment was too small to be detected.

b, This fragment was detected, but was too small for an accurate molecular weight determination.

*, Values calculated from Fig. 5.6 assuming pAB224 was the circularised EcoRI fragment A of pAB124.

Table 5.8. Fragments of pAB224 produced by digestion with Restriction Endonucleases

Enzyme (sequence)	Fragments		Sum of fragment sizes (Md)
	No.	Size (Md)	
AluI (AG/CT)	3	1.43 (0.52)[a]	1.95
CauI ($GG^{A}_{T}CC$)	3	1.20, 0.50, 0.25	1.95
HinfI (G/ANTC)	>3	0.70, 0.48, 0.45 ( )[c]	(1.63)
Sau3A (/GATC)	4	1.23, 0.38, 0.21 (0.14)[b]	1.96
TaqI (T/CGA)	>4	1.03, 0.31, 0.26, 0.25	(1.85)

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using pAB224 DNA isolated as described in Chapter 5 (5.2).

a, this fragment was over intense and assumed to be a "double".

b, this fragment was too small for an accurate molecular weight determination.

c, it was assumed there must be one, or more, undetected fragments present due to the low value for the sum of the fragment sizes.

Table 5.9. Fragments produced by digestion of pAB224 with two Restriction Endonucleases, one of which is CauI

Enzyme pair	Fragment size (Md) A	B	C	D	Sum of fragment sizes (Md)
CauI	1.20	0.50	0.25		1.95
CauI + BstEII	1.20	(0.25)[a]			1.95
CauI + CauII	1.20	0.50	(0.15)[b]	(0.10)[b]	1.95
CauI + HpaI	1.00	0.50	0.25	(0.20)[b]	1.95
CauI + EcoRI	1.20	0.35	0.25	(0.15)[b]	1.95
CauI + HhaI	1.10	0.50	0.25	(0.10)[b]	1.95

Double digests and agarose-gel electrophoresis was carried out as described in Methods (2.13, 2.14) using pAB224 DNA isolated as described in Chapter 5 (5.2).

a, this fragment was very intense and assumed to be a "triple"

b, these fragments were too small for an accurate molecular weight determination.

Figure 5.8. Restriction Endonuclease Cleavage Site Map of pAB224

The restriction endonuclease cleavage site map was constructed as described in Chapter 5 (5.3).

Map distances

CauII & HpaII, 0/1.95; CauI, 0.15; ThaI, 0.23; HhaI & HaeIII, 0.25; BalI, 0.30; PvuII, 0.31; HindII, 0.33; HphI, 0.34; HpaI, 0.35; BclI, 0.38; HphI, 0.40; HaeIII, 0.53; CauI, 1.35; EcoRI, 1.55; BstEII, 1.60; HindII, 1.67; BclI, 1.68; HphI, 1.79; HphI, 1.89.

FIG. 5.8

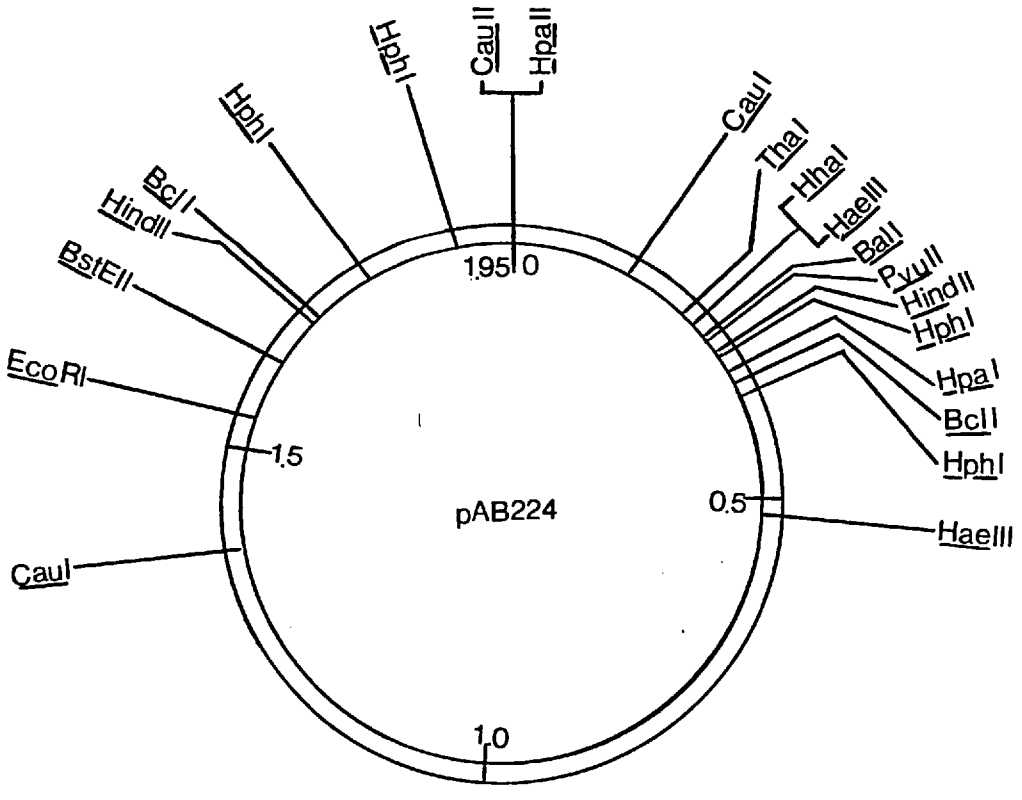
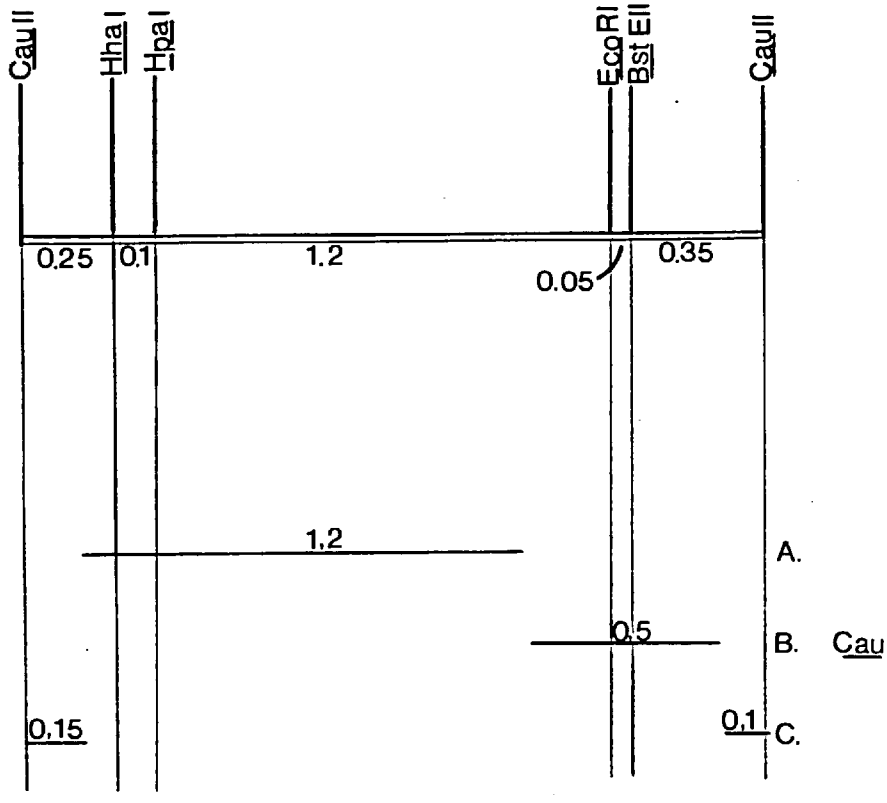

CauII
HpaII
HphI
HphI
BclI
HindII
BstEII
EcoRI
CauI
CauI
ThaI
HhaI
HaeIII
BalI
PvuII
HindII
HphI
HpaI
BclI
HphI
HaeIII
1.95
0
1.5
0.5
1.0
pAB224

Figure 5.9. Relative Position of Fragments Produced by Digestion of pAB224 with CauI Restriction Endonuclease

The relative position of the CauI fragments was determined from the results of double-digest experiments (Table 5.9) as described in Chapter 5 (5.3).

FIG. 5.9


CauII
HhaI
HpaI
EcoRI
BstEII
CauII
0.25
0.1
1.2
0.05
0.35
1.2
A.
0.5
B. CauI
0.15
0.1
C.

5.4. Characterisation of pAB524

The restriction endonuclease cleavage sites present in pAB524 (Table 5.6) was consistent with it being the circularised EcoRI fragments (A + C) of pAB124 (Fig 5.7). However, there are two possible orientations of the fragments within pAB524 (Fig 5.10), each clone containing pAB524 (Table 5.5) was examined.

The two orientations can easily be identified since each will give different sized fragments when digested with HpaII or HhaI and all the clones contained the plasmid of orientation A (Table 5.10), identical to that of pAB124 (Fig 5.7). This plasmid is not as useful for gene-cloning as pAB224 nor pAB124 since it has 2 EcoRI sites and only 6 restriction endonucleases with 1 cleavage site, all of which produce blunt-ended termini.

A third deletion variant of pAB124 could be constructed in a similar manner to pAB224 and pAB524, by the circularisation of EcoRI fragments A + B of pAB124 from pHII (Table 5.4) so retaining the single XbaI site. However repeated ligations of EcoRI cut pHII failed to generate this plasmid on transformation into B. subtilis IG20, only circularisation of fragment A (pAB224) and insertion of fragment A into pUB1654 to form pHI was obtained (Table 5.5). This was very surprising since it would be expected circularisation of pAB124 EcoRI fragments A + B would be approximately as efficient as circularisation of EcoRI A + C to form pAB524 (Table 5.5).

Figure 5.10. Possible Orientations of the Fragments Produced by Digestion of pAB524 with EcoRI Restriction Endonuclease

Plasmid pAB524 was constructed as described in Chapter 5 (5.2) and the orientation of the two EcoRI fragments determined from restriction endonuclease digests (Table 5.10).

FIG. 5.10

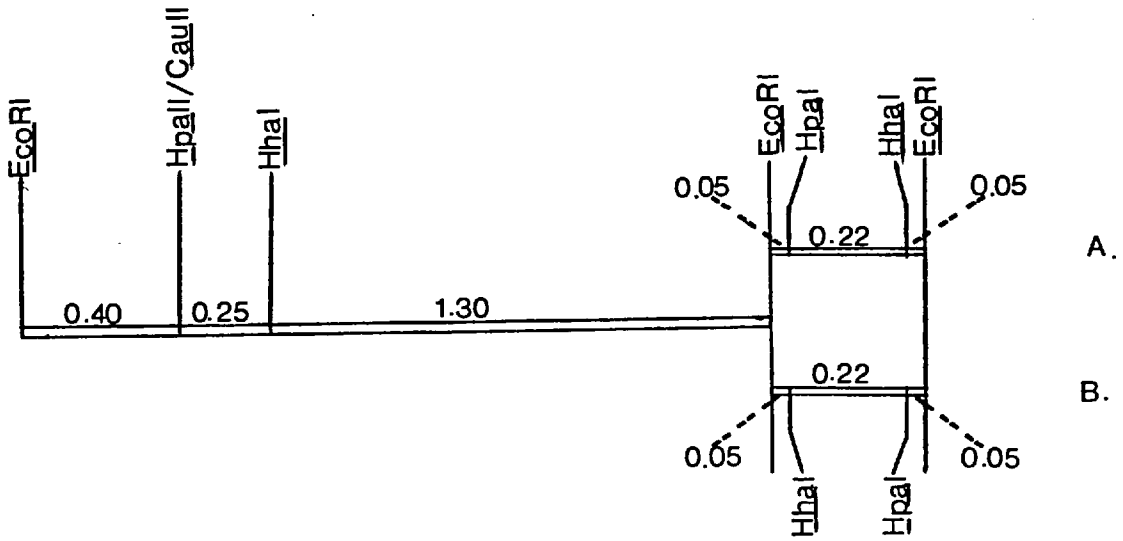

EcoRI
HpaII/CauII
HhaI
0.40
0.25
1.30
EcoRI
HpaI
HhaI
EcoRI
0.05
0.22
0.05
A.
0.22
B.
0.05
0.05
HhaI
HpaI

Table 5.10. Digestion of pAB524 with HpaII and HhaI Restriction Endonucleases

Orientation*	Fragment sizes (Md)	
	HpaII	HhaI
A	1.60	1.57
	0.67	0.70
B	1.82	1.35
	0.45	0.92
Observed	1.60	1.58
	0.68	0.72

Digestions and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using pAB524 DNA isolated as described in Chapter 5 (5.2).

*See Fig. 5.9.

5.5. Cloning of BclI fragments of pAB124

The cloning of the EcoRI fragments of pAB124 proved very useful from two aspects. It located the Tc^r genes in a 1.95 Md region and allowed the construction of deletion plasmids pAB224 and pAB524. A similar experiment was therefore attempted with the BclI fragments (1.63, 0.68, 0.58 Md) of pAB124; two BclI sites lying within the 1.95 Md Tc^r region (Fig 5.6).

The Staphylococcal plasmid pUB110 (Neo^r) was used, which has a single BamHI cleavage site which does not lie within the neomycin resistance gene (Gryczan & Dubnau, 1978). Since BclI produces the same cohesive terminus as BamHI (see appendix Fig 1) recombinant molecules of pUB110 and the BclI fragments of pAB124 can be constructed.

pUB110 (1 µg) was digested with BamHI and then heated to 66°C for 10 min to inactivate the enzyme. pAB124 (5 µg) was digested with BclI. Since BclI is active at 66°C the enzyme was removed by phenol extraction. Trace amounts of phenol were then removed by several diethyl ether extractions (Methods 2.7.6) before mixing the two species of DNA. After ligation with T_4 DNA-ligase (Methods 2.16) the recombinant molecules were transformed into competent B. subtilis IG20 cells and Tc^r Neo^r transformants selected on TSBA containg tetracycline (12 $\mu g.ml^{-1}$) or neomycin (12 $\mu g.ml^{-1}$). All the transformatns were then tested for the $Neo^r.Tc^r$ phenotype by replica plating (Collins & Lyne, 1970). No $Neo^r.Tc^r$ clones were identified, only Neo^r (2.5×10^2 per µgDNA) and Tc^r (15 per µg DNA) transformants were obtained.

This result could be explained if all 3 BclI fragments were required for tetracycline resistance. Two of the BclI sites lie within the 1.95 Md fragment associated with Tc^r (Fig 5.7). If they are situated within the Tc^r genes or a promotor for this region then all three BclI fragments would be required. The chance of ligating all 3 BclI fragments in the correct orientations within the BamHI site of pUB110 is very low (approximately 1 in 4096), and since a dimer of such a molecule would be needed (1 in 16×10^6 chance) to transform competent cells (Canosi et. al., 1978) the failure of this cloning experiment was not surprising.

5.6. Heavy metal ion and antibiotic resistance associated with pAB124

The results described in Section 5.2 indicated that a 0.95 Md fragment of pAB124 could be deleted without any apparent loss of expression of tetracycline resistance. This was tested by examining Tc^r expression of the deletion variants in comparison to pAB124 in B. subtilis and B. stearothermophilus (Table 5.11). It was found that B. subtilis containing pAB124 could tolerate a higher level of tetracycline than the TB124/TB144 strains. Also the tolerance was reduced slightly (150 $\mu g.ml^{-1}$ to 100 $\mu g.ml^{-1}$) when fragments of pAB124 were cloned in pUB1654 (pHI-pHIV). The deletion plasmids pAB224 and pAB524 showed no reduction in the ability to tolerate high levels of tetracycline when compared to pAB124. The conclusion, therefore, must be that the 0.95 Md fragment deleted in pAB224 is not necessary for full expression of the Tc^r phenotype, and cloning of pAB224 in pUB1654 (pHI) does not significantly impair expression of the tetracycline resistance genes.

The function of the 'non-essential' region of pAB124 (0.95 Md) does not seem to be associated with any additional antibiotic resistance. Strains bearing pAB124 are only resistant to tetracyclines (Table 3.7). However, heavy metal ion resistance has been associated with an Ap^r. Staphylococcal plasmid pI258, tolerant to Cadmium, bismuth, lead, mercury, arsenate/arsenite (Novick et. al., 1979) and strains bearing pAB124 and derivatives were tested against several heavy metal ions. The results (Table 5.12) show that generally the thermophilic bacilli were more sensitive to heavy metal ions than B. subtilis and that Cadmium and Mercury salts were much more toxic to bacilli than arsenic salts, cobalt and lead. However, the presence of pAB124 or its derivatives in no way afforded any resistance to heavy metal ions to the Bacilli strains. In conclusion the region of pAB124 deleted to form pAB224 (0.95 Md) does not have any identifiable genotypic function.

SUMMARY

(1) A restriction endonuclease cleavage site map was constructed for pAB124.

(2) EcoRI fragments of pAB124 were cloned in pUB1654 and tetracycline resistance attributed to a 1.95 Md region of pAB124.

(3) Two deletion variants (pAB224, pAB524) were constructed from cloned pAB124 EcoRI fragments in pUB1654.

(4) No identifiable genetic trait was associated with the 0.95 Md region deleted from pAB124 to form pAB224.

Table 5.11. Maximum level of tetracycline resistance associated with plasmid pAB124

Strain	Tetracycline concentration ($\mu g.ml^{-1}$) 50	75	100	125	150	175
TB124 (pAB124)	+	+	+	W	-	-
TB144 (pAB124)	+	+	+	W	-	-
B. subtilis (pAB124)	+	+	+	+	+	W
B. subtilis (pHI)*	+	+	+	W	-	-
B. subtilis (pHII)*	+	+	+	W	-	-
B. subtilis (pHIII)*	+	+	+	W	-	-
B. subtilis (pHIV)*	+	+	+	W	-	-
B. subtilis (pAB224)	+	+	+	+	+	W
B. subtilis (pAB524)	+	+	+	+	+	W

Each strain was inoculated onto TSBA plates containing levels of tetracycline shown above and incubated at 37°C overnight (TB124, TB144 incubated at 50°C). Good growth was recorded as +, and weak by W.

*EcoRI fragments of pAB124 cloned in pUB1654, see table 5.4.

Table 5.12. Tolerance of strains containing pAB124 and derivatives to heavy metal ions

	B. subtilis strains				Thermophilic Bacilli	
	IG20	pAB124	pAB224	pAB524	TB124 (pAB124)	TB144 (pAB124)
Cadmium nitrate (μM)						
0.02	+	+	+	+	+	+
0.2	+	+	+	+	+	+
2.0	+	+	+	W	+	W
10	W	+	+	W	W	-
20	W	W	W	W	-	-
50	-	-	-	-	-	-
Mercurous nitrate (μM)						
0.02	+	+	+	+	+	+
0.2	+	+	+	+	+	+
2.0	+	+	+	+	+	+
10	W	W	W	W	W	W
20	-	W	-	-	-	W
50	-	-	-	-	-	-
Sodium arsenite (mM)						
0.4	+	+	+	+	+	+
0.8	+	+	+	+	+	+
1	W	+	W	W	+	+
4	W	W	W	W	W	W
6	-	-	-	-	W	-
0.2 (arsenate)	+	+	+	+	+	+
1	+	+	+	+	+	+
2	+	+	+	+	+	+
4	W	W	W	W	W	W
10	-	-	-	-	-	-
Cobalt chloride (mM)						
0.1	+	+	+	+	+	+
0.4	+	+	+	+	+	+
0.8	+	+	+	+	W	W
1	W	W	W	W	-	-
2	-	-	-	-	-	-

Table 5.12 (Cont'd)

	B. subtilis strains				Thermophilic Bacilli	
	IG20	pAB124	pAB224	pAB524	TB124 (pAB124)	TB144 (pAB124)
Lead nitrate (mM)						
0.2	+	+	+	+	W	+
0.4	+	+	+	+	W	+
0.8	+	+	+	+	W	+
2	+	+	+	+	W	+
5	W	W	W	W	-	W
10	W	W	W	W	-	-

A fresh overnight culture in TSB was used as a standard inoculum (0.1 ml) for TSBA plates containing one of the heavy metal ions at varying concentrations shown in the table. Plates were incubated overnight at 37°C except for thermophilic bacilli (50°C) and good growth was recorded by +, and weak by W.

CHAPTER SIX

CLONING IN pAB124, pAB224 AND EXPRESSION IN BACILLUS SUBTILIS

6.1. Cloning of Staphylococcal plasmid pUB110 in pAB124 and characterisation of the chimeric plasmid

The characterisation of pAB124 (Chapter 5) revealed that the single XbaI site of pAB124 lies in a region not essential for tetracycline resistance, therefore, it should be possible to clone at this site without inactivating replication or tetracycline resistance. Several plasmids have been isolated from Staphylococcus species that contain a single XbaI site (Chapter 1) and the most useful of these is pUB110 (Neo^r) since it is a multi-copy plasmid (20-40 copies per cell) and insertion of DNA at its XbaI site does not inactivate the neomycin resistance gene (Gryczan & Dubnau, 1978). Only a few restriction endonuclease cleavage sites have been mapped on pUB110 (Gryczan et. al., 1978) and before cloning it was further characterised with several other restriction endonucleases (Table 6.1). The AvaI, CauII, ThaI and HaeII sites were added to the map of pUB110, already published, and the results of these double digest experiments are shown in Table 6.2.

An XbaI + AvaI digest gave no additional fragment indicating that the AvaI site was very close to the XbaI site and its precise position was determined from a AvaI + BamHI and AvaI + EcoRI digest. Small fragments of 0.15 and 0.45 Md were generated respectively so locating the AvaI site 0.04 Md to the right of the XbaI site (Fig 6.1). This position was consistent with the AvaI + BglII digest.

Double digests of CauII + BamHI and CauII + BglII generated small fragments of 0.7 and 0.3 Md respectively and as a result the CauII site can only be situated between the BamHI and BglII sites, i.e. 0.7 Md to the right of the BamHI site. The ThaI site could be positioned in a similar manner since ThaI + BamHI and ThaI + BglII digests generated small fragments of 0.47 and 0.53 Md respectively and therefore the ThaI site lies between the BamHI and BglII sites, 0.47 Md to the right of the BamHI site. The position of the CauII and ThaI sites (Fig 6.1) was consistent with all the other double digests.

Double digests involving HaeII indicated the small HaeII fragment (0.28 Md) contained the CauII site while the large fragment (2.7 Md) contained all other single sites. The exact location of the small fragment over the CauII site could not be determined since the fragments produced by a HaeII + CauII digest were too small for accurate molecular weight estimation. However, the position of this fragment could be inferred from the location of the large fragment which starts

0.16 Md to the left of the site CauII and runs to 0.12 Md to the left of the site BglII.

A comprehensive restriction endonuclease cleavage site map of pUB110 is given in Fig 6.2.

A hybrid plasmid of pAB124 and pUB110 was constructed using their XbaI sites. DNA (1 µg) of each plasmid was digested with XbaI, mixed and ligated with T_4-DNA ligase after heat inactivation as described in Methods (2.14, 2.16) and then transformed into competent B. subtilis IG20 cells (Methods 2.20.1). Neo^r, Tc^r and Neo^r Tc^r transformants were selected on TSBA plates containing the respective antibiotics at 12 $\mu g.ml^{-1}$ or 25 $\mu g.ml^{-1}$. As a control a mixture of pUB110 (1 µg) and pAB124 (µg) was transformed into competent B. subtilis IG20 cells. The results are shown in Table 6.3 and all apparent Neo^r or Tc^r transformants were tested for a Neo^r Tc^r phenotype by replica plating (Collins & Lyne, 1970). The results were not as expected since only 6 Neo^r Tc^r transformants were identified. If insertion of plasmid DNA at the XbaI site of both plasmids does not impair expression of either antibiotic resistance function, as all the previous evidence suggests, then a high frequency of Neo^r Tc^r transformants should be obtained. Plasmid DNA was extracted from all the Neo^r Tc^r transformants using the method described in Chapter 2 (9.2) and examined with BamHI which can identify specifically pUB110 and BstEII which can identify specifically pAB124.

Digestion with BamHI gave two bands one at 3.0 Md and one at 6.0 Md, and digestion with BstEII only generated a 6.0 Md band without digesting the fastest running species (CCC) in undigested plasmid samples. This indicated that a hybrid species (6.0 Md) was present but there was also substantial quantities of independent pUB110 molecules (3.0 Md). No independent pAB124 molecules were detected indicating that the hybrid must be responsible for the Tc^r phenotype and digestion with XbaI only generated two fragments of 2.9 and 3.0 suggesting the pUB110: pAB124 hybrid had not undergone any deletion or insertion.

There was about four times as much pUB110 DNA than hybrid molecules present in the Neo^r Tc^r clones, indicating that the hybrid was under the control of the pAB124 replicon (6-9 copies per cell) not the pUB110 replicon (30-40 copies per cell).

The presence of pUB110 in Neo^r Tc^r transformants suggested that the hybrid could not express the Neo^r genes and had to incorporate pUB110 molecules as well as the hybrid during transformation, in order to have a Neo^r Tc^r phenotype. This explains the low numbers of Neo^r Tc^r transformants obtained, since uptake of two plasmids would

be expected to occur at a low frequency.

These conclusions were tested by removing the CCC species of pUB110 and hybrid from agarose gels using the electrophoretic elution method (Chapter 2.13), so isolating the two plasmids, and carrying out a second transformation. As a control, a mixture of hybrid and pUB110 was also transformed into competent B. subtilis cells. The results in Table 6.4 show that Neo^r Tc^r transformants were obtained when transforming with the mixture of pUB110 and hybrid DNA molecules. Plasmid DNA from several of these transformants (20) was examined and revealed the presence of two plasmids; the hybrid and pUB110. Transformation of the CCC species of pUB110 isolated from the gel only gave Neo^r transformants, while the hybrid plasmid CCC species mainly gave Tc^r transformants confirming that the hybrid can not express the Neo^r genes of pUB110. In 2 cases only, were Neo^r Tc^r transformants obtained with the hybrid plasmid. Plasmid DNA was isolated from these clones (H11, H24) as described in Methods (2.9.2), and examined with several restriction endonucleases (Table 6.5). No independent pUB110 molecules were detected in either case, and also, different results were obtained with the restriction endonuclease analysis than would be expected for a hybrid of pUB110 and pAB124. This would suggest that these plasmids, called pAB324, had probably undergone some degree of deletion, a common occurrence when constructing chimeric plasmids in B. subtilis (Gryczan & Dubnau, 1978; Gryczan et. al., 1980a). Transformation of this new plasmid (pAB324) into competent B. subtilis IG20 cells gave 3050 Neo^r and 3250 Tc^r transformants all of which were shown to have a Neo^r Tc^r phenotype by replica plating (Collins & Lyne, 1970). The pAB324 plasmid, therefore, has gained the ability to express the Neo^r genes, presumably as a result of the deletion of part of the hybrid. Plasmid pAB324 was characterised with restriction endonucleases (Table 6.6) in order to determine the extent and position of the deletion(s).

A restriction endonuclease cleavage site map of all the enzymes having just one site in pAB324 was first constructed. The results of the double digest experiments involving AvaI, BamHI, BalI, BstEII, BglII, HgaI, HpaI, HindIII, XbaI and XhoI are shown in Table 6.7.

All the restriction endonuclease cleavage sites were mapped with the XbaI site as reference point. All double digests involving AvaI, BamHI, BglII and XbaI generated small fragments of identical size with those produced in the same digests of pUB110. To be consistent with this the four sites must be in the same relative position as in

pUB110 (Fig 6.2) as described previously. Double digests involving HindIII indicated that this site was not close to the XbaI site since a HindIII + BamHI digest generated a small fragment of 2.3 Md while a HindIII + BglII digest gave a 1.3 Md fragment, the HindIII site must lie 2.3 Md to the right of the BamHI site ie 1.3 Md to the right of the BglII site (Fig 6.3). The HpaI site was positioned in a similar manner; double digests with XbaI, BamHI and BglII indicated it must lie 3.6 Md to the right of the BamHI site ie 2.6 Md to the right of the BglII site (Fig 6.3). A BalI + HpaI digest indicated that the two sites were very close together and double digests with XbaI, BglII and HindIII positioned the BalI site 0.05 Md to the right of the HpaI site consistent with its position in pAB124 (Fig 5.5). In a similar manner the position of the BstEII site was determined as 0.74 Md from the HpaI site and 0.66 Md from the BalI site consistent with its position in pAB124 (Fig 5.5). Also, digests with HgaI were consistent with the HgaI site located at 0.52 Md to the right of the BstEII site and 1.20 Md to the right of the HpaI site identical to its position in pAB124 (Fig 5.5). An XbaI + XhoI digest indicated that the two sites were quite close together (0.70 Md). If the XhoI site lies to the right of the XbaI site it would then be close to the BamHI and BglII sites, 0.5 Md and 0.3 Md respectively. Since 0.9 and 1.9 Md fragments were generated in double digests this clearly is not the case. The XhoI site therefore lies 0.7 Md to the left of the XbaI site and as a result 0.76 Md to the right of the BstEII site. The relative positions of all the restriction endonucleases with one site in pAB324 is shown in Fig 6.3.

Using this map, the fragments produced by BclI, CauII, EcoRI, HaeII, HaeIII, HgiAI, HindII, PvuII were then psoitioned and the results of the double digest experiments are shown in Table 6.7. A HaeII digest of pAB324 generated a small fragment of identical size (0.28 Md) to that produced in pUB110 suggesting that the two HaeII sites were in the same relative position in pAB324 as in pUB110. This was confirmed by the double digests, one HaeII site being 0.6 Md to the right of the BamHI site and the other 0.12 Md to the left of the BglII site (Fig 6.4). Double digests with CauII indicated that the larger fragment contains the BglII, HindIII and HpaI sites but not the BstEII, XhoI and XbaI sites. Therefore the large 2.75 Md fragment produced by a BglII + CauII digest must lie to the right of the BglII site with the 0.3 Md fragment produced to the left (Fig 6.4). The resulting position of the two CauII sites was consistent with all the double digest experiments. A double digest of BclI + HpaI and BclI + BstEII indicated that the BclI sites were very close to the HpaI and

BstEII sites and their precise positions (Fig 6.4) were determined from the other double digests. The position of the BclI sites were consistent with that in pAB124 (Fig 5.5). A PvuII + HpaI digest gave no additional fragments indicating that one PvuII site is close to the HpaI site as is the case in pAB124 (Fig 5.5), and that the second PvuII site is located close to the BamHI site since a PvuII + XbaI digest gave a small fragment of 0.25 Md while a PvuII + BglII digest gave a 0.95 Md fragment. This placed the second PvuII site 0.05 Md to the right of the BamHI site (Fig 6.4) and is consistent with all the double digest data.

The large EcoRI fragment (A) contained the XbaI, XhoI and BglII sites but not the BstEII, HindIII or HpaI sites. To be consistent with these data only one orientation of the fragment is possible in which the 1.4 Md fragment of a BglII + EcoRI digest lies to the right of the BglII site and the 2.23 Md fragment produced, to the left (Fig 6.4). The EcoRI fragment B was therefore positioned to the right of fragment A since it contained the HindIII and HpaI sites. This leaves the small fragment (0.32 Md) overlying the CauII site (Fig 6.4). The position of the fragments was consistent with all the double digests.

The position of the HgiAI and ThaI fragments was determined in a similar manner. The large HgiAI fragment, A (3.2 Md) over-lies the HindIII, HpaI and BstEII sites with HgiAI fragment B to the right containing the XhoI, XbaI and BamHI sites; and HgiAI fragment C to the left of HgiAI A overlying the BglII site (Fig 6.4). The large Tha I fragment, (A) 3.4 Md over-lies the HindIII site with ThaI (B) to the left containing the BamHI, XbaI and XhoI sites and therefore ThaI (C) lies to the right of ThaI (A), containing the BstEII site (Fig 6.4). The precise position of the HgiAI and ThaI fragments was determined from all the double digest experiments.

Only one orientation of the large HaeIII fragment A (2.90 Md) is possible since it contains the XbaI, XhoI, BstEII, BamHI sites but not the BglII, HindIII or HpaI sites (Fig 6.4). The HaeIII fragment C contains the BglII site and therefore lies to the left of HaeIII fragment B which over-lies the HindIII site (Fig 6.4). This leaves the smallest fragment HaeIII (D) to the right of HaeIII (B) since it contains the HpaI site (Fig 6.4). The HindII fragments were positioned in a similar manner. The large fragment (A) over-lies the BamHI, XbaI and XhoI sites and only one orientation is possible. One HindII site is close to the BglII site and another close to the HpaI site (Table 6.7) since HindII (B) contains the HindIII site

the positions of the HindII fragments B, C and D could easily be determined (Fig 6.4).

The relative positions of all the restriction endonuclease cleavage sites in pAB324 are shown in Fig 6.5. As mentioned previously, pAB324 does not contain the same number of cleavage sites as would be expected for a simple hybrid of pAB124 and pUB110 (Table 6.5) and comparison of the cleavage site maps of the expected hybrid (Fig 6.6) and pAB324 (Fig 6.5) indicated two regions of homology as shown in Fig 6.7. Between these two regions a deletion has occurred where one EcoRI, one XbaI and one HaeIII sites have been lost, and this region now contained a HindIII site not present in either "parent" plasmid (Fig 6.7). At the extreme right of the molecule (Fig 6.7) a small deletion had occurred where one ThaI site and one BclI site were lost, but in addition, a quite large insertion was present. This insert was shown to contain a unique XhoI site. It is not likely that this insert originated from either pAB124 or pUB110 since it contained no restriction endonuclease cleavage sites characteristic of these plasmids and presumably therefore was derived from the B. subtilis chromosome. If this was the case there should be some hybridisation between B. subtilis chromosomal DNA and pAB324 DNA on nitrocellulose filters. Using the method described in Methods (2.23) B. subtilis chromosomal DNA fragments generated by EcoRI digestion were separated in agarose gels, denatured with alkali, transferred to nitrocellulose filters, and 'nick' translated pAB324 (^{32}P) DNA added. Hybridisation of the radioactive pAB324 to the filter was detected by autoradiography. The results (Plate 6.1) show hybridisation to B. subtilis chromosomal DNA in a region of the filter, corresponding to 1.05 Md.

The presence of this chromosomal insert in pAB324 could possibly be associated with expression of the neomycin resistance genes not expressed in a chimeric plasmid of pAB124: pUB110. However, it appears that the insert is not physically close to the Neo^r genes which are thought to be located around the BglII site of pUB110 since insertion of DNA at the BglII site of pUB110 inactivates the Neo^r genes (A. Docherty, pers. comm.). This position was confirmed by removing the BamHI to BglII (1.0 Md) region of pAB324. Since both enzymes produce the same cohesive terminus (see Appendix) digestion of pAB324 (1 µg) with BamHI, BglII followed by ligation with T_4 DNA-ligase (Methods 2.14, 2.16) will generate some molecules where the BamHI cohesive terminus will anneal with the BglII cohesive terminus of

the large fragment (5.0 Md) so deleting the 1.0 Md fragment. The ligated DNA molecules were transformed into competent B. subtilis IG20 cells and Tc^r transformants selected. Of the 2.3×10^3 Tc^r transformants obtained (per µg DNA) at 12 $\mu g.ml^{-1}$ tetracycline, 5.5×10^2 were Tc^r Neo^s and plasmid DNA from 30 of these clones was extracted (Methods 2.9.2) and analysed with several restriction endonucleases. Two types of plasmids were obtained (Table 6.8), termed pAB624 (5.0 Md) and pAB724 (6.0 Md) and as expected neither contained a BamHI or BglII site. In comparison to pAB324, pAB624 contained one less site for CauII, HindII, HaeIII, HgiAI, PvuII, ThaI and two less for HaeII, as would be expected for a derivative of pAB324 where the 1.0 Md fragment of a BamHI + BglII digest had been deleted. Several double-digests were carried out in order to confirm this (Table 6.9). A cleavage site map of pAB624 was constructed as described previously for pAB324 and is shown in Fig 6.8. Plasmid pAB724 contains all the cleavage sites present in pAB324 (except BamHI, BglII) and is the same size. Therefore it does not appear to have deleted the BamHI + BglII 1.0 Md fragment. The only possible explanation is that the fragment has been inverted; this would destroy the BamHI and BglII cleavage sites and generate a Neo^s Tc^r phenotype. This was confirmed by double-digest experiments (Table 6.10). Double-digests using enzymes having just one cleavage site in pAB724 gave identically sized fragments to that generated with pAB324. The inversion of the 1.0 Md BamHI + BglII fragment will result in different sized fragments to that of pAB324 with PvuII, HgiAI, HaeIII, HindII, ThaI and CauII, and a cleavage site map was constructed (Fig 6.8), as described previously for pAB324 using the results shown in Table 6.10.

Table 6.1. Additional Restriction Endonuclease cleavage sites in pUB110

Enzyme (sequence)	Fragments No.	Fragments Size (Md)	Sum of fragment sizes (Md)
AvaI (G/G$^{A}_{T}$CC)	1	3.00	3.00
BclI (T/GATCA)	0	-	-
BstEII (G/GTNACC)	0	-	-
CauII (CC$^{C}_{G}$GG)	1	3.00	3.00
HpaI (GTT/AAC)	0	-	-
HaeII (PuGCGC/Py)	2	2.70, 0.28	2.98
ThaI (CG/CG)	1	3.00	3.00

Digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).

Table 6.2. Fragments produced by digestion of pUB110 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md) A	B	C	Sum of fragment sizes (Md)
AvaI + BamHI	2.85	(0.15)[a]		3.00
AvaI + BglII	1.85	1.15		3.00
AvaI + EcoRI	2.53	0.45		2.98
AvaI + XbaI	3.00	-		3.00
CauII + BamHI	2.30	0.70		3.00
CauII + BglII	2.70	0.30		3.00
CauII + EcoRI	1.70	1.30		3.00
CauII + XbaI	2.10	0.90		3.00
ThaI + BamHI	2.62	0.37		2.99
ThaI + BglII	2.37	0.63		3.00
ThaI + EcoRI	2.10	0.87		2.97
ThaI + XbaI	2.43	0.57		3.00
HaeII	2.70	0.28		2.98
HaeII + BamHI	2.10	0.60	0.28	2.98
HaeII + BglII	2.50	0.28	0.20	3.00
HaeII + EcoRI	1.30	1.40	0.28	2.98
HaeII + XbaI	1.90	0.80	0.28	2.98
HaeII + CauII	2.70	(0.12)[a]	(0.16)[a]	2.98

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).

a, this fragment was too small for an accurate molecular weight determination.

Figure 6.1. Relative Positions of Fragments Produced by Digestion of pUB110 with Several Restriction Endonucleases

The relative positions of the fragments were determined from the results of double-digest experiments (Table 6.2) as described in Chapter 6 (6.1) and Gryczan et. al., (1978).

FIG. 6.1

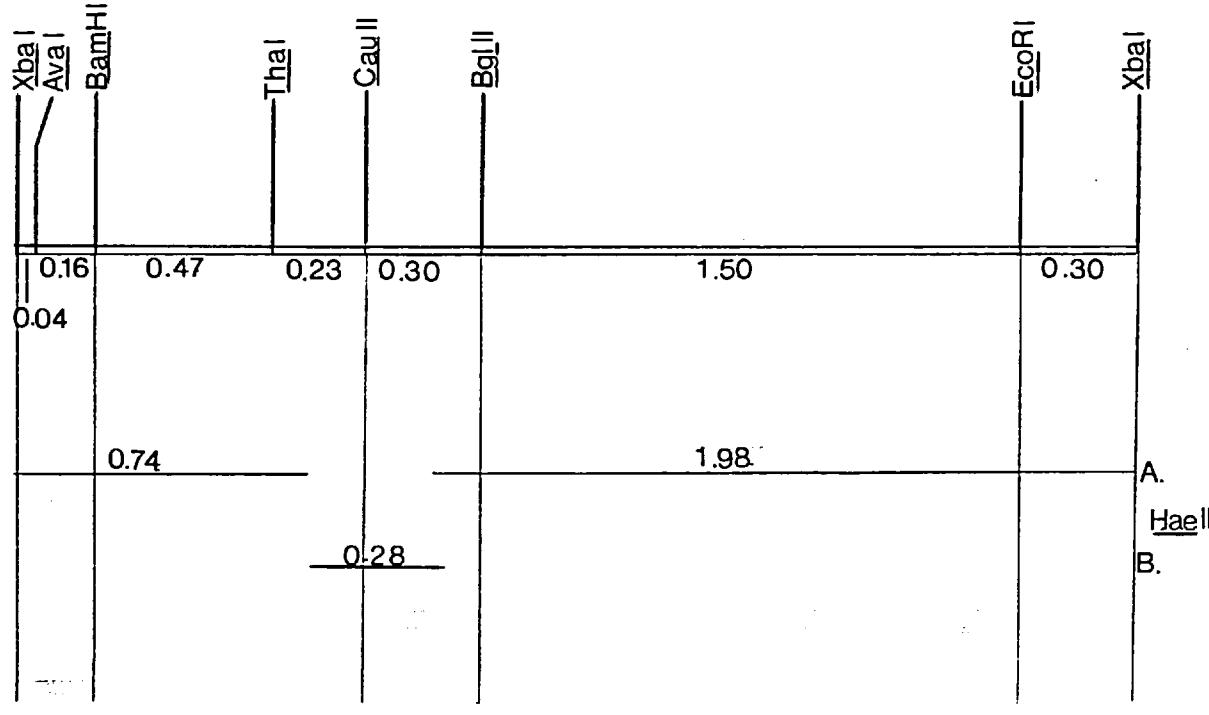

Xba I
Ava I
BamHI
Tha I
Cau II
Bgl II
EcoRI
Xba I
0.16
0.47
0.23
0.30
1.50
0.30
0.04
0.74
1.98
A.
Hae II
0.28
B.

Figure 6.2. Restriction Endonuclease Cleavage Site Map of pUB110

The restriction endonuclease cleavage site map was constructed as described in Chapter 6 (6.1) from data shown in Table 6.2 and from Gryczan et. al., 1978.

Map distances

XbaI, 0/3.0; AvaI, 0.04; BamHI, 0.20; HpaII, 0.30; ThaI, 0.55; HaeII, 0.74; CauII, 0.90; HaeIII, 0.95; HpaII, 0.97; HaeII, 1.02; HindII, 1.17; BglII, 1.20; HindII, HpaII & HaeIII, 1.40; EcoRI, 2.70.

FIG. 6.2

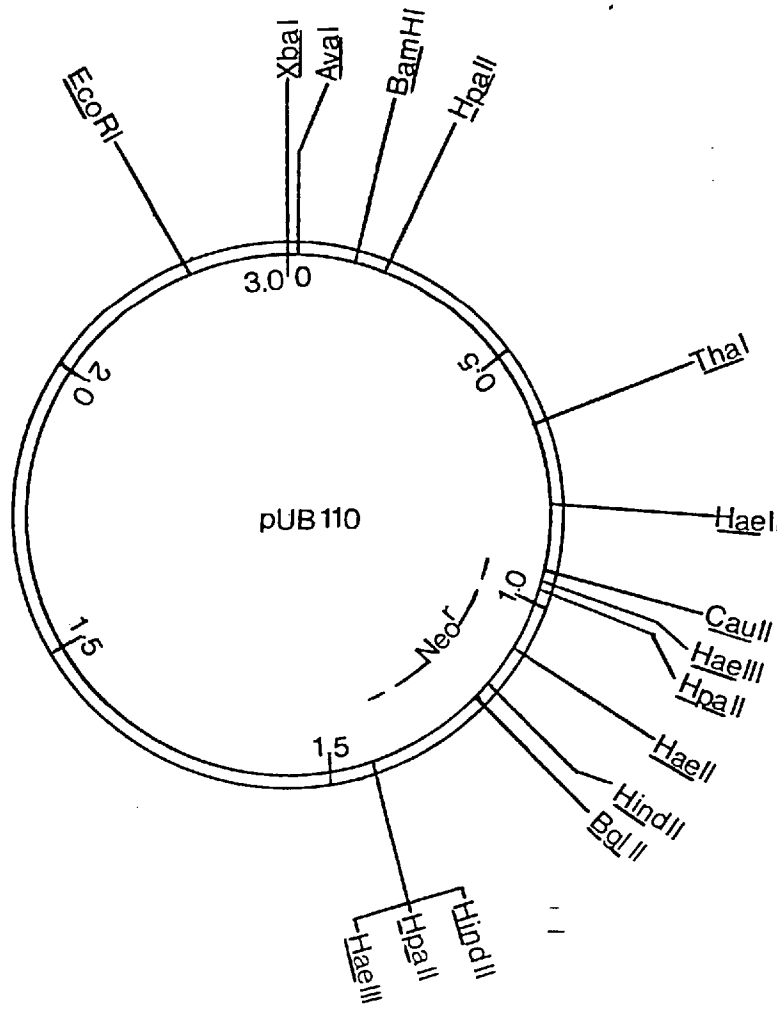

EcoRI
XbaI
AvaI
BamHI
HpaII
3.0
0
5.0
2.0
ThaI
HaeII
pUB 110
1.0
CauII
HaeIII
HpaII
Neo r
1.5
HaeII
1.5
HindII
BglII
HpaII
HaeIII
HindII

Table 6.3. Transformants obtained with a hybrid plasmid molecule of pAB124: pUB110

	Antibiotic selection ($\mu g.ml^{-1}$)	No. Transformants $\mu g\ DNA^{-1}$	No. $Neo^r Tc^r$ clones on replica plating
(A) Recombinant Plasmid			
	Neo(25)	850	2
	Neo(12)	925	1
	Tc(25)	300	4
	Tc(12)	450	2
	NeoTc(25)	0	0
	NeoTc(12)	6	6
(B) Mixture of pUB110, pAB124			
	Neo(25)	10,800	0
	Neo(12)	11,150	0
	Tc(25)	850	0
	Tc(12)	10,550	0
	NeoTc(25)	0	0
	NeoTc(12)	0	0

The pUB110/pAB124 hybrid was constructed *in vitro* as described in Chapter 6 and transformed into competant *B. subtilis* IG20 cells (Methods 2.20).

Table 6.4. Transformants obtained with the hybrid plasmid pAB124 : pUB110 and pUB110 isolated from $Neo^r Tc^r$ clones

Clone	Plasmid species	Antibiotic selection	No. transformants $\mu g\ DNA^{-1}$
I	CCC hybrid	Neo	0
		Tc	870
		Neo, Tc	0
	CCC pUB110	Neo	8,550
		Tc	0
		Neo, Tc	0
	Mixture	Neo	10,100
		Tc	1,500
		Neo, Tc	90
IV	CCC hybrid	Neo	2
		Tc	952
		Neo, Tc	2
	CCC pUB110	Neo	9,655
		Tc	0
		Neo, Tc	0
	Mixture	Neo	9,850
		Tc	2,100
		Neo, Tc	105

The two species of plasmid were separated in agarose gels and isolated using the electrophoretic elution method (Chapter 2.13.3) and transformed into competent B. subtilis IG20 cells (Chapter 2.20). Transformants were selected on TSBA containing the relevant antibiotic(s) at 12 $\mu g.ml^{-1}$.

Table 6.5. Restriction Endonuclease analysis of plasmid DNA from Neo^rTc^r clones derived from hybrid plasmid pAB124: pUB110

Enzyme (sequence)	No. cleavage sites H11	H24	pAB124: pUB110*
EcoRI (G/AATTC)	3	3	4
ThaI (CG/CG)	3	3	4
CauII ($CC\overset{C}{\underset{G}{}}GG$)	2	2	2
HpaI (GTT/AAC)	1	1	1
XbaI (T/CTAGA)	1	1	2

Digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).

*Values derived from a composite map of pAB124/pUB110 hybridised at their XbaI sites.

Table 6.6. Fragments produced by digestion of pAB324 with several Restriction Endonucleases

Enzyme	No. fragments	Fragment sizes (Md)	Sum of fragment sizes (Md)
AvaI	1	6.00	6.00
AccI	0	-	-
BamHI	1	6.00	6.00
BalI	1	6.00	6.00
BclI	2	5.30, 0.68	5.98
BstEII	1	6.00	6.00
BglII	1	6.00	6.00
BglI	0	-	-
CauII	2	3.30, 2.70	6.00
EcoRI	3	3.63, 2.10, 0.32	6.05
EcoRII	0	-	-
HaeII	2	5.70, 0.28	5.98
HaeIII	4	2.90, 2.30, 0.50, 0.30	6.00
HgaI	1	6.00	6.00
HgiAI	3	3.20, 1.60, 1.20	6.00
HphI	>5	2.30, 1.70, 0.82, 0.51, 0.40, ( )[a]	5.73
HpaI	1	6.00	6.00
HindII	4	2.60, 2.50, 0.65, 0.25	6.00
HindIII	1	6.00	6.00
KpnI	0	-	-
MboII	>6	1.15, 0.55, 0.45, 0.39, 0.30, 0.28 ( )[a]	3.12
MnlI	>6	0.80, 0.53, 0.50, 0.45, 0.30, 0.25 ( )[a]	2.83
PvuI	0	-	-
PvuII	2	3.70, 2.30	6.00
PstI	0	-	-
SalI	0	-	-
SacI	0	-	-
Sau961	>5	2.35, 1.15, 0.80, 0.54, 0.30 ( )[a]	5.14
ThaI	3	3.40, 1.95, 0.64	5.99
XbaI	1	6.00	6.00
XhoI	1	6.00	6.00

Table 6.6 (Cont'd)

Digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).
a, Several more fragments were assumed to be present although not detected.

Table 6.7. Fragments produced by digestion of pAB324 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
XbaI + AvaI	6.00	-				6.00
XbaI + BamHI	5.80	0.20				6.00
XbaI + BglII	4.80	1.20				6.00
AvaI + BamHI	5.80	(0.15)[b]				5.95
AvaI + BglII	4.85	1.15				6.00
BamHI + BglII	5.00	1.00				6.00
HindIII + XbaI	3.30	2.70				6.00
HindIII + BamHI	3.50	2.50				6.00
HindIII + BglII	4.50	1.50				6.00
HindIII + HpaI	4.80	1.20				6.00
HpaI + XbaI	3.85	2.12				5.97
HpaI + BamHI	3.70	2.30				6.00
HpaI + BglII	3.30	2.70				6.00
HpaI + HindIII	4.80	1.20				6.00

Table 6.7 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
BalI + XbaI	4.00	2.05				6.05
BalI + BglII	3.25	2.75				6.00
BalI + HindIII	4.75	1.25				6.00
BalI + HpaI	6.00	-				6.00
BstEII + XbaI	4.55	1.45				6.00
BstEII + BalI	5.30	0.65				5.95
BstEII + HpaI	5.30	0.70				6.00
BstEII + HindIII	4.10	1.90				6.00
BstEII + XhoI	5.25	0.75				6.00
HgaI + BstEII	5.50	0.50				6.00
HgaI + HpaI	4.80	1.25				6.05
HgaI + HindIII	3.60	2.40				6.00
HgaI + XbaI	5.10	0.90				6.00
XhoI + XbaI	5.30	0.68				5.98
XhoI + BstEII	5.22	0.74				5.96
XhoI + HpaI	4.55	1.44				5.99
XhoI + BglII	4.15	1.85				6.00
XhoI + HindIII	3.35	2.65				6.00

Table 6.7 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
HaeII	5.70	0.28				5.98
HaeII + BamHI	5.10	0.60	0.28			5.98
HaeII + BglII	5.55	2.80	(0.15)[b]			5.98
HaeII + XbaI	4.90	0.80	0.28			5.98
HaeII + HindIII	4.35	1.65	0.28			5.98
CauII	3.30	2.70				6.00
CauII + BamHI	3.30	2.00	0.70			6.00
CauII + BglII	3.00	2.70	0.30			6.00
CauII + HpaI	2.90	2.70	0.35			5.95
CauII + BstEII	3.30	2.35	0.35			6.00
CauII + XbaI	3.30	0.95	1.75			6.00
CauII + HindIII	2.70	1.85	1.45			6.00
BclI	5.30	0.68				5.98
BclI + XhoI	4.45	0.82	0.68			5.95
BclI + HindIII	4.10	1.20	0.68			5.98
BclI + BglII	2.70	2.60	0.68			5.98
BclI + XbaI	3.80	1.50	0.68			5.98
BclI + HpaI	4.10	1.20	0.68			5.98

Table 6.7 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
BclI + BstEII	5.20	0.68	(0.10)[b]			5.98
PvuII	3.70	2.30				6.00
PvuII + BamHI	3.70	2.25	(0.05)[c]			6.00
PvuII + BglII	2.75	2.30	0.95			6.00
PvuII + BstEII	3.70	1.95	0.35			6.00
PvuII + HpaI	3.70	2.30				6.00
PvuII + XhoI	3.70	1.35	0.95			6.00
EcoRI	3.63	2.10	0.32			6.05
EcoRI + XbaI	2.60	2.10	1.03	0.32		6.05
EcoRI + XhoI	3.25	2.10	0.35	0.32		6.02
EcoRI + BglII	2.10	2.23	1.40	0.32		6.05
EcoRI + HindIII	3.63	2.00	0.32	(0.10)[b]		6.05
EcoRI + HpaI	3.63	1.35	0.75	0.32		6.05
EcoRI + BstEII	3.63	2.05	0.32	(0.05)[c]		6.05
HgiAI	3.20	1.60	1.20			6.00
HgiAI + BstEII	2.85	1.60	1.20	0.35		6.00
HgiAI + HpaI	2.15	1.60	1.20	1.05		6.00

Table 6.7 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
HgiAI + HindIII	2.20	1.60	1.20	1.00		6.00
HgiAI + BglII	3.20	1.60	0.70	0.53		6.03
HgiAI + XbaI	3.20	1.20	1.10	0.50		6.00
ThaI	3.40	1.95	0.64			5.99
ThaI + BamHI	3.40	1.60	0.64	0.35		6.00
ThaI + XbaI	3.40	1.35	0.64	0.56		5.95
ThaI + HindIII	2.20	1.95	1.20	0.64		5.99
ThaI + HpaI	3.30	1.95	0.64	(0.10)[b]		5.99
ThaI + BstEII	3.40	1.85	0.64	(0.10)[b]		5.99
ThaI + XhoI	3.40	1.30	(0.64)[a]			5.98
HaeIII	2.90	2.30	0.50	0.30		6.00
HaeIII + BamHI	2.30	2.20	0.70	0.50	0.30	6.00
HaeIII + BglII	2.90	2.30	(0.30)[a]	0.20		6.00
HaeIII + BstEII	(2.30)[a]	0.60	0.50	0.30		6.00
HaeIII + HindIII	2.90	1.30	1.00	0.50	0.30	6.00
HaeIII + HpaI	2.90	2.30	0.50	(0.20)[b]	(0.10)[b]	6.00
HaeIII + XbaI	2.30	2.00	0.90	0.50	0.30	6.00
HaeIII + XhoI	2.30	1.55	1.33	0.50	0.30	5.98

Table 6.7 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
HindII	2.60	2.50	0.65	0.25		6.00
HindII + BamHI	2.50	1.65	0.95	0.65	0.25	6.00
HindII + BglII	2.60	2.50	0.65	(0.20)[b]	(0.05)[c]	6.00
HindII + BstEII	2.60	2.50	0.60	0.25	(0.05)[c]	6.00
HindII + HindIII	2.60	1.30	1.20	0.65	0.25	6.00
HindII + HpaI	2.60	2.50	0.65	0.25	-	6.00
HindII + XbaI	2.50	1.45	1.15	0.65	0.25	6.00
HindII + XhoI	2.50	1.80	0.80	0.65	0.25	6.00

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).

a, This fragment was over intense and assumed to be a "double".

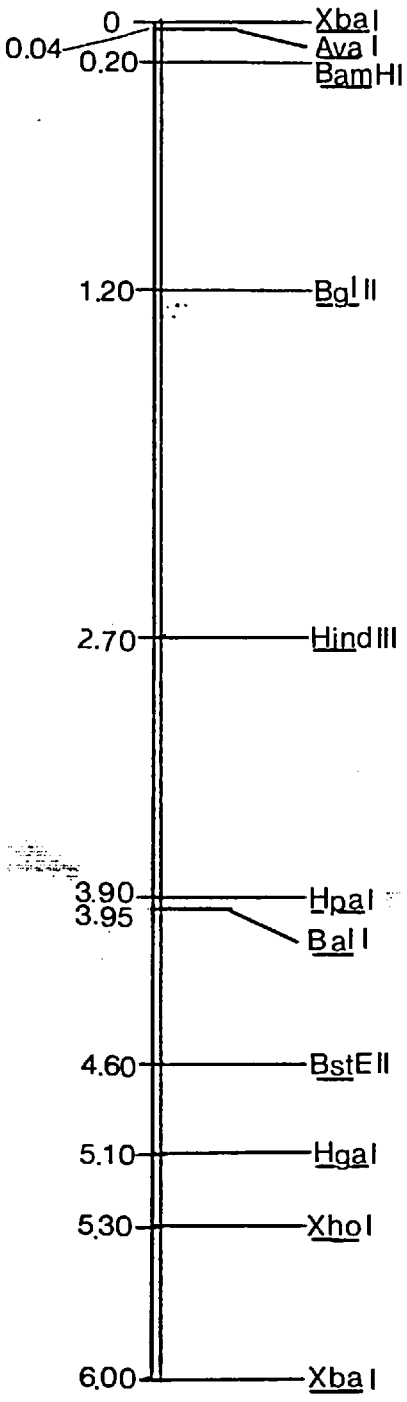
b, This fragment was too small for an accurate molecular weight determination.

c, This fragment was not detected but its presence can be predicted.

Figure 6.3. Relative Positions of Restriction Endonucleases Having Just one Cleavage Site in pAB324

The relative positions of the restriction endonuclease cleavage sites were determined from the results of double-digest experiments (Table 6.7) as described in Chapter 6 (6.1).

FIG. 6.3


0
0.04
0.20
1.20
2.70
3.90
3.95
4.60
5.10
5.30
6.00
Xba I
Ava I
BamHI
Bgl II
Hind III
Hpa I
Bal I
BstE II
Hga I
Xho I
Xba I

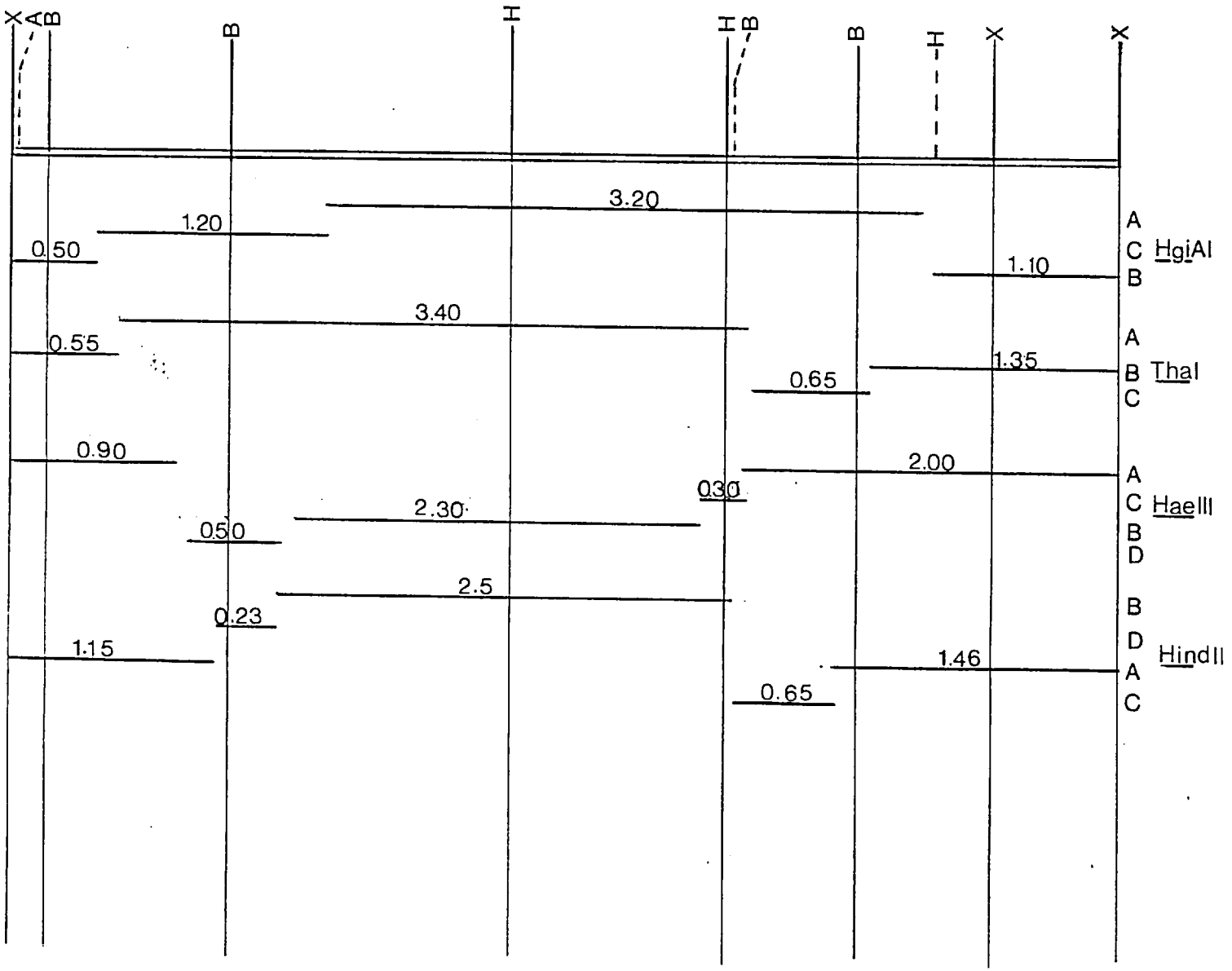

X
A
B
B
H
H
B
B
H
X
X
3.20
1.20
0.50
1.10
3.40
0.55
1.35
0.65
0.90
2.00
0.30
2.30
0.50
2.5
0.23
1.15
1.46
0.65
A C B HgiAI
A B C ThaI
A C B D HaeIII
B D A C HindII

FIG. 6.4

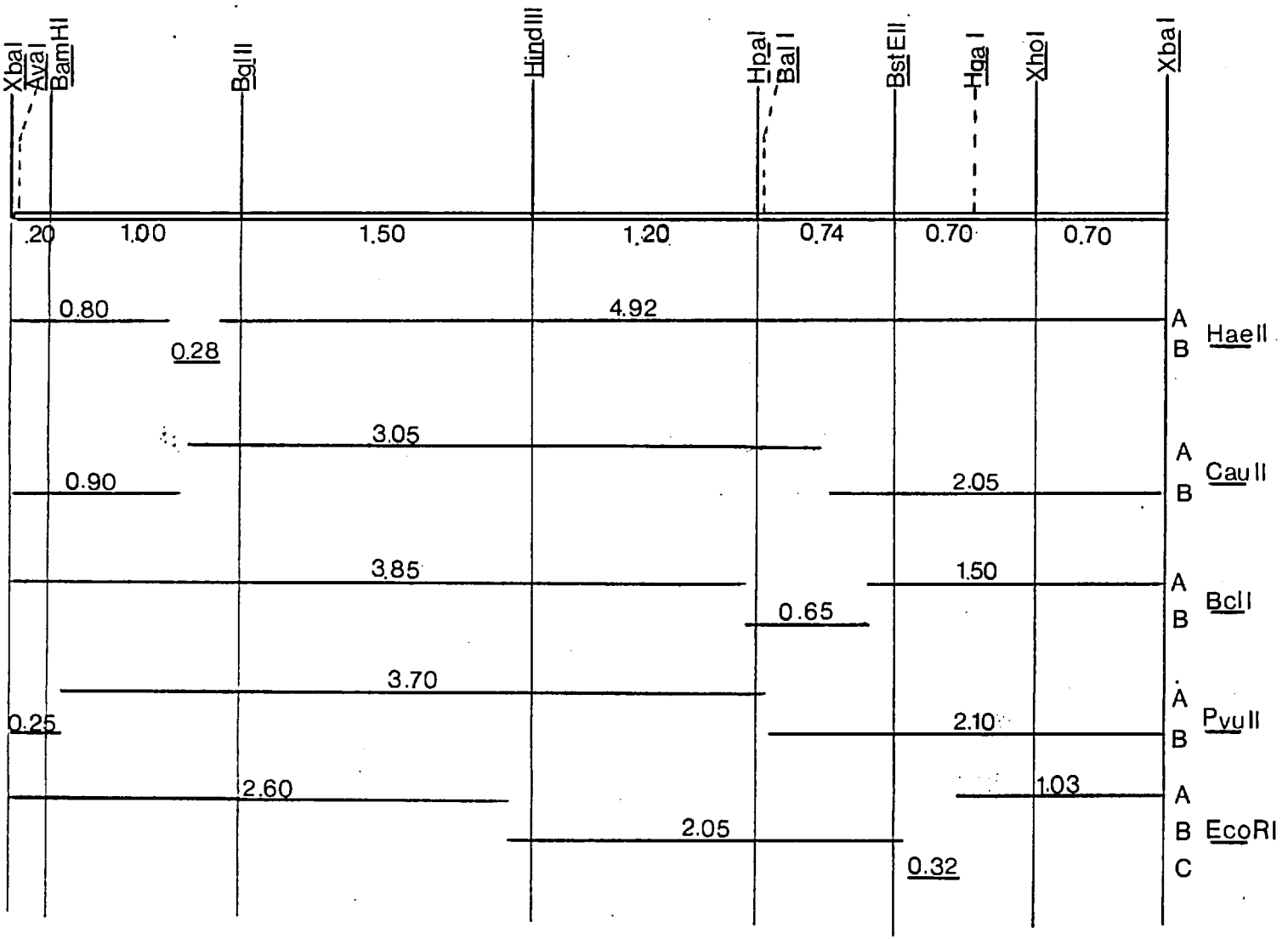

XbaI
AvaI
BamHI
BglII
HindIII
HpaI
BalI
BstEII
HgaI
XhoI
XbaI
.20
1.00
1.50
1.20
0.74
0.70
0.70
0.80
4.92
A
B
HaeII
0.28
3.05
A
B
CauII
0.90
2.05
3.85
1.50
A
B
BclI
0.65
3.70
A
B
PvuII
0.25
2.10
2.60
1.03
A
B
EcoRI
2.05
0.32
C

FIG.6.4

Figure 6.4. Relative Positions of Fragments Produced by Digestion of pAB324 with Several Restriction Endonucleases

The relative positions of the fragments was determined from the results of double-digest experiments (Table 6.7) as described in Chapter 6 (6.1).

Figure 6.5. Restriction Endonuclease Cleavage Site Map of pAB324

The restriction endonuclease cleavage site map was constructed as described in Chapter 6 (6.1) from data shown in Table 6.7.

Map distances

XbaI, 0/6.0; AvaI, 0.04; BamHI, 0.20; PvuII, 0.25; HgiAI, 0.50; ThaI, 0.55; HaeII, 0.80; CauII, 0.90; HaeIII, 0.95; HaeII, 1.08; HindII, 1.15; BglII, 1.20; HaeIII, HindII, 1.40; HgiAI, 1.70; EcoRI, 2.60; HindIII, 2.70; HaeIII, 3.75; BclI, 3.85; HpaI, HindII, 3.90; BalI, PvuII, 3.95; HaeIII, 4.00; ThaI, 4.05; CauII, 4.25; BclI, 4.50; HindII, 4.55; BstEII, 4.60; EcoRI, 4.65; ThaI, 4.72; HgiAI, 4.90; EcoRI, 4.97; HgaI, 5.00; XhoI, 5.30; XbaI, 6.00.

FIG. 6.5

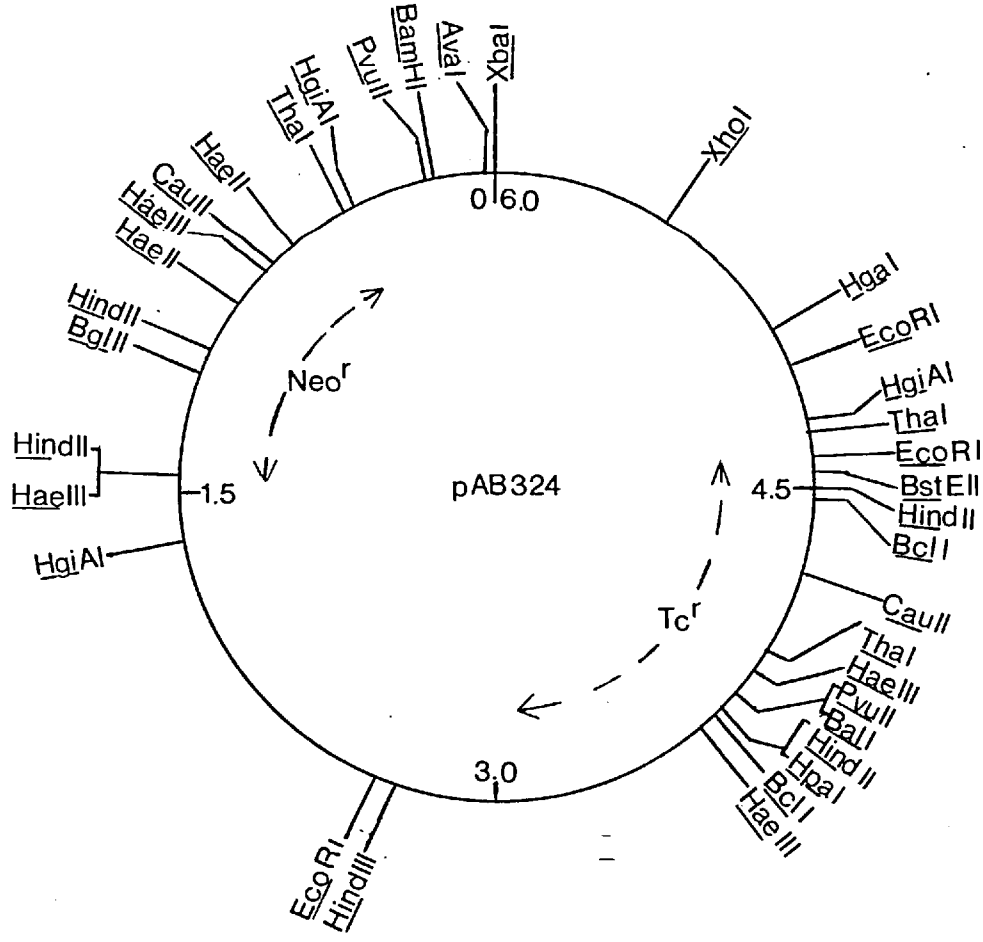

pAB324
0
6.0
1.5
4.5
3.0
Neo^r
Tc^r
XbaI
AvaI
BamHI
PvuII
HgiAI
ThaI
HaeII
CauII
HaeIII
HaeII
HindII
BglII
HindII
HaeIII
HgiAI
EcoRI
HindIII
XhoI
HgaI
EcoRI
HgiAI
ThaI
EcoRI
BstEII
HindII
BclI
CauII
ThaI
HaeIII
PvuII
BalI
HindII
HpaI
BclI
HaeIII

Figure 6.6. Restriction Endonuclease Cleavage Site Map of the Expected Hybrid of Staphylococcus aureus plasmid pUB110 (Neo^r) and B. stearothermophilus plasmid pAB124 (Tc^r)

Restriction endonuclease cleavage site map based on maps of pAB124 (Fig 5.5) and pUB110 (Fig 6.1) for their orientation in pAB324 (Fig 6.5). The double line represents pAB124 and the single line pUB110.

FIG. 6.6

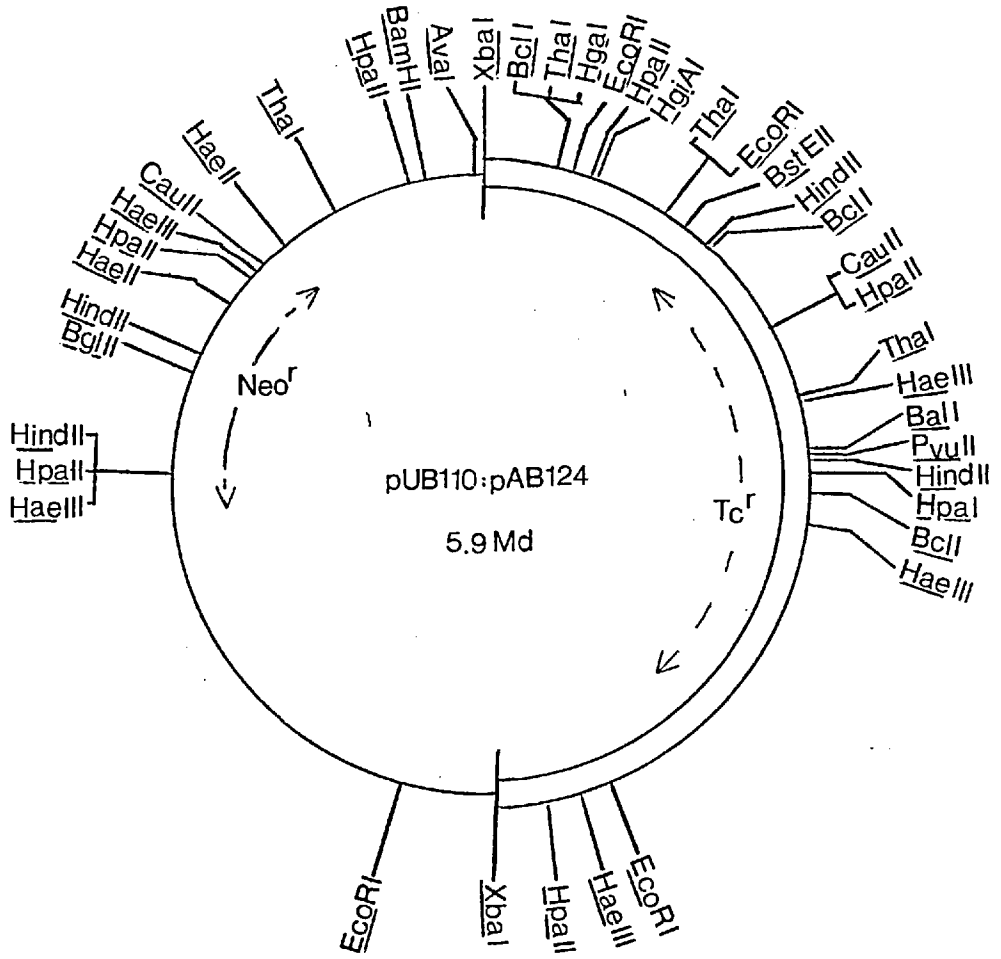

pUB110:pAB124
5.9 Md
Neo^r
Tc^r
BamHI
HpaII
AvaI
XbaI
BclI
TthI
HgaI
EcoRI
HpaII
HgiAI
TthI
EcoRI
BstEII
HindIII
BclI
CauII
HpaII
TthI
HaeIII
BalI
PvuII
HindII
HpaI
BclI
HaeIII
EcoRI
XbaI
HpaII
HaeIII
EcoRI
HindII
HpaII
HaeIII
HindII
BglII
HaeII
HpaII
HaeIII
CauII
HaeII
TthI

Figure 6.7. Comparison of the Restriction Endonuclease Cleavage Maps of pAB324 and the Hybrid of pUB110 and pAB124

This comparison is based on Figures 5.5, 6.2, 6.5 and 6.6. The single line represents pUB110, the wide double line, pAB124 and the narrow double line pAB324.

FIG.6.7

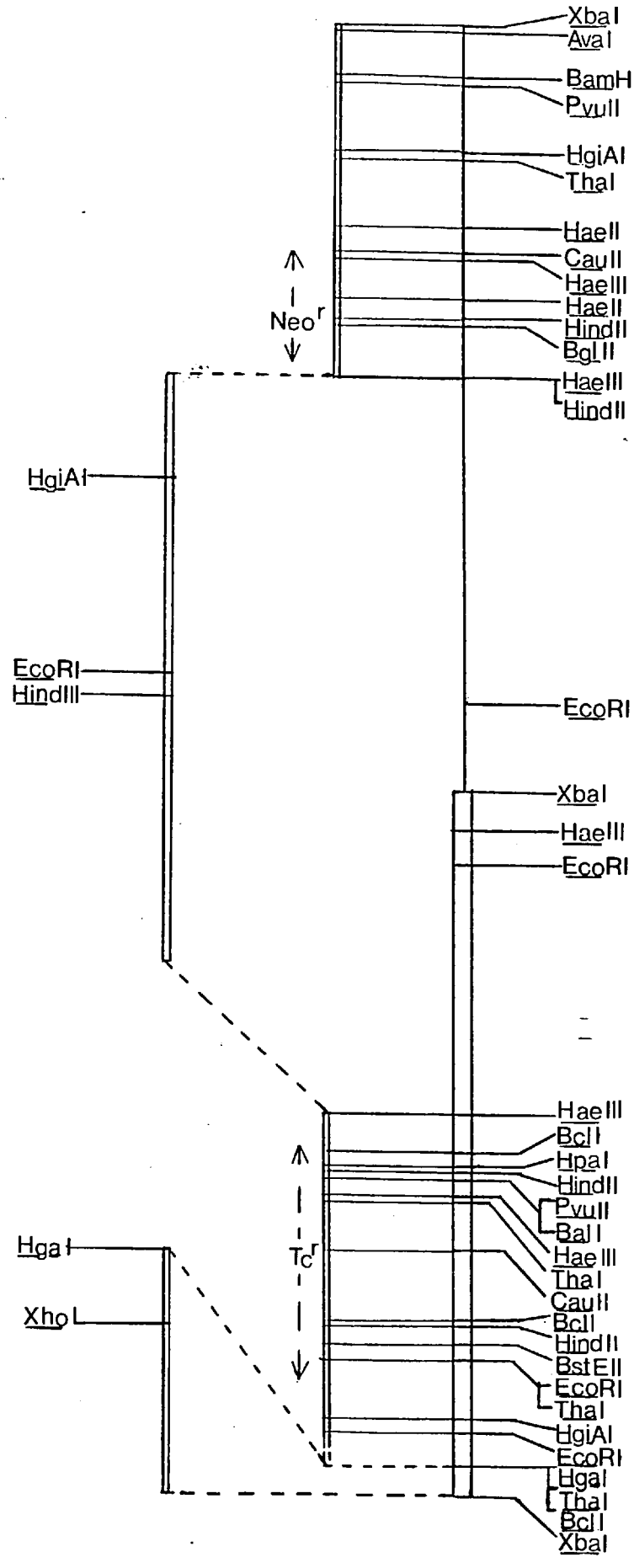
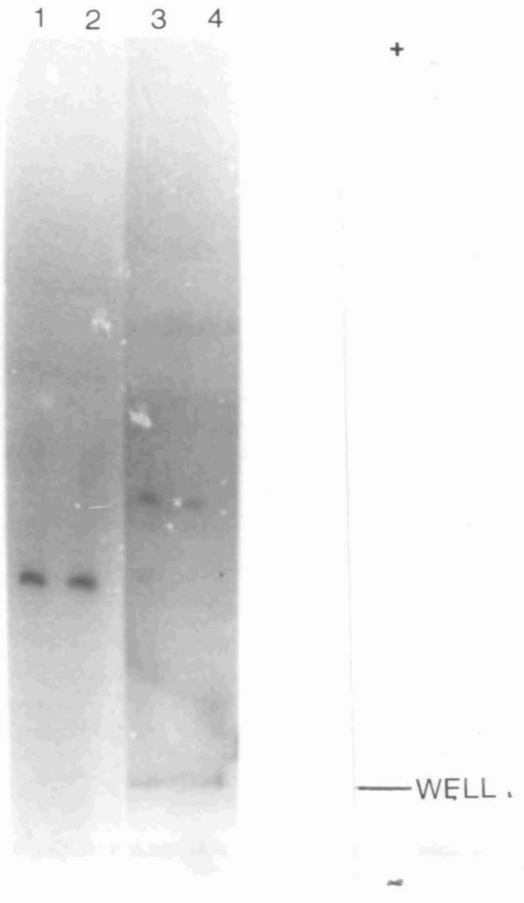

XbaI
AvaI
BamHI
PvuII
HgiAI
ThaI
HaeII
CauII
HaeIII
HaeII
HindII
BglII
HaeIII
HindII
Neo^r
HgiAI
EcoRI
HindIII
EcoRI
XbaI
HaeIII
EcoRI
HaeIII
BclI
HpaI
HindII
PvuII
BalI
HaeIII
ThaI
CauII
BclI
HindII
BstEII
EcoRI
ThaI
HgiAI
EcoRI
HgaI
ThaI
BclI
XbaI
Tc^r
HgaI
XhoI

Plate 6.1. X-ray plate of a Nitrocellulose filter where pAB324 ^{32}P DNA was hybridised to B. subtilis chromosomal DNA and pAB124



1: pAB124 digested with XbaI.

2: " " " "

3: B. subtilis chromosomal DNA digested with EcoRI

4: " " " " " "

Plasmid DNA from TB124 and B. subtilis chromosomal DNA were isolated, digested with XbaI and EcoRI respectively, and subjected to agarose-gel electrophoresis as described in Chapter 2 (2.7., 2.11., 2.14., 2.13.). The DNA was transferred to a nitrocellulose filter and hybridised to ^{32}P pAB324 as described in 2.23.

Table 6.8. Restriction Endonuclease cleavage sites in pA324 and derivatives pAB624 and pAB724

Enzyme	No. cleavage sites Type I recombinant (pAB624)	Type II recombinant (pAB724)	pAB324
BamHI	0	0	1
BglII	0	0	1
CauII	1	2	2
HindII	3	4	4
HaeII	0	2	2
HaeIII	3	4	4
HgiAI	2	3	3
PvuII	1	2	2
ThaI	2	3	3
AvaI	1	1	1
HindIII	1	1	1
HpaI	1	1	1
BalI	1	1	1
BstEII	1	1	1
HgaI	1	1	1
XbaI	1	1	1
XhoI	1	1	1

Digests and gel-electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from $Neo^{s}Tc^{r}$ transformants of B. subtilis (2.9.2).

Table 6.9. Fragments produced by digestion of pAB624 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md) A	B	C	D	Sum of fragment sizes (Md)
CauII + PvuII	4.70	0.30			5.00
CauII + HindIII	3.45	1.55			5.00
CauII + HpaI	4.65	0.35			5.00
CauII + BalI	4.70	0.30			5.00
CauII + BstEII	4.65	0.35			5.00
CauII + HgaI	4.25	0.75			5.00
CauII + XbaI	3.25	1.75			5.00
CauII + XhoI	3.95	1.05			5.00
PvuII + HindIII	3.75	1.25			5.00
PvuII + HpaI	4.95	(0.05)[a]			5.00
PvuII + BalI	5.00	-			5.00
PvuII + BstEII	4.35	0.65			5.00
PvuII + HgaI	3.95	1.05			5.00
PvuII + XbaI	2.92	2.05			4.98
PvuII + XhoI	3.60	1.38			4.98
HgiAI	3.20	1.83			5.03
HgiAI + XbaI	3.20	1.10	0.70		5.00
HgiAI + HindIII	2.25	1.83	0.95		5.03
HgiAI + HpaI	2.15	1.83	1.05		5.03
ThaI	4.30	0.68	-		4.98
ThaI + CauII	4.30	0.45	0.25		5.00
ThaI + HindIII	3.30	1.30	0.68		4.98
ThaI + XbaI	2.95	1.35	0.68		4.98
HindII	2.50	1.85	0.65		5.00
HindII + XbaI	2.50	1.47	0.65	0.38	5.00
HindII + HindIII	1.85	1.30	1.20	0.65	5.00
HindII + CauII	2.50	1.85	0.35	0.30	5.00
HaeIII	2.40	2.30	0.30		5.00
HaeIII + XbaI	2.30	2.00	0.40	0.30	5.00
HaeIII + HindIII	2.40	1.30	1.00	0.30	5.00
HaeIII + HpaI	2.40	2.30	0.20	(0.10)[a]	5.00

Table 6.9 (Cont'd)

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using plasmid DNA isolated from B. subtilis (2.9).

a, This fragment was not detected but its presence can be predicted.

Table 6.10. Fragments produced by digestion of pAB724 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
HindIII + XbaI	3.30	2.70				6.00
HindIII + HpaI	4.80	1.20				6.00
HpaI + BalI	6.00	-				6.00
BalI + BstEII	5.30	0.65				5.95
BstEII + HgaI	5.60	0.40				6.00
HgaI + XhoI	5.65	0.35				6.00
XhoI + XbaI	5.30	0.68				5.98
PvuII	3.20	2.80				6.00
PvuII + HindIII	3.20	1.55	1.25			6.00
PvuII + BalI	3.20	2.80	-			6.00
PvuII + XhoI	2.80	1.86	1.35			6.01
CauII	3.75	2.27				6.02
CauII + XbaI	3.75	1.75	0.52			6.02
CauII + HindIII	2.27	2.20	1.55			6.02
CauII + BstEII	3.75	1.90	0.35			6.00
HgiAI	3.20	2.00	0.82			6.02
HgiAI + XbaI	3.20	1.10	0.90	0.82		6.02
HgiAI + XhoI	3.20	1.60	0.82	0.40		6.02
HgiAI + HindIII	2.00	1.20	1.00	0.82		6.02
ThaI	3.15	2.20	0.64			5.99
ThaI + HindIII	2.20	1.85	1.30	0.65		6.00
ThaI + HpaI	3.05	2.20	0.64	(0.10)[b]		5.99
ThaI + XhoI	3.15	1.55	(0.64)[a]			5.98
ThaI + XbaI	3.15	1.35	0.85	0.64		5.99
HaeIII	2.50	2.30	0.90	0.30		6.00
HaeIII + HpaI	2.50	2.30	0.90	(0.20)[b]	(0.10)[b]	6.00
HaeIII + HindIII	2.50	1.30	1.00	0.90	0.30	6.00
HaeIII + XbaI	2.30	2.00	0.90	0.50	0.30	6.00
HaeIII + XhoI	2.30	1.33	1.17	0.90	0.30	6.00

Table 6.10 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	Sum of fragment sizes (Md)
HindII	2.50	1.70	1.15	0.65		6.00
HindII + HindIII	1.70	1.30	1.20	1.15	0.65	6.00
HindII + BalI	2.50	1.70	1.15	0.60	(0.05)[c]	6.00
HindII + XhoI	2.50	1.15	1.05	(0.65)[a]		5.98
HindII + XbaI	2.50	1.45	1.15	0.65	0.25	6.00

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) with plasmid DNA isolated from B. subtilis (2.9.1).

a, This fragment was over intense and assumed to be a "double".

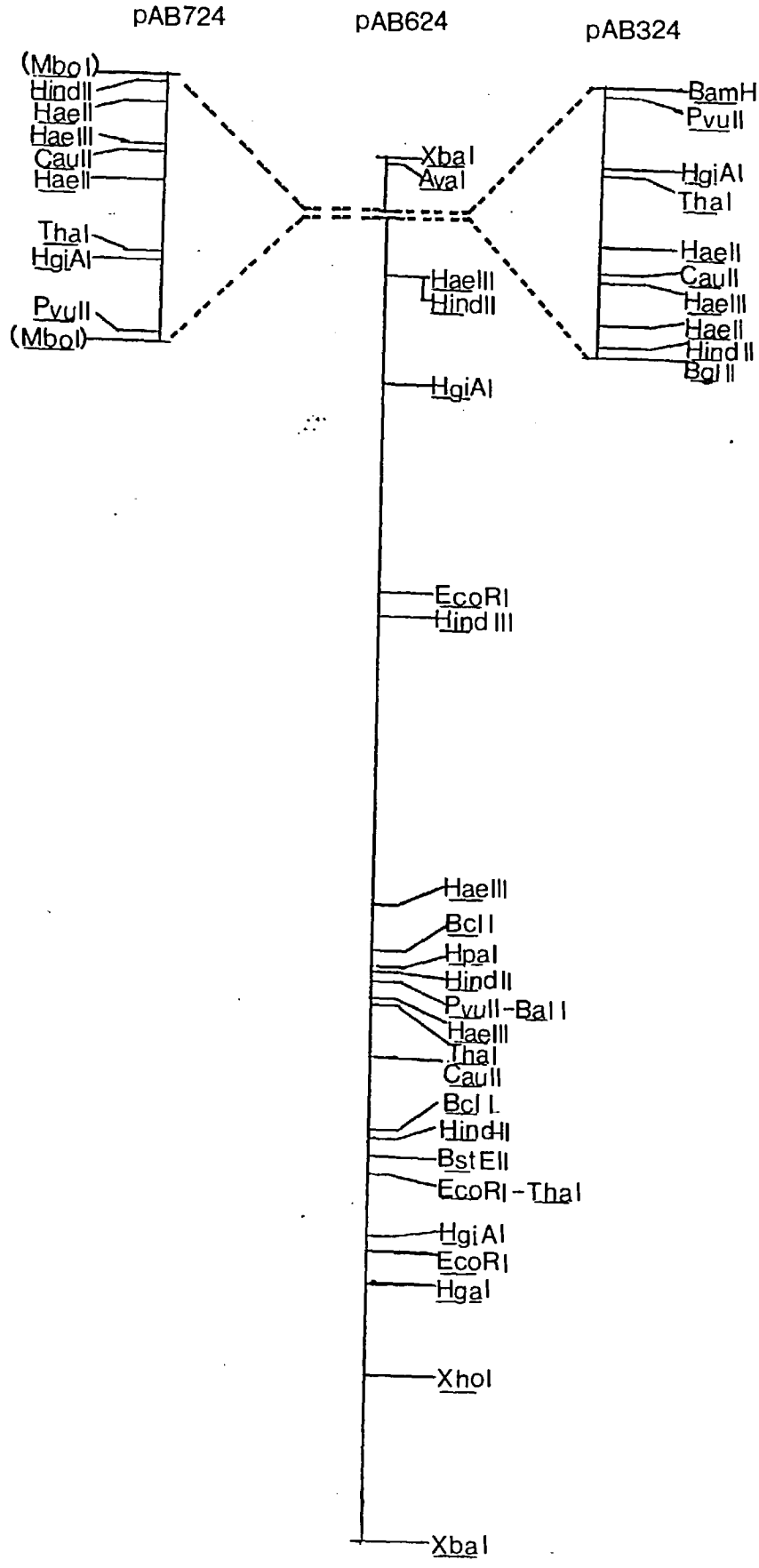
b, This fragment was too small for an accurate molecular weight determination.

c, This fragment was not detected but its presence can be predicted.

Figure 6.8. Restriction Endonuclease Cleavage Site Maps of pAB324 pAB624 and pAB724

The restriction endonuclease cleavage site map of pAB324 was constructed as described in Chapter 6 (6.1), see Fig 6.5. Plasmid pAB624 was constructed by deleting the BamHI-BglII 1 Md fragment of pAB324 while pAB724 was constructed by inversion of this fragment (Chapter 6).

FIG. 6.8


pAB724
pAB624
pAB324
(MboI)
HindII
HaeII
HaeIII
CauII
HaeII
ThaI
HgiAI
PvuII
(MboI)
XbaI
AvaI
HaeIII
HindII
HgiAI
EcoRI
HindIII
HaeIII
BclI
HpaI
HindII
PvuII-BalI
HaeIII
ThaI
CauII
BclI
HindII
BstEII
EcoRI-ThaI
HgiAI
EcoRI
HgaI
XhoI
XbaI
BamHI
PvuII
HgiAI
ThaI
HaeII
CauII
HaeIII
HaeII
HindII
BglII

6.2. Cloning of E. coli plasmid pROG29 in pAB124 and characterisation of the chimeric plasmid (pAR2)

This work was done in association with Dr. R.F. Sherwood. A fragment of yeast 2 micron DNA containing the yeast leu2 gene was cloned into the HindIII site of pBR322 (Tc^r Ap^r, Bolivar et. al., 1977b) so inactivating the Tc^r genes (R.F. Sherwood and R. Gibson, unpublished). The resulting plasmid, pROG29 (Fig 6.9) had an Ap^r Tc^s $leuB^+$ phenotype in E. coli transformants. Restriction endonuclease analysis of pROG29 revealed a single XbaI cleavage site within the yeast 2 micron DNA, and pAB124 was cloned at this site. 1 µg of each plasmid was digested with XbaI, mixed and ligated with T_4-DNA ligase after heat inactivation (Methods 2.14, 2.16). The recombinant molecules were transformed into E. coli C600 ($RecA^-$. $leuB^-.r_k^-m_k^+$) as described in Methods (2.21), and Ap^r Tc^r $leuB^+$ transformants identified (1.4×10^4 per µg DNA).

Several Ap^r Tc^r $leuB^+$ clones were examined for the presence of the chimeric plasmid (9.2 Md) as described in Methods (2.10) and 3 were identified that all contained the same plasmid of approximately 8 Md. A large-scale preparation of this plasmid, called pAB2, was carried out (Methods 2.10.1) and then analysed with several restriction endonucleases (Table 6.11). The fragments produced appear consistent with what would be generated by constructing a chimeric plasmid of pAB124 and pROG29. A restriction endonuclease cleavage site map of pAR2 was constructed from the results of double-digest experiments (Table 6.12). The relative positions of the single sites for BamHI, PstI and SalI (Fig 6.10) were consistent with their position in pROG29 (Fig 6.9).

The large AvaI fragment (A) contained the BamHI, SalI sites but not the PstI site, therefore only one orientation of the fragments is possible (Fig 6.10). Double digests involving HindIII revealed that the small fragment contained the BamHI, PstI and SalI sites. Since a HindIII + BamHI digest yields a small fragmentof 0.28 Md, and a HindIII + SalI digest generated one of 0.49 Md, only one orientation of the HindIII fragments is possible (Fig 6.10) and is consistent with a HindIII + PstI digest. The HpaI and XbaI fragments (Fig 6.10) were positioned in the same way since only one of the two fragments (HpaI (A), XbaI (A)) contained the BamHI, PstI and SalI sites. The large EcoRI fragment, A (3.70 Md) was positioned on the extreme

right of the molecule (Fig 6.10) since it contained the BamHI, PstI and SalI sites. The EcoRI fragment B (1.95 Md) was positioned to the left of EcoRI (A) since it was shown to contain a HpaI site but not an AvaI or XbaI site. This leaves a gap between EcoRI (A) and EcoRI (B) 0.31 Md into which the EcoRI (E) fragment fits (Fig 6.10). The EcoRI fragment C (1.68 Md) was positioned to the left of EcoRI (B) since it contained an XbaI, AvaI and HpaI site. (Fig 6.10.) EcoRI fragments D (0.50) and F (0.15) were therefore positioned to the left of EcoRI (C) (Fig 6.10) as in pROG29 (Fig 6.9). The relative positions of the cleavage sites in pAR2 are shown in Fig 6.11 and show that the chimeric plasmid of pROG29 and pAB124 (pAR2) had not undergone any detectable deletion or insertion upon transformation into E. coli.

The pAB124 part of pAR2 was removed from the chimeric plasmid by digestion with XbaI, followed by ligation with T_4 DNA ligase after heat inactivation. To prevent reconstitution of pAR2 the plasmid was treated prior to XbaI digestion in the following manner. Plasmid (2 µg) was digested with 2 units of BamHI and SalI for 60 min at 37°C (Methods 2.14) followed by 10 units of S1 nuclease (Uniscience Ltd., Cambridge) for 5 min at 37°C. After heating the digested DNA to 66°C for 15 min the plasmid was precipitated with cold (-20°C) ethanol, suspended in XbaI assay buffer, and digested with 2 units XbaI for 60 min at 37°C. After heat inactivation the DNA was ligated with T_4 DNA-ligase and transformed into competent B. subtilis IG20 cells. Tc^r transformants were selected and 6.5×10^2 .µg DNA^{-1} at 12 µg.ml^{-1} tetracycline were obtained. Plasmid DNA was extracted from several clones (Methods 2.9.2) and digestion of the plasmid preparations with 10 restriction endonucleases generated fragments of identical size to that of pAB124 confirming the presence of pAB124 in pAR2.

Within E. coli, pAR2 confers a Tc^r Ap^r phenotype and complementation of the leuB mutation from the yeast DNA insert. The enzyme responsible for this complementation is α-Hydroxy-β carboxy-isocaproic acid dehydrogenase and is deleted in B. subtilis leuC7 mutants. pAR2 DNA was therefore transformed into competent B. subtilis CU741 (TrpC2, LeuC7) cells and Tc^r transformants selected. Of the 1.4×10^4 (µg DNA^{-1}) Tc^r transformants obtained (12 µg.ml^{-1} tetracycline) none complemented the LeuC7 mutation or expressed ampicillin resistance. Indicating that the Ap^r genes can not be expressed in B. subtilis as already reported (Goebel et. al., 1979) and that the yeast leu2 gene can not be expressed in B. subtilis.

Figure 6.9. Restriction Endonuclease Cleavage Site Map of pROG29
(Dr. R. Gibson & Dr. R.F. Sherwood, unpublished)

The single line represents pBR322 and the double line yeast 2 μ DNA containing the yeast leu2 genes (XXXX).

Map distances

EcoRI, 0/5.30; HindIII, 0.07; EcoRI, 0.14; EcoRI, 0.63; HpaI, 1.26; AvaI, 1.47; XbaI, 1.96; HindIII, 2.31; BamHI, 2.59; SalI, 2.80; AvaI, 3.29; PstI, 4.83.

FIG. 6.9

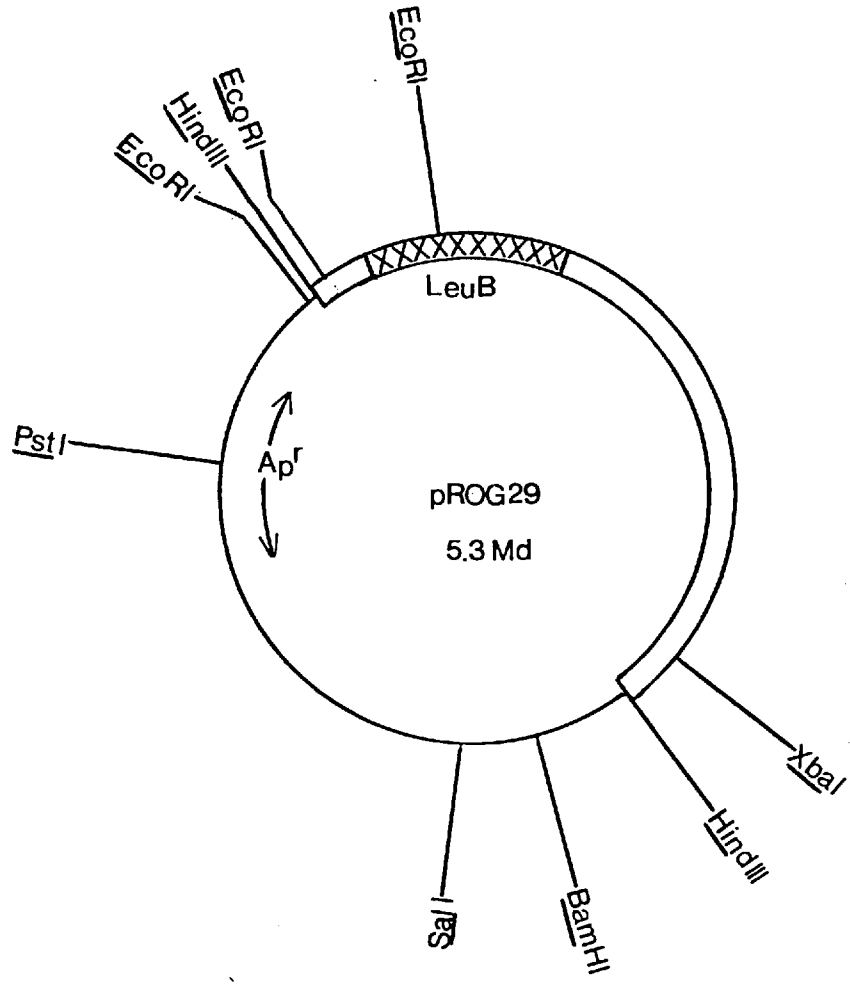

EcoRI
EcoRI
HindIII
EcoRI
LeuB
PstI
Ap^r
pROG29
5.3 Md
XbaI
HindIII
BamHI
SalI

Table 6.11. Fragments produced by digestion of pAR2, pROG29 and pAB124 with several Restriction Endonucleases

Enzyme	Fragment sizes (Md) pROG29	pAB124	pAR2
AvaI	3.50, 1.82	-	3.50, 4.72
BamHI	5.30	-	8.20
EcoRI	4.65, 0.50, (0.15)[a]	1.95, 0.61, 0.33	3.70, 1.95, 1.70, 0.50, 0.33 (0.15)[a]
HindIII	2.24, 3.05	-	5.15, 3.05
HpaI	5.30	2.90	5.90, 2.30
PstI	5.30	-	8.20
SalI	5.30	-	8.20
XbaI	5.30	2.90	5.30, 2.90

Digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using pROG29, pAR2 DNA isolated from E. coli (2.10.1) and pAB124 DNA isolated from B. subtilis (2.9.1).

a, This fragment was too small for an accurate molecular weight determination.

Table 6.12. Fragments produced by digestion of pAR2 with two Restriction Endonucleases

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	F	G	H	Sum of fragment sizes (Md)
BamHI + PstI	5.95	2.25							8.20
BamHI + SalI	8.00	(0.20)[b]							8.20
PstI + SalI	6.15	2.05							8.20
AvaI	4.70	3.50							8.20
AvaI + PstI	4.70	1.55	1.95						8.20
AvaI + BamHI	4.00	3.50	0.70						8.20
AvaI + SalI	4.20	3.50	0.49						8.19
HindIII	5.15	3.05							8.20
HindIII + PstI	5.15	2.50	0.55						8.20
HindIII + BamHI	5.15	2.75	0.28						8.18
HindIII + SalI	5.15	2.56	0.49						8.20
HpaI	5.90	2.30							8.20
HpaI + PstI	4.10	2.30	1.75						8.15
HpaI + BamHI	3.80	2.30	2.10						8.20
HpaI + SalI	3.60	(2.30)[a]							8.20

Table 6.12 (Cont'd)

Enzyme pair	Fragment sizes (Md) A	B	C	D	E	F	G	H	Sum of fragment sizes (Md)
XbaI	5.30	2.90							8.20
XbaI + PstI	2.90	2.85	2.45						8.20
XbaI + BamHI	4.65	2.90	0.65						8.15
XbaI + SalI	4.45	2.90	0.85						8.20
EcoRI	3.70	1.95	1.68	0.50	0.31	(0.15)[b]			8.29
EcoRI + PstI	3.20	1.95	1.68	(0.50)[a]	0.31	(0.15)[b]			8.29
EcoRI + BamHI	2.75	1.95	1.68	0.95	0.50	0.31	(0.15)[b]		8.26
EcoRI + SalI	2.50	1.95	1.68	1.20	0.50	0.31	(0.15)[b]		8.29
EcoRI + AvaI	2.02	1.95	1.68	(0.84)[a]	0.50	0.31	(0.15)[b]		8.29
EcoRI + HpaI	3.70	1.20	1.05	0.77	0.60	0.50	0.31	(0.15)[b]	8.26
EcoRI + XbaI	3.35	1.95	1.30	0.50	(0.35)[a]	0.31	(0.15)[b]		8.26

Double digests and agarose-gel electrophoresis were carried out as described in Methods (2.13, 2.14) using DNA isolated from E. coli (2.10.1).

a, This fragment was over intense and assumed to be a "double".

b, This fragment was too small for an accurate molecular weight determination.

Figure 6.10. Relative Positions of Fragments Produced by Digestion of pAR2 with Several Restriction Endonucleases

The relative position of the fragments was determined from the results of double-digest experiments (Table 6.12) as described in Chapter 6 (6.2).

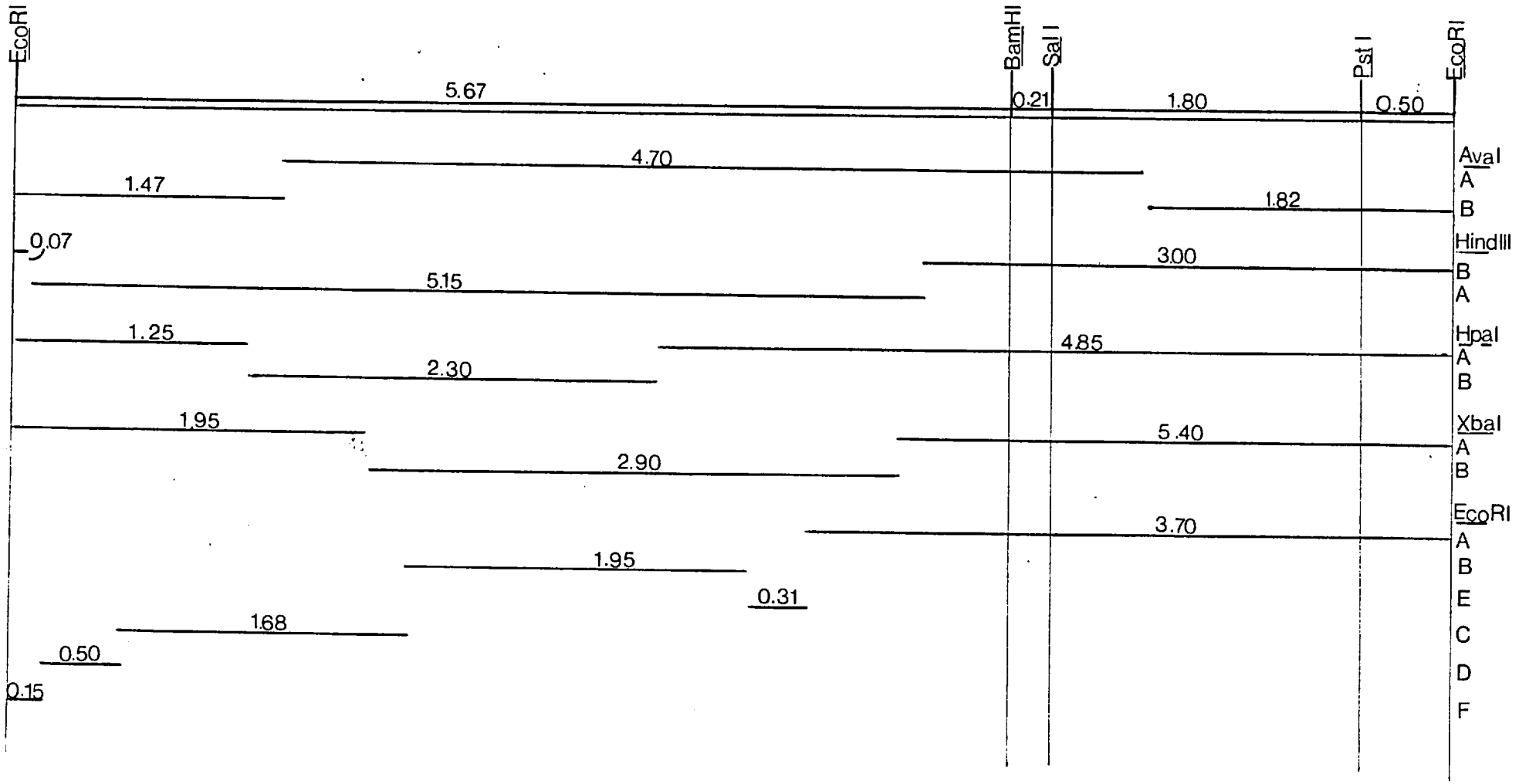

EcoRI
BamHI
Sal I
Pst I
EcoRI
5.67
0.21
1.80
0.50
4.70
1.47
1.82
0.07
3.00
5.15
1.25
4.85
2.30
1.95
5.40
2.90
3.70
1.95
0.31
1.68
0.50
0.15
AvaI
A
B
HindIII
B
A
HpaI
A
B
XbaI
A
B
EcoRI
A
B
E
C
D
F

FIG. 6.10

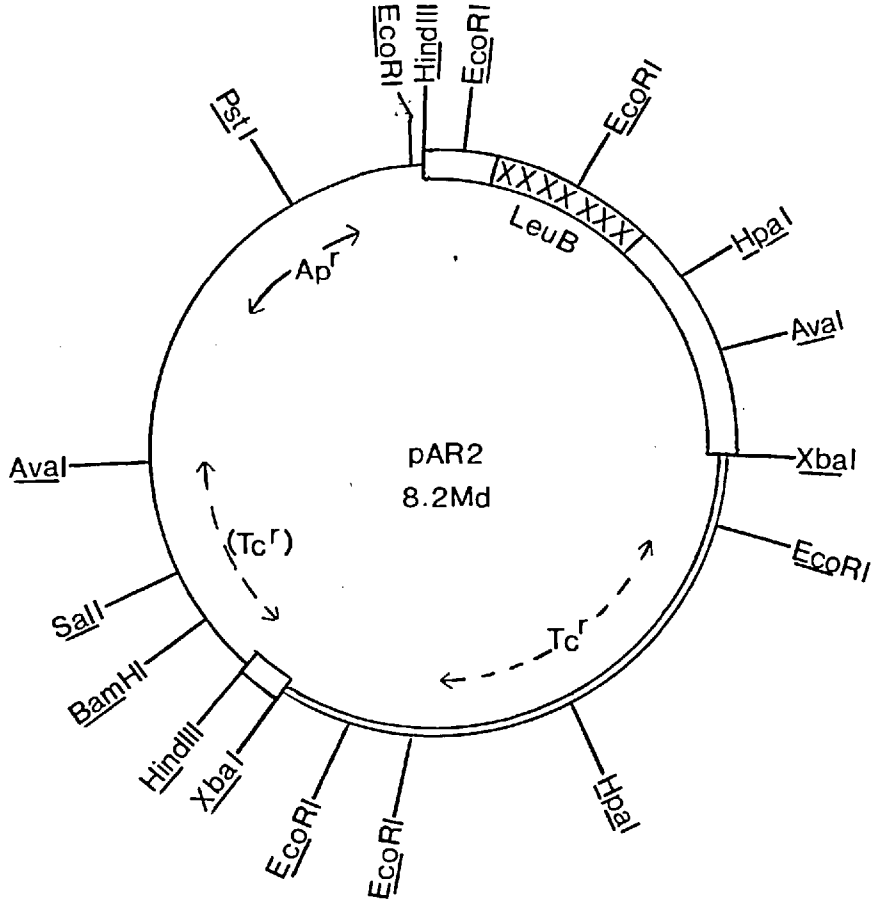
Figure 6.11. Restriction Endonuclease Cleavage Site Map of pAR2

The restriction endonuclease cleavage site map was constructed as described in Chapter 6 (6.2) from data shown in Table 6.12. The single line represents pBR322; the wide double line, yeast 2 μ DNA containing the yeast leu2 genes (XXXX) and the narrow double line, pAB124.

Map distances

EcoRI, 0/8.2; HindIII, 0.07; EcoRI, 0.14; EcoRI, 0.63; HpaI, 1.26; AvaI, 1.47; XbaI, 1.95; EcoRI, 2.31; HpaI, 3.35, EcoRI, 4.26; EcoRI, 4.55; XbaI, 4.80; HindIII, 5.15; BamHI, 5.45; SalI, 5.65; AvaI, 6.38; PstI, 7.70.

FIG. 6.11


EcoRI
HindIII
EcoRI
EcoRI
Pst I
XXXXXXXX
LeuB
HpaI
AvaI
XbaI
EcoRI
HpaI
EcoRI
EcoRI
XbaI
HindIII
BamHI
SalI
AvaI
Ap^r
(Tc^r)
Tc^r
pAR2
8.2Md

6.3. Other cloning experiments involving pAB124 and pAB224

Attempts were made to clone the β-lactamase gene from the Staphylococcal plasmid pI258 which confers penicillin and erythromycin resistance and tolerance to several heavy metal ions (Peyru et. al., 1969). The β-lactamase gene lies between two EcoRI sites of pI258 (Novick et. al., 1979) and was ligated into EcoRI digested pAB224 as described in Methods (2.14, 2.16). The recombinant molecules were transformed into competent B. subtilis IG20 cells and Tc^r transformants (12 $\mu g.ml^{-1}$ tetracycline) selected. Of the 4.9×10^3 transformants obtained ($\mu g\ DNA^{-1}$) no Tc^r Ap^r clones were identified. The reason for this failure to clone the Ap^r gene is explained below when discussing failed 'shot gun' clonings.

Several attempts were made to clone chromosomal genes from B. subtilis, B. stearothermophilus and yeast into the XbaI site of pAB124 and the EcoRI site of pAB224 and are summarised in Table 6.13. These were all unsuccessful: none of the transformants obtained that could complement an auxotrophic mutation contained any inserted DNA in the vector. This inability to clone chromosomal genes in the vectors in B. subtilis was explained by the examination of the transforming efficiency of different plasmid species (Canosi et. al., 1978). Canosi and co-workers found that the monomeric plasmid species transformed at an extremely low efficiency, in fact one thousandth of that of a dimer and a hundred thousandth of that of a trimer. Shot gun experiments to clone chromosomal fragments will tend to produce mainly recombinant molecules of one copy of the plasmid plus a chromosmal insert. The possibility of transforming competent B. subtilis cells with such a molecule is virtually zero and therefore 'shot gun' experiments will only produce transformants of the vector recircularised during ligation to produce a dimer or other oligomer. These problems in cloning chromosmal genes has also been found by other workers (Gryczan et. al., 1980). There has been only one report of cloning chromosmal genes in B. subtilis by transformation of competent cells, where the trp genes were cloned in the Staphylococcal vector pUB110 (Keggins et. al., 1978). An alternative method of transforming B. subtilis with recombinant molecules is therefore required.

The high frequency transformation of B. subtilis protoplasts by plasmid DNA was reported by Chang and Cohen (1979). Using their

method 10^5, Tc^r transformants(per μg pAB124 DNA)in B. subtilis IG20 was obtained with about 40% regeneration of protoplasts. Incorporation of the modifications recommended by DeCastro-Costa & Landman (1977) and Gabor & Hotchkins (1979) improved regeneration to 80-85% and transformation to 10^6.μg DNA^{-1}. The procedure is described fully in Methods (2.20.2). Time did not allow the use of this method for cloning B. subtilis chromosomal genes since substantial development would be required. Chang & Cohen (1979) noted that plasmid DNA that had been cut with a restriction endonuclease and then ligated transformed protoplasts at one hundreth the frequency of untreated plasmid. Also regeneration of protoplasts requires a highly complex medium making initial selection of transformants containing recombinant plasmids complementing auxotrophic requirements impossible. No regeneration in a minimal medium has been reported.

SUMMARY

(1) A hybrid plasmid of pUB110 (Neo^r) and pAB124 (Tc^r) was constructed and shown to have a Neo^s Tc^r phenotype.

(2) A derivative of the pUB110: pAB124 hybrid was isolated, pAB324, and shown to have a Neo^r Tc^r phenotype.

(3) A restriction endonuclease cleavage site map of pAB324 was constructed and by comparison with the pUB110: pAB124 hybrid was shown to have undergone a minimum of 2 deletions and 1 insertion. The inserted DNA was shown to originate from the B. subtilis chromosome.

(4) An E. coli recombinant plasmid containing a region of yeast DNA (leu2 genes present) was cloned in pAB124 to produce pAR2. Expression of the yeast leu2 genes in B. subtilis was not obtained.

(5) Attempts to clone B. subtilis chromosomal functions in the vectors pAB124, pAB224 by transformation of recombinant molecules into competent B. subtilis cells were not successful.

Table 6.13. Summary of Cloning experiments involving Chromosomal DNA and pAB124 and pAB224

Strain	Host Genotype	Requirements	Vector (endonuclease)	Source of DNA	Selection of Transformants[a]
IG20	trpC2 r⁻.m⁻.	trp	pAB124 (XbaI)	(1) B. subtilis	trp⁺.Tc^r
				(2) RS93	
			pAB224 (EcoRI)	(1) B. subtilis[1]	
				(2) RS93	
QB944 (K1)	purA16	adenine	pAB124 (XbaI)	(1) Yeast	ade⁺.Tc^r
	cysA14	cys		(2) RS93	cys⁺.Tc^r
	trpC2	trp	pAB224 (EcoRI)	(1) Yeast	trp⁺.Tc^r
				(2) RS93	
QB928 (K2)	aroI906	phe+tyr+trp	pAB124 (XbaI)	(1) B. subtilis	phe⁺.tyr⁺.trp⁺.Tc^r.
	purB33	adenine		(2) RS93[2]	ade⁺.Tc^r
	dal	D-ala		(3) Yeast	D-ala⁺.Tc^r
	trpC2	trp			trp⁺.Tc^r
QB935 (K6)	aroD120	phe+tyr+trp	pAB124 (XbaI)	(1) B. subtilis	phe⁺.tyr⁺.trp⁺.Tc^r
	lys1	lys		(2) RS93	lys⁺.Tc^r
	trpC2	trp	pAB224 (EcoRI)	(1) B. subtilis	trp⁺.Tc^r
				(2) RS93[3]	

Table 6.13 (Cont'd)

Strain	Host Genotype	Requirements	Vector (endonuclease)	Source of DNA	Selection of Transformants[a]
QB936 (K7)	leuA8	leu	pAB124 (XbaI)	(1) B. subtilis[4]	$leu^+.Tc^r$.
	aroG932	phe+tyr		(2) RS93	$phe^+.tyr^+.Tc^r$
	ald	L-ala		(3) Yeast	$L\text{-}ala^+.Tc^r$
	trpC2	trp	pAB224 (EcoRI)	(1) B. subtilis[5]	$trp^+.Tc^r$
				(2) RS93	
				(3) Yeast	
QB917 (K8)	hisA1	his	pAB124 (XbaI	(1) RS93	$his^+.Tc^r$
	thr5	thr		(2) Yeast	$thr^+.Tc^r$
	trpC2	trp	pAB224 (EcoRI	(1) RS93	$trp^+.Tc^r$
				(2) Yeast	
BD224	thr5	thr	pAB124 (XbaI)	(1) B. subtilis[6]	$thr^+.Tc^r$
	trpC2	trp		(2) RS93	$trp^+.Tc^r$.
	recE4			(3) Yeast	
			pAB224 (EcoRI)	(1) B. subtilis	
				(2) RS93	
				(3) Yeast	

Legend to Table 6.13

Plasmid DNA was purified from B. subtilis QB936(K7) as described in Methods (2.9.1). B. subtilis and B. stearothermophilus RS93 chromosomal DNA was purified as described in Methods (2.11). Yeast DNA from Saccharomyces cerevisiae was kindly provided by Dr R.F. Sherwood.

Endonuclease digestions and ligations with T_4 DNA ligase were carried out as described previously (2.14, 2.16). Transformations were carried out with competent B. subtilis cells as described in Methods (2.20.1), and selection was carried out in SMS defined medium containing appropriate additions.

(a) Tc^r transformants were also selected and the number obtained varied from $1.0 \times 10^3 - 2.5 \times 10^4$ ($\mu g \cdot DNA^{-1}$) for the experiments carried out.

(1) 5 trp^+Tc^r transformants were obtained, but the vector contained no inserted DNA.

(2) 1, ala^+Tc^r, 2 trp^+Tc^r transformants were obtained but the vector contained no inserted DNA.

(3) 2, ala^+Tc^r, 1 trp^+Tc^r transformants were obtained but the vector contained no inserted DNA.

(4) 2 trp^+Tc^r transformants were obtained but the vector contained no inserted DNA.

(5) 1 trp^+Tc^r transformant was obtained but the vector contained no inserted DNA.

(6) 4 trp^+Tc^r transformants were obtained but the vector contained no inserted DNA.

CHAPTER SEVEN

TRANSFORMATION OF BACILLUS STEAROTHERMOPHILUS WITH PLASMID DNA

7.1. Transformation of thermophilic bacilli by competence induction and $CaCl_2$ treatment

Two strains of thermophilic bacilli with known auxotrophic markers were examined, B. stearothermophilus NCA1503 (met^- ilv^- bio^- nic^- thi^-) and B. stearothermophilus RS93 (his^- pro^- arg^-), R.J. Sharp, pers. comm.

Each strain was grown in 200 ml AD defined medium (Methods 2.1) containing 0.5 ml or 1.0 ml Casein hydrolysate (5% W/V) at 50°C (above this temperature growth was too rapid). The optical density (550 nm) was monitored at 30 min intervals and when the rate of increase was decreasing (Fig 7.1) 0.5 ml samples of the culture were taken at 30 min intervals and transformed with pAB124 (1 µg) as described for B. subtilis (2.20.1); except, incubations were carried out at 50°C. Transformants were selected on TSBA plates containing tetracycline at 6 $\mu g.ml^{-1}$ or 12 $\mu g.ml^{-1}$. No Tc^r transformants were obtained with either strain. This might suggest that induction of competence in these strains can not be achieved in the same way as for B. subtilis.

Each strain was grown in 50 ml TYS and 50 ml AD medium containing 1 ml glucose (40% W/V) overnight at 50°C. 10 ml was used to inoculate 90 ml fresh medium and incubation continued for 60 min at 50°C. The cells were harvested by centrifugation (6000 xg for 15 min) and treated with $CaCl_2$ as described for E. coli (Methods 2.21). Transformation of the cells (0.4 ml) with 2 µg pAB124 and recovery (50°C) was carried out as described in Methods (2.21). Substantial lysis of the cells during the recovery step occurred and no transformants were obtained on TSBA plates containing tetracycline (6 $\mu g.ml^{-1}$ or 12 $\mu g.ml^{-1}$). Several variations of this procedure were attempted. Firstly, the conditions of the heat-shock step were varied; 1 min at 37°C, 5 min at 37°C, 10 min at 37°C, 1 min at 50°C, 5 min at 50°C and 10 min at 50°C were used. No reduction in lysis was observed, nor any Tc^r transformants obtained. Secondly, the amount of $CaCl_2$ present during the transformation, normally 75 mM, was varied and the following concentrations used; 100 mM, 75 mM, 50 mM, 25 mM, 10 mM and none. No reduction in lysis was observed unless $CaCl_2$ was omitted, nor any Tc^r transformants obtained.

It was therefore assumed that the E. coli transformation procedure could not be applied to B. stearothermophilus due to the

$CaCl_2$ promoted autolysis of the cells.

FIG. 7.1a (RS93)

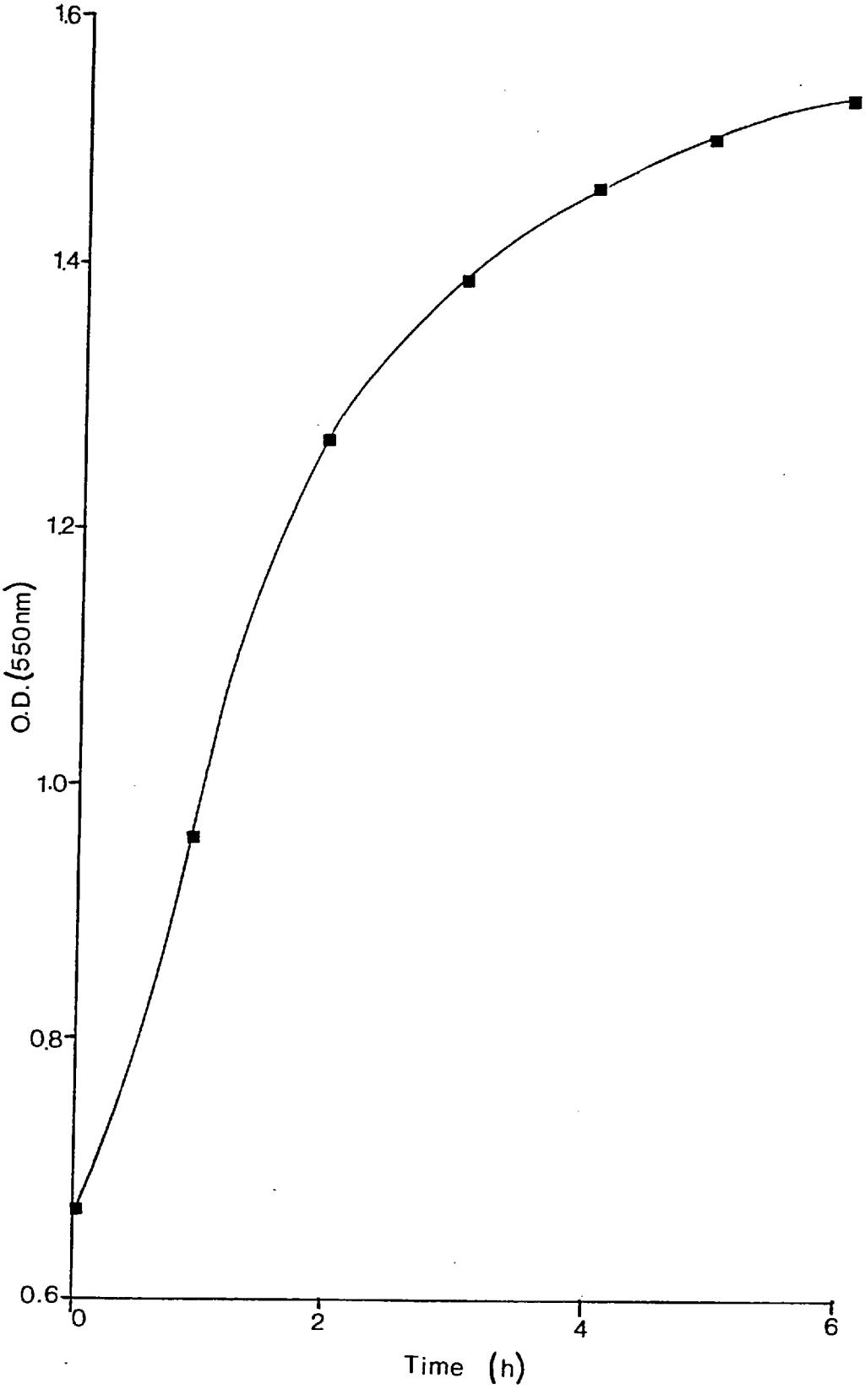


FIG. 7.1b (NCA1503)

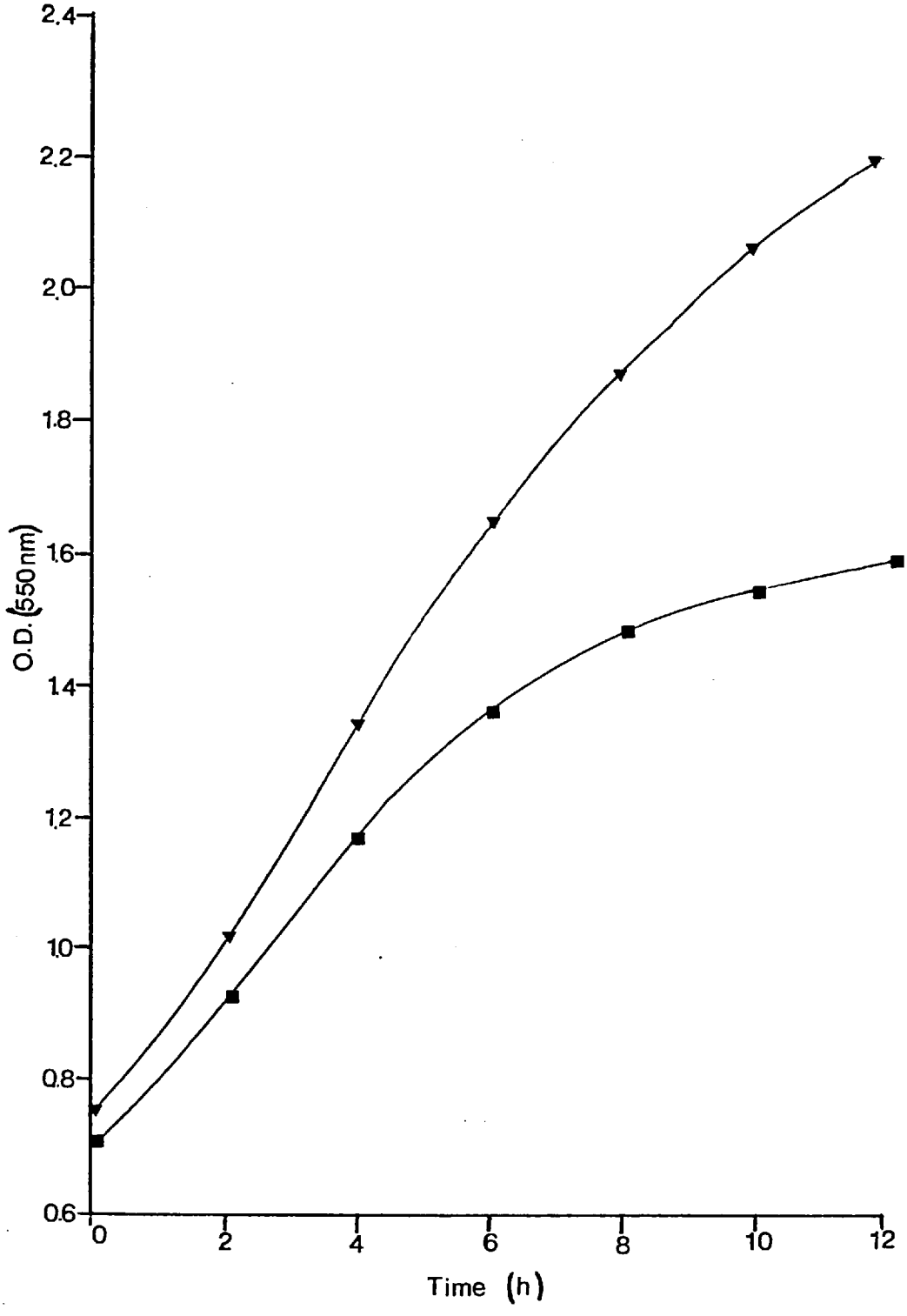


Figure 7.1. Growth Cycle of *B. stearothermophilus* NCA1503 and *B. stearothermophilus* RS93 in Minimal Medium

Cultures were grown in 200 ml of AD medium (2.1.1.) containing either 0.5 ml (■———■) or 1.0 ml (▼———▼) casein hydrolysate (5% w/v) as described in Chapter 7 (7.1).

7.1.a. *B. stearothermophilus* RS93

7.1.b. *B. stearothermophilus* NCA1503

7.2. Preparation of protoplasts of thermophilic bacilli strains

The conditions for protoplast formation of mid-log cells of *B. subtilis* are the addition of 2 $mg.ml^{-1}$ lysozyme and incubation at 37°C for 30 min (Chang & Cohen, 1979) or 200 $\mu g.ml^{-1}$ lysozyme incubated at 42°C for 30 min (Gabor & Hotchkins, 1979) in SMM medium (Methods 2.1).

The amount of lysozyme required and length of incubation was determined for the following four strains, *B. stearothermophilus* NCA1503, *B. stearothermophilus* RS93, *B. stearothermophilus* LO2 (TB124 cured of plasmid pAB124; Chapter 4) and *B. caldovelox*.

A fresh overnight culture (55°C) in TYS (20 ml) was used to inoculate 200 ml of fresh medium and incubation continued. When the OD_{550} reached 0.8-1.0 (mid-log) 50 ml of culture was centrifuged at 6000 xg for 10 min (20°C) and the cells resuspended in 2 ml SMM. Lysozyme was then added (see below) and the suspension incubated at 37°C or 42°C. Protoplast formation was observed under the microscope by conversion of the rods to spheres.

B. stearothermophilus strains NCA1503, LO2 and RS93 all gave a similar result when mid-log cells grown in TYS medium were treated with lysozyme. At 37°C a 2-3 h incubation with 4 $mg.ml^{-1}$ lysozyme gave only about 10% protoplast formation. Increasing the amount of lysozyme to 10 $mg.ml^{-1}$ gave no significant improvement. When the incubation temperature was increased to 42°C about 25% protoplast formation was achieved with 2 $mg.ml^{-1}$ lysozyme. *B. caldovelox* however was much more sensitive to lysozyme. Incubation at 42°C with 10 $\mu g.ml^{-1}$ lysozyme caused substantial lysis, indicating that there was not sufficient osmotic stabilisation in SMM. Increasing the concentration of sucrose in SMM from 0.5 M to 0.6 M (SMM 6) prevented lysis with *B. caldovelox*. Complete protoplast formation was achieved by 30 min incubation at 42°C with 100 $\mu g.ml^{-1}$ lysozyme in SMM6.

Samples of the protoplast suspensions of the four strains were diluted in SMM or SMM6 and plated on the regeneration medium(DM3) of Chang & Cohen (1979) containing succinate at 0.5 M or 0.6 M. The results are shown in Table 7.1. No regeneration of protoplasts was achieved with *B. stearothermophilus* strains NCA1503, RS93 or *B. caldovelox*; cells could not grow on DM3 (0.6 M succinate) plates. With *B. stearothermophilus* LO2 a 10 fold increase in cell count was obtained on DM3. To overcome the inhibition of growth on DM3 plates exhibited by *B. stearothermophilus* NCA1503, RS93 and *B. caldovelox* a variety of

different chemicals for osmotic production were examined for their effect on growth of the strains.

An overnight culture of each strain in TYS was used to inoculate (0.05 ml) 10 ml of TYS containing varying concentrations of osmotic stabiliser (0.1-1.5 M) prepared from stock solutions of double strength TYS (containing 1% glucose) and 3 M osmotic stabiliser solution in H_2O. Cultures were incubated overnight at 55°C and the optical density measured. The results are shown in Fig 7.2 for glucose, glycerol, glutamate, sorbitol, succinate, sucrose, lactose, rhamnose, inositol and mannitol.

Growth of B. stearothermophilus NCA1503 and B. caldovelox appeared to be less inhibited by glucose and glycerol at the concentrations that might be expected to stabilise protoplasts ie 0.6 M (Fig 7.2). However, on solid media (TSBA) NCA1503 would not grow in the presence of 0.2 M glucose or glycerol. Growth of strain LO2 was not substantially inhibited by glucose, glycerol, glutamate, sucrose, succinate or sorbital (Fig 7.2).

The stabilisation of B. caldovelox protoplasts by glucose and glycerol was examined. A mid-log culture was prepared as described previously. Samples (30 ml) of the culture were centrifuged (6000 xg for 10 min) and the cells resuspended in 20 mM Tris-HCl pH 7.5, 20 mM maleic acid containing glycerol at concentrations ranging from 0.2-2.0 M. Lysozyme (1 mg) was added and the suspension incubated at 42°C for 30 min. No concentration of glycerol was found to stabilise the protoplasts, lysis being evident after 10 min incubation. This suggested that the protoplasts were sensitive to glycerol even at a concentration likely to provide osmotic protection. A similar experiment with glucose indicated that 0.6 M gave osmotic protection to the protoplasts of B. caldovelox and was used as the basis of a regeneration medium. DM3 regeneration medium was used where succinate was replaced by 0.6 M glucose (DM3-G). The concentration of glucose in these plates was slightly inhibitory to growth. Cells produced only very small colonies after 24 h incubation (55°C) and no significant amount of protoplast regeneration was obtained. Cells (10^9) in SMM6 were treated with 10 µg. ml^{-1} lysozyme at 42°C for 30 min and dilutions in SMM6 made. TSBA plates gave a cell count of $2.2 \times 10^5 . ml^{-1}$ while DM3-G gave 2.0×10^6 colonies. ml^{-1}. The addition of BSA, 1% (w/v) or gelatine, 0·5% (w/v) to the protoplast dilution medium and regeneration environment (Gabor & Hotchkins, 1979) increased the regeneration slightly, 2.6×10^6 colonies. ml^{-1} were obtained. The addition of dead B. caldovelox cells (autoclaved) to DM3-G plates (Clive & Landman, 1970), or high gelatine concentrations

10-20% (w/v), (Landman et. al., 1968) did not induce reversion to the bacillary form.

Using the optimum conditions for B. caldovelox protoplast formation and growth on DM3-G several transformations were carried out with pAB124 DNA using the PEG induced method for B. subtilis (Methods 2.20.2). No Tc^r transformants were obtained.

Growth of B. stearothermophilus LO2 was not inhibited by high concentrations of most of the osmotic stabilisers used to the same extent as B. stearothermophilus NCA1503 and B. caldovelox (Fig 7.2). The production of stable protoplasts in the presence of sorbitol, succinate and glutamate was examined in preference to glucose, glycerol, or sucrose since the latter three promote polysaccharide production by LO2. It was found that protoplasts of B. stearothermophilus LO2 could not be stabilised by any concentaration of sorbitol like B. caldovelox protoplasts with glycerol. However, both succinate and glutamate could be used for preparing protoplasts by strain LO2.

A protoplast suspension in SMM6 was prepared from mid-log cells as described in Methods (2.22) and dilutions made in SMM6 + 1% (w/v) BSA. A sample (0.1 ml) of each dilution was spread on DM4 plates containing differing concentrations of glutamate, and DM5 plates containing differing concentrations of succinate. The results (Table 7.2) indicate that 0.6 M succinate and 0.9 M glutamate were optimum for protoplast regeneration.

Table 7.1. Regeneration of protoplasts of thermophilic bacilli strains on DM3 regeneration medium

Protoplasts	Colonies (ml^{-1})		
	DM3 (0.5 M Succinate)	DM3 (0.6 M Succinate)	TSBA[a]
NCA1503	0	0	2.8×10^5
RS93	0	0	1.5×10^5
LO2	1.3×10^4	1.5×10^5	1.2×10^4
B. caldovelox	0	0	1.4×10^3

Protoplasts were prepared from 1×10^9 cells as described in text using 2 $mg.ml^{-1}$ lysozyme for NCA1503, RS93, LO2 and incubation at 42°C for 60 min; and 100 $\mu g.ml^{-1}$ lysozyme for B. caldovelox with incubation at 42°C for 30 min.

a, This represents osmatically insensitive rods.

FIG. 7.2a (NCA1503)

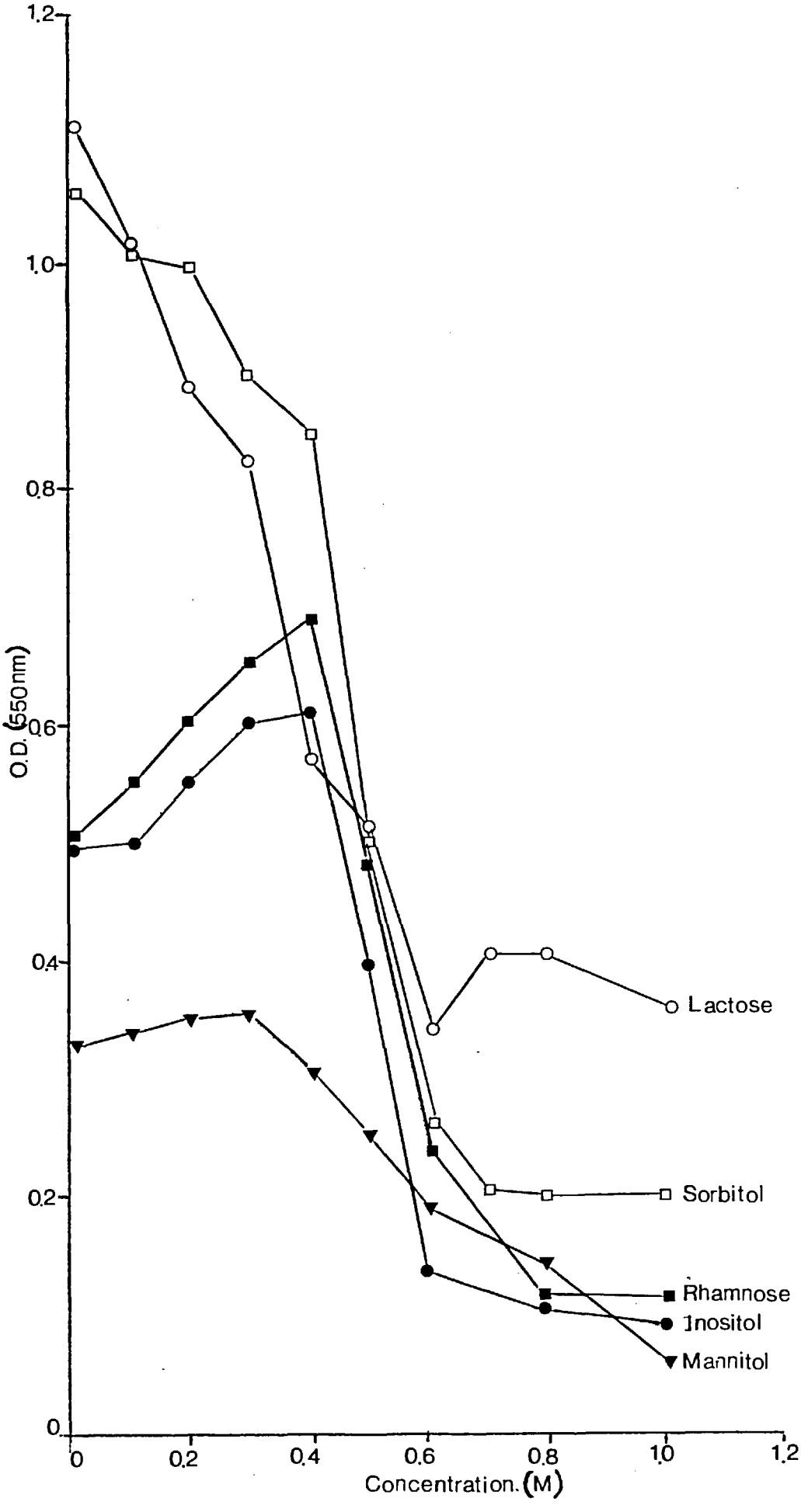


FIG. 7.2b (NCA1503)

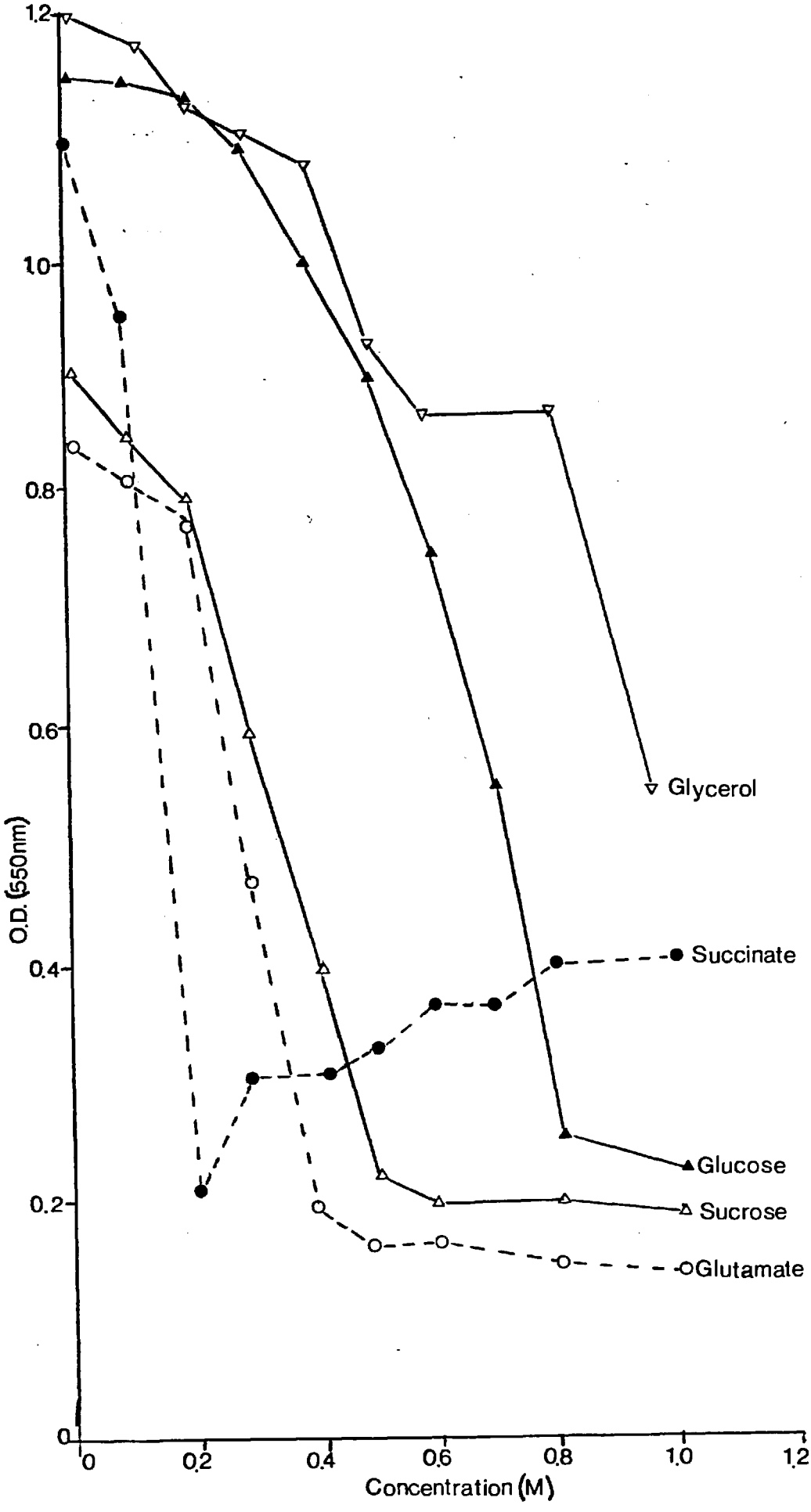


FIG. 7.2c (B caldovelox)

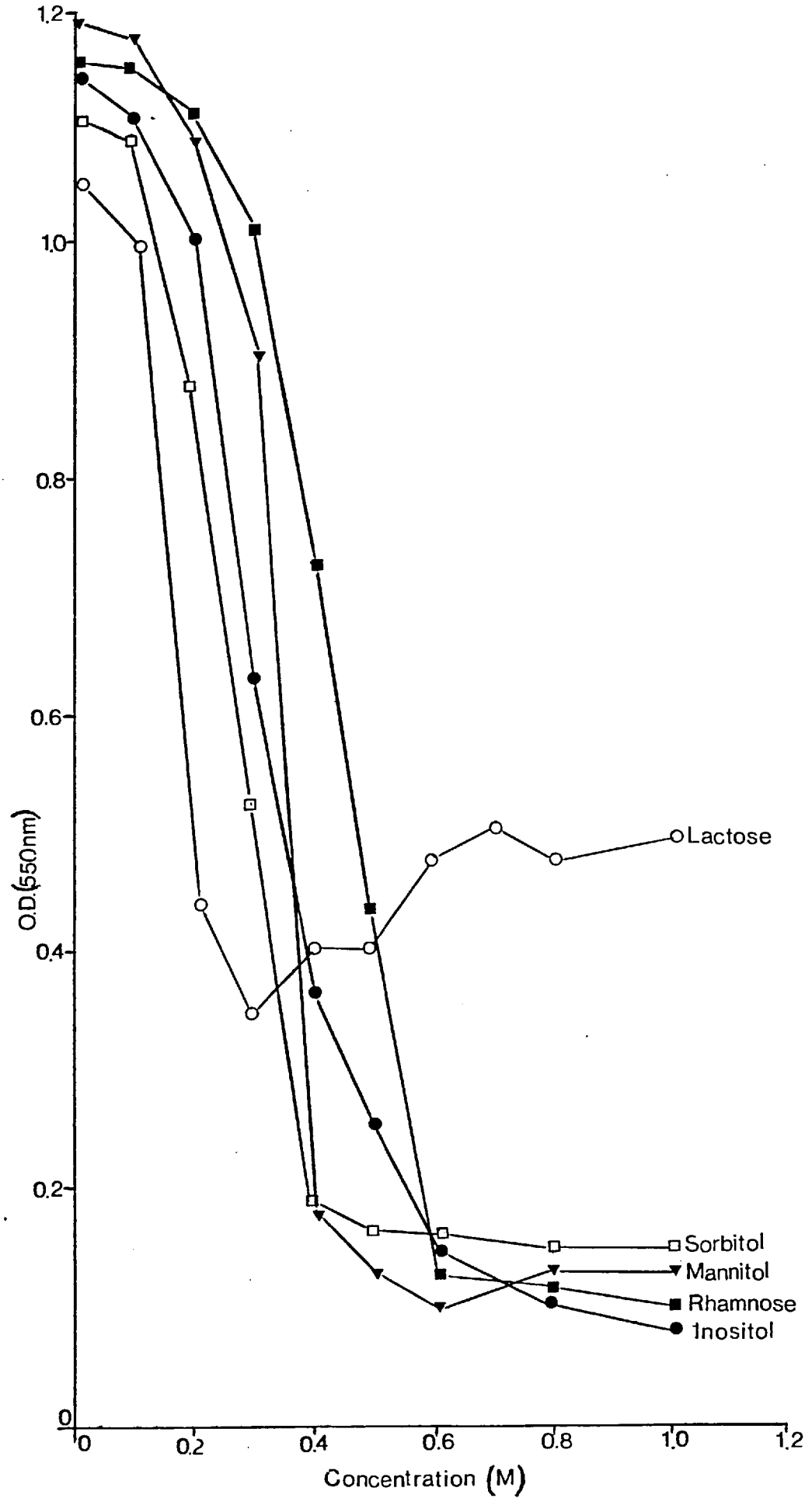


FIG. 7.2d (B.caldovelox)

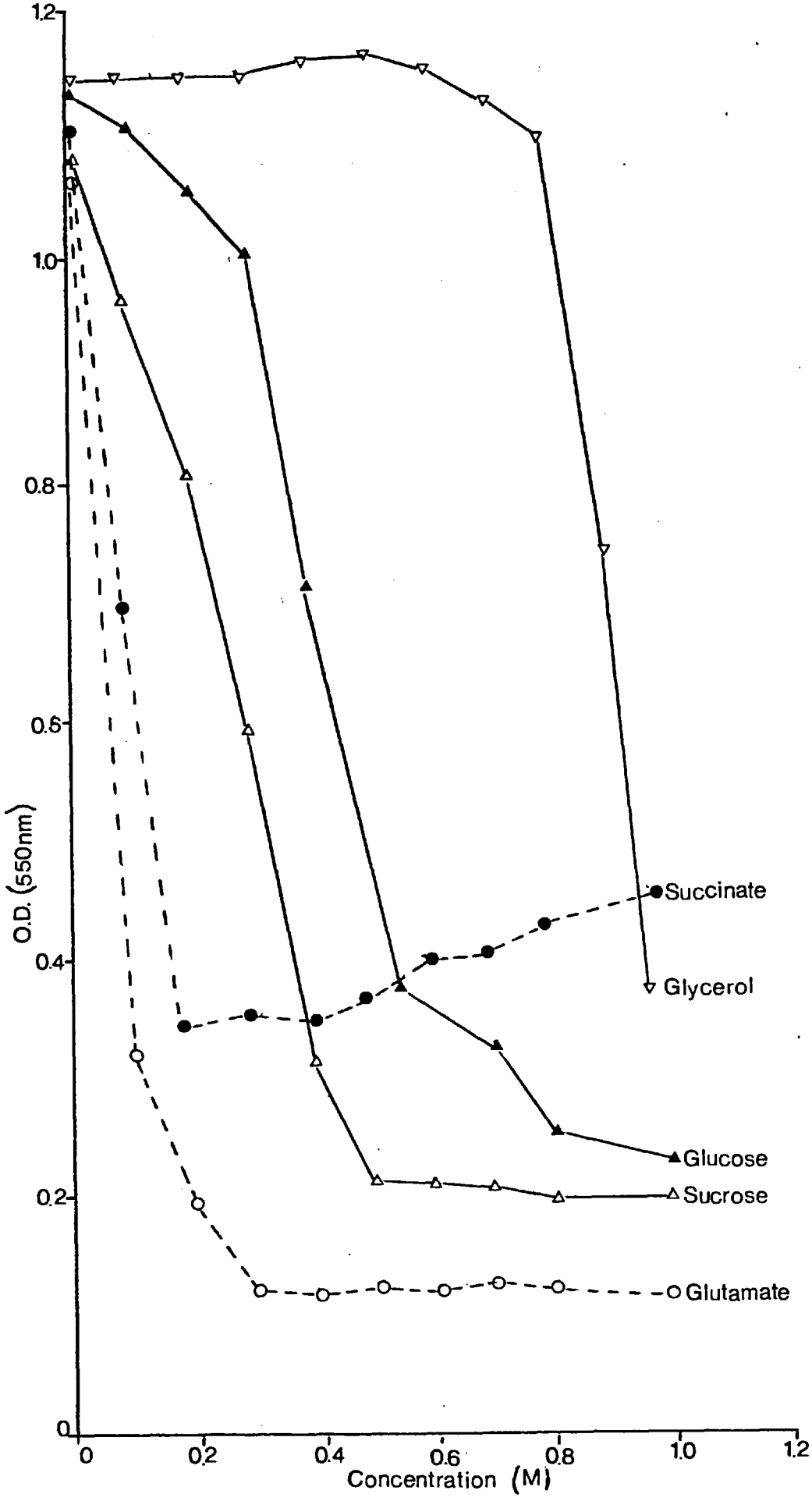


FIG. 7.2e (LO2)

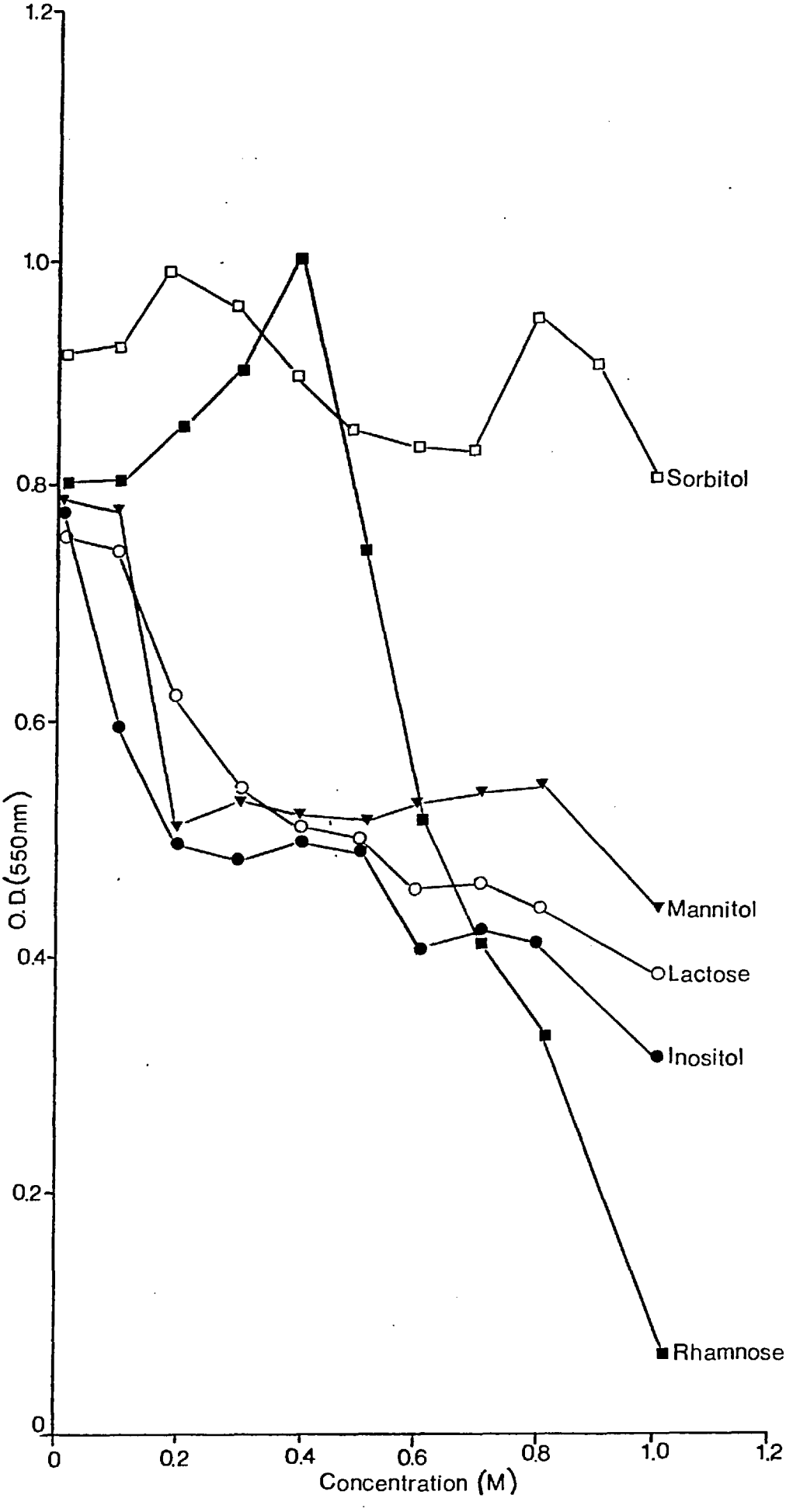

1.2
1.0
0.8
0.6
0.4
0.2
0
O.D.(550nm)
0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration (M)
Sorbitol
Mannitol
Lactose
Inositol
Rhamnose

FIG. 7.2f (LO2)

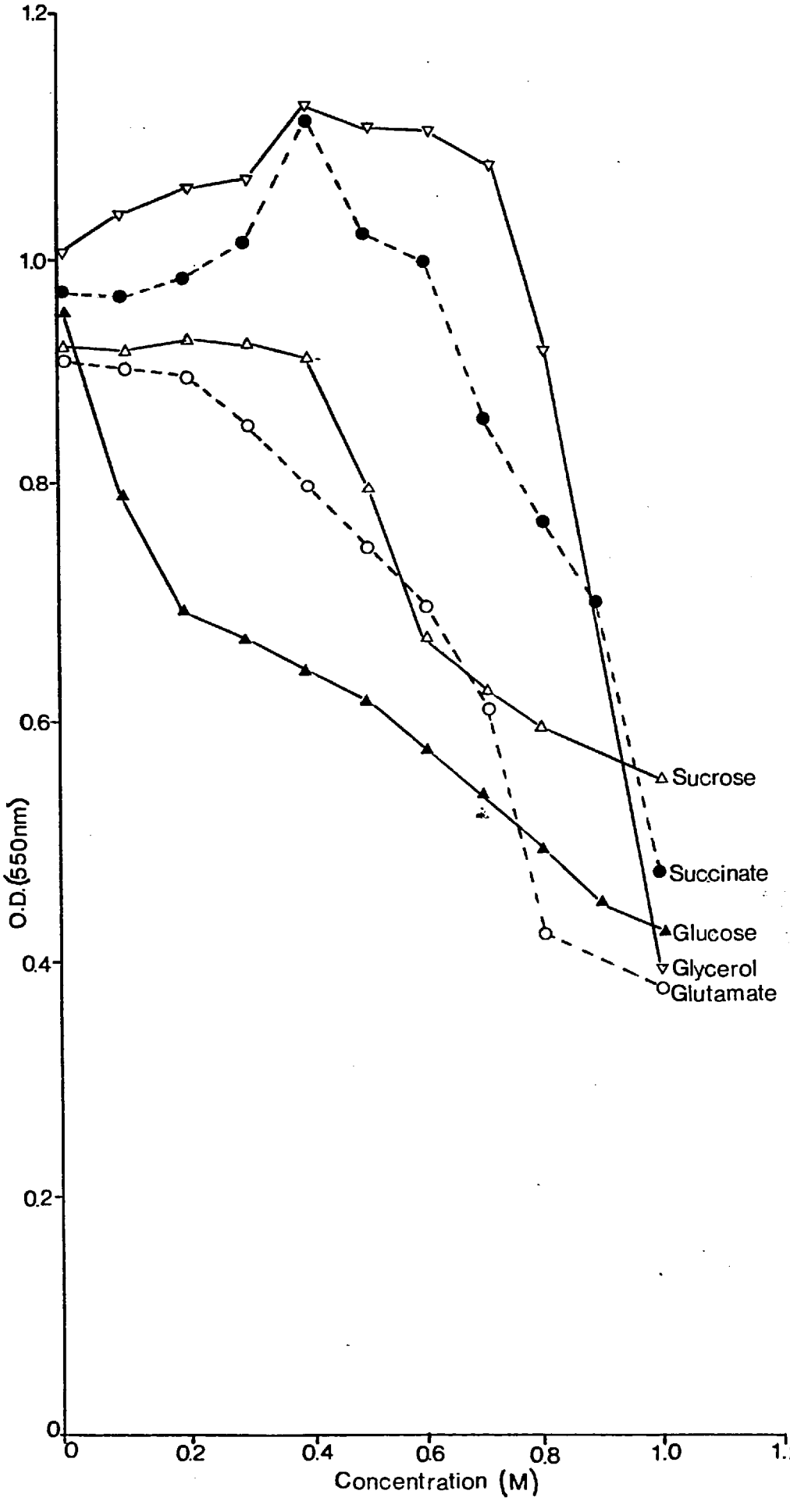

1.2
1.0
0.8
0.6
0.4
0.2
0
O.D.(550nm)
0
0.2
0.4
0.6
0.8
1.0
1.2
Concentration (M)
Sucrose
Succinate
Glucose
Glycerol
Glutamate

Figure 7.2. Growth of B. stearothermophilus NCA1503, B. stearothermophilus LO2 and B. caldovelox in the Presence of Chemicals used for Osmotically Stabilising Protoplasts

Cultures were grown as described in Chapter 7 (7.2) with varying concentrations of: Inositol (●———●)

Lactose (o———o)

Rhamnose (■———■)

Sorbitol (□———□)

Mannitol (▼———▼)

Glycerol (▽———▽)

Glucose (▲———▲)

Sucrose (△———△)

Succinate (●-------●)

Glutamate (o-------o)

7.2.a., 7.2.b. B. stearothermophilus NCA1503

7.2.c., 7.2.d. B. caldovelox

7.2.e., 7.2.f. B. stearothermophilus LO2

Table 7.2. Effect of succinate or glutamate concentration on strain LO2 protoplast regeneration

Concentration (M)	Colonies (ml^{-1})	
	DM4 + Glutamate	DM5 + Succinate
0	3.0×10^3	3.0×10^3
0.4	3.0×10^3	3.0×10^3
0.5	3.0×10^3	2.0×10^6
0.6	3.0×10^3	5.0×10^7
0.7	5.1×10^3	3.1×10^6
0.8	4.0×10^4	2.0×10^5
0.9	1.1×10^7	3.0×10^4
1.0	8.0×10^6	3.0×10^3
1.1	5.0×10^6	3.0×10^3
1.2	2.1×10^6	3.0×10^3

Protoplasts were prepared from 1×10^9 cells as described in Methods (2.22); dilutions made in SMM6 + 1% BSA and samples (0.1 ml) spread on DM4 or DM5 plates with varying concentrations of glutamate or succinate. Plates were incubated for 2 days at $50^{\circ}C$.

7.3 Transformation of B. stearothermophilus LO2 protoplasts with plasmid DNA

Protoplasts of strain LO2 were prepared with 1 mg.ml^{-1} lysozyme as described in the previous section and Methods (2.22), and transformed with 1 µg pAB124 DNA isolated from TB124 as described in Methods (2.22). After incubation in SMM6P for 1-5 h to allow for phenotypic expression of the genetic determinants carried on the plasmid dilutions were made in SMM6 + 1% (w/v) BSA and samples (0.1 ml) spread on DM5 plates (0.6 M succinate) and DM5 + tetracycline (6 µg.ml^{-1} and 12 µg.ml^{-1}). Tc^r transformants did appear after overnight incubation at 50°C, however a 20-fold increase in Tc^r transformants was obtained if plates were incubated for 36 h. The results are summarised in Table 7.3. 7.0 x 10^3 transformants µg.DNA^{-1} were obtained (tetracycline at 6 µg.ml^{-1}) with 5% protoplast regeneration. All transformants were resistant to 25 µg.ml^{-1} tetracycline when replica-plated (Collins & Lyne, 1970) onto TSBA plates containing the antibiotic. Several transformants were screened for the presence of plasmid DNA as described in Methods (2.6.2) and plasmid was detected in all cases.

It was found that if regenerated protoplasts from the DM5 plates from the previous experiment were replica-plated (Collins & Lyne, 1970) onto TSBA + tetracycline (12 µg.ml^{-1}) 4.0 x 10^4 Tc^r transformants per µg DNA were obtained. This was significantly higher than obtained by direct selection (Table 7.3) and would suggest that the period of incubation after transformation was not long enough to allow full phenotypic expression of the Tc^r genes. The transformation experiment was therefore repeated and the length of incubation varied. The results, shown in Table 7.4 indicated that 2-2.5 h was optimum for obtaining the maximum number of transformants. However, since protoplast regeneration also increased with the length of incubation it might indicate that the increase in Tc^r transformants was related to the increase in protoplast regeneration.

When preparing protoplasts with 1 mg.ml^{-1} lysozyme it was found that although all lysozyme treated cells were osmotically sensitive (TSBA plates were sterile) a quite high number of rods were visualised under the microscope. This indicated that protoplast formation of strain LO2 was not efficient using the amount of lysozyme used. The results in Table 7.5 summarise a transformation experiment where the amount of lysozyme used to prepare the protoplasts was varied. The number of transformants obtained was dependent of the quantity of lysozyme used, ie

the degree of protoplast formation. No improvement in protoplast formation was observed with concentrations of lysozyme above 2 $mg.ml^{-1}$. In fact at high concentrations it appeared that the activity of the lysozyme was impaired. This effect was also noted when lysing thermophilic bacilli strains for detection of plasmid DNA. At levels above 5 $mg.ml^{-1}$ lysozyme lysis was significantly impaired.

As mentioned previously not all osmotically sensitive cells appeared as spheroplasts under the microscope when using the optimum conditions described above. It was hoped that the sensitivity of *B. stearothermophilus* LO2 to lysozyme could be increased, and therefore the transformation frequency, by growing the cells with low levels of ampicillin in an osmotically stabilised medium. This antibiotic prevents cross-linking of the cell wall peptidoglycan (Gale *et. al.*, 1972) in growing cells and should render the peptidoglycan more accessable to lysozyme.

The minimal inhibitory concentration of ampicillin in solid media was determined as 10 $\mu g.ml^{-1}$ for strain LO2. This was a quite high value, particularly when compared with the values determined for *B. caldovelox* (0.03 $\mu g.ml^{-1}$) and *B. stearothermophilus* NCA1503 (0.015 $\mu g.ml^{-1}$). The effect of ampicillin on an actively growing culture was determined by following the optical density of the culture. Inhibitory levels of ampicillin will cause lysis and therefore a drop in optical density (Gale *et. al.*, 1972). Four mid-log cultures (100 ml) of each strain were prepared and different concentrations of ampicillin added to three. The results shown in Fig 7.3 show that addition of ampicillin does cause lysis for *B. caldovelox*, while inhibiting growth of *B. stearothermophilus* NCA1503. With strain LO2 however, only a slight 'lag-phase' was caused by ampicillin after which growth proceeded normally. This might suggest that a β-lactamase was induced by the addition of ampicillin. This antibiotic therefore could not be used for increasing lysozyme sensitivity.

The optimum conditions for transformation of strain LO2 are described fully in Methods (2.22) and in later experiments 1.2×10^5 transformants per μg pAB124 was obtained with 90% protoplast regeneration. Using these conditions, LO2 was also transformed by pAB124 isolated from *B. subtilis* and the deletion variants pAB224 and pAB524 both isolated from *B. subtilis*. The results are shown in Table 7.6. When strain LO2 was transformed with plasmid DNA from *B. subtilis* the number of transformants was dramatically reduced. 1.0×10^5 .μg DNA^{-1} were obtained with pAB124 from *B. stearothermophilus* TB124 but only 3.0×10^3 .μg DNA^{-1} pAB124 isolated from *B. subtilis*. This suggested

that LO2 is restricting DNA from B. subtilis, and can modify DNA in the cell. DNA was isolated from LO2 (Methods 2.7) and then transformed into LO2 a second time, 1.1×10^5 transformants per μg DNA was obtained indicating the presence of a restriction and modification system in B. stearothermophilus LO2.

It was not possible to obtain any Tc^r or Neo^r transformants with pAB324 which does not have the thermostable replicon of pAB124. (Chapter 6.)

SUMMARY

(1) It was not possible to transform B. stearothermophilus NCA1503 or B. stearothermophilus RS93 with plasmid DNA by inducing competence or treatment with $CaCl_2$.

(2) Conditions for the preparation of protoplasts of B. caldovelox, B. stearothermophilus NCA1503 and B. stearothermophilus LO2 were developed. Only B. stearothermophilus LO2 protoplasts could be regenerated to the bacillary form using an osmotically stabilised medium.

(3) Transformation of B. stearothermophilus LO2 protoplasts by plasmid DNA to a high frequency was achieved. The vectors pAB124, pAB224 and pAB524 all transformed this strain while pAB324 could not.

Table 7.3. Transformation of B. stearothermophilus LO2 protoplasts with pAB124

	Colonies (ml^{-1})				
	DM5	DM5 + Tc (6 $\mu g.ml^{-1}$)	DM5 + Tc (12 $\mu g.ml^{-1}$)	TSBA[a]	TSBA + Tc[a] (12 $\mu g.ml^{-1}$)
Before lysozyme	1.0×10^9	0	0	1.0×10^9	0
After lysozyme	5.0×10^7	0	0	0	0
After transformation	4.8×10^7	7.0×10^3	5.0×10^3	0	0

Protoplasts were prepared and transformed with 1 µg pAB124 isolated from TB124 as described in Methods (2.22) except 1.5h incubation (50°C) for phenotypic expression was used. Plates were incubated for 36h at 50°C.

a, This represents osmotically insensitive rods.

Table 7.4. Phenotypic expression of tetracycline resistance in transformants of B. stearothermophilus

Incubation time (h)	Colonies (ml^{-1})			
	DM5	DM5 + Tc (6 $\mu g.ml^{-1}$)	DM5 + Tc (12 $\mu g.ml^{-1}$)	TSBA[a]
0	1.7×10^5	0	0	6.0×10^3
0.5	6.0×10^5	0	0	6.0×10^3
1.0	1.1×10^6	1.2×10^2	1.0×10^2	7.8×10^3
1.5	2.5×10^6	2.3×10^3	2.0×10^3	1.4×10^4
2.0	3.8×10^7	9.5×10^3	7.4×10^3	2.0×10^4
2.5	4.0×10^7	5.0×10^4	4.8×10^4	3.5×10^4
3.0	4.2×10^7	5.1×10^4	4.7×10^4	8.0×10^4

Protoplasts were prepared with 500 $\mu g.ml^{-1}$ lysozyme from 1×10^9 cells and transformed with 1 µg pAB124 isolated from TB124 as described in Methods (2.22).

a, This represents osmotically insensitive rods.

Table 7.5. Effect of Lysozyme concentration on protoplast formation and transformation

Lysozyme concentration ($mg.ml^{-1}$)	Colonies (ml^{-1})		
	TSBA[a]	DM5	DM5 + Tc ($12\ \mu g.ml^{-1}$)
0.01	1.1×10^6	1.6×10^7	1.1×10^2
0.1	1.0×10^5	2.2×10^7	8.2×10^2
1.0	2.0×10^4	1.0×10^8	3.8×10^4
2.0	1.2×10^4	1.2×10^8	3.5×10^4
5.0	2.6×10^5	1.1×10^6	1.0×10^4
10	2.5×10^5	2.8×10^5	5.5×10^3
20	2.0×10^5	2.2×10^5	6.0×10^3

Protoplasts were prepared in SMM6 from 1×10^9 cells with varying amounts of lysozyme, incubated at $42^{\circ}C$ for 30 min, and transformed with 1 µg pAB124 isolated from TB124 as described in Methods (2.22).
a, This represents osmotically insensitive rods.

FIG. 7.3a (NCA1503)

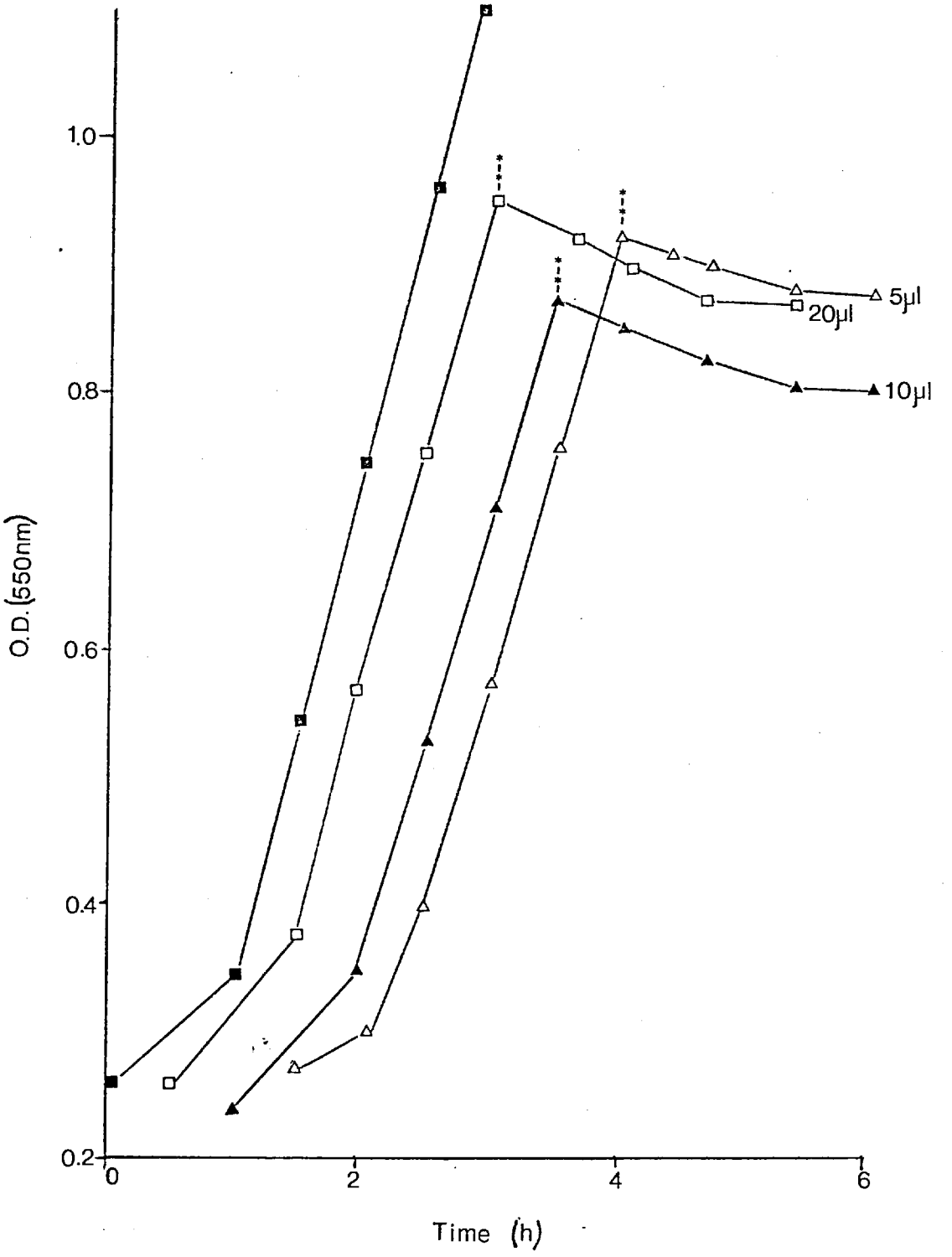


FIG. 7.3b (B.caldovelox)

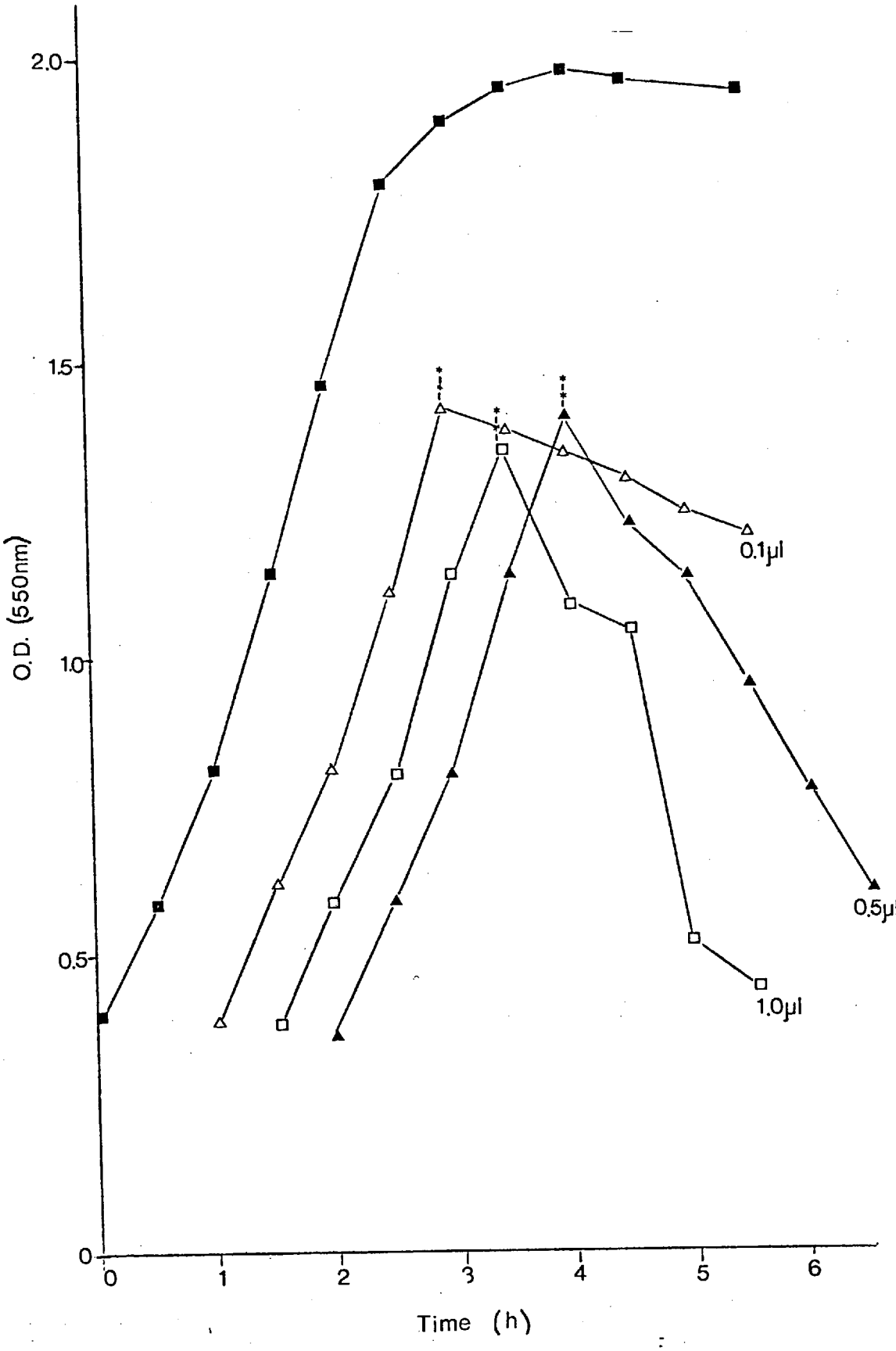


FIG.7.3c (LO2)

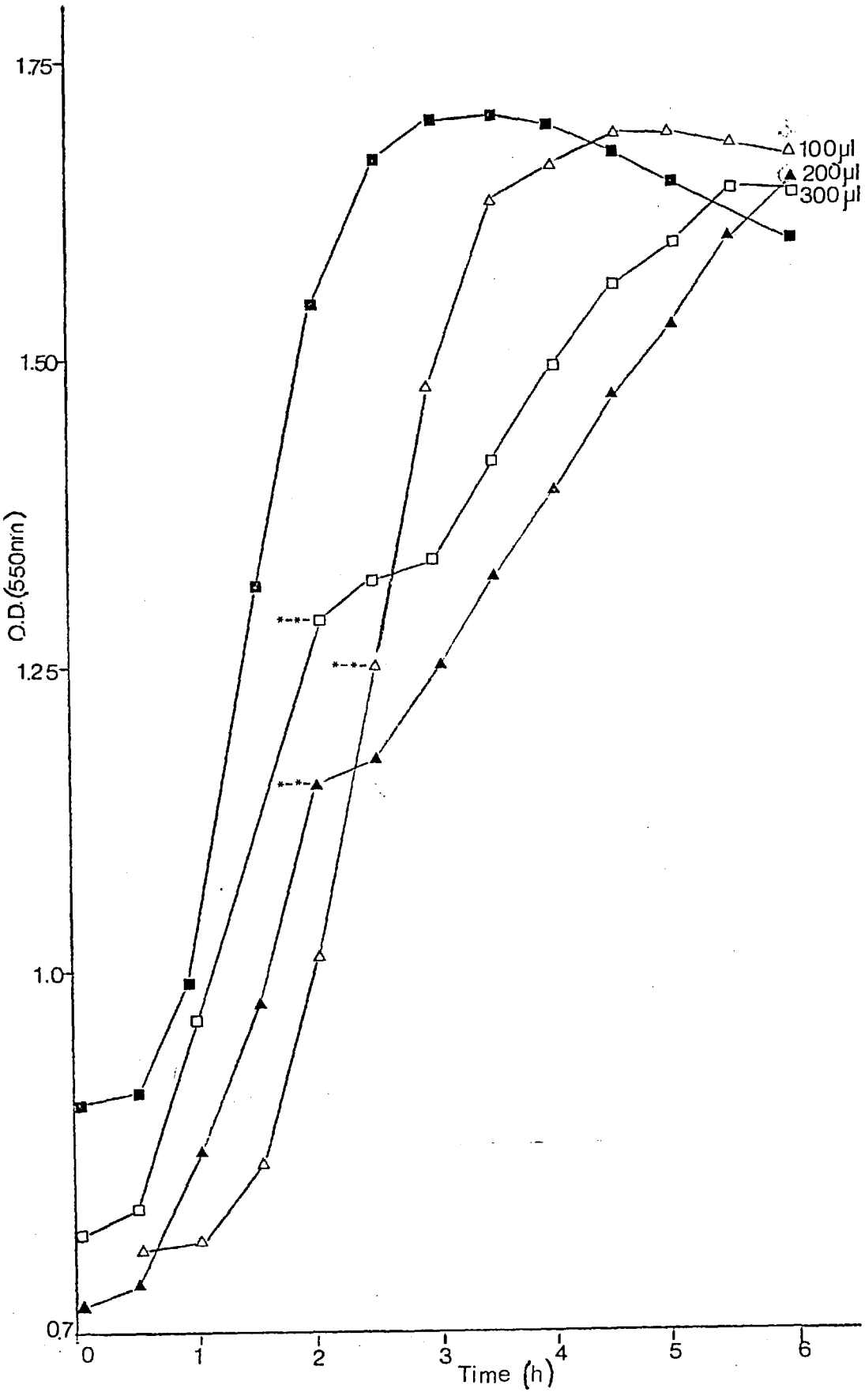


Figure 7.3. Effect of Ampicillin on the growth of B. stearothermophilus NCA1503, B. stearothermophilus LO2 and B. caldovelox

Cultures were grown to mid-log and varying amounts of Ampicillin (5 $mg.ml^{-1}$ in H_2O) added (-*-*) as described in Chapter 7 (7.3).

7.3.a., B. stearothermophilus NCA1503

■———■, no ampicillin added; □———□, 20 µl added; ▲———▲, 10 µl added; △———△, 5 µl added.

7.3.b., B. caldovelox

■———■, no ampicillin added; □———□, 1 µl added; ▲———▲, 0.5 µl added; △———△, 0.1 µl added.

7.3.c., B. stearothermophilus LQ2:

■———■, no ampicillin added; □———□, 300 µl added; ▲———▲, 200 µl added; △———△, 100 µl added.

Table 7.6. Transformation of *B. stearothermophilus* LO2 protoplasts with pAB124, pAB224 and pAB524

Plasmid (Source)	Colonies (ml^{-1})		
	TSBA[a]	DM5	DM5 + TC (12 $\mu g.ml^{-1}$)
pAB124 (TB124)	3.0×10^2	3.3×10^8	1.0×10^5
pAB124 (*B. subtilis* IG20)	3.0×10^2	3.0×10^8	3.0×10^3
pAB224 (*B. subtilis* IG20)	3.0×10^2	3.3×10^8	1.5×10^2
pAB524 (*B. subtilis* IG20)	3.0×10^2	3.5×10^8	1.0×10^4

Protoplasts were prepared from 1.0×10^9 cells and transformed with plasmid DNA as described in Methods (2.22).

a, This represents osmotically insensitive rods.

CHAPTER EIGHT

DISCUSSION

The ease with which it was possible to isolate thermophilic bacilli from a wide variety of environmental samples might indicate that these strains are widely distributed in nature. The three taxonomic characters used to classify thermophilic bacilli as either B. coagulans or B. stearothermophilus, ie growth at 65°C, growth in azide and growth on Sabouraud agar (Buchanan & Gibbons, 1974) could not be used effectively for the RS or TB strains isolated. Very few strains were identifiable as characteristic B. coagulans or B. stearothermophilus. The problems in classifying thermophilic bacilli were also illustrated by Sharp et. al., (1980) who found that one strain (136) previously classified as B. stearothermophilus, in fact was more related to B. coagulans than to 10 other known strains of B. stearothermophilus on the basis of biochemical tests, DNA-DNA hybridisation and % G + C content.

Examination of the RS strains showed rather surprisingly that over half the strains had some degree of bacteriocin production, with some 10% being extremely potent producers. Evidence for bacteriocins produced by the thermophile B. stearothermophilus has previously been presented. (Shafia, 1966; Yule & Barridge, 1976). The frequent occurrence of bacteriocin producing thermophilic bacilli could indicate that there is significant competition between thermophiles in the environment. Strains producing a bacteriocin effective against other thermophiles would have a distinct advantage. Characterisation of the bacteriocin from RS93 showed that it was a small, heat-stable protein only effective against other thermophiles (Sharp et. al., 1979).

With the exception of naladixic acid, which is known to have little effect against gram-positive bacteria (Gale et. al., 1972), resistance to antibiotics is not common amongst the strains tested. Only 19 of 100 RS strains were identified and these are resistant only to very low levels of the antibiotics (1-2 $\mu g_{eq}.ml^{-1}$), erythromycin, chloramphenicol and ampicillin. It is likely therefore that these antibiotic resistant RS strains are tolerant to the antibiotic by means of a permeability effect, rather than plasmid mediated enzyme hydrolysis or modification. Thermophilic bacilli resistant to high levels of antibiotic or a heavy metal-ion were successfully isolated by direct selection at a slightly lower temperature (55°C) and the large number obtained (32) from one sample of silage and river sludge would suggest a high distribution in some environments. The numbers of antibiotic resistant and heavy metal-ion tolerant thermophilic bacilli isolated from the river sludge (Table 2.1) could be indicative of the degree of river pollution. The sample was taken quite close, downstream, from a paint factory and therefore isolation of heavy metal-ion tolerant bacteria was not altogether

surprising.

At the start of this investigation no plasmids had been identified in strains of thermophilic bacilli. A few plasmids from other Bacilli species had been isolated; B. megaterium (Carlton & Helinski, 1969), B. pumilus (Lovett, 1973; Lovett & Burdick, 1973; Lovett & Bramucci, 1974 and 1975; Lovett et. al., 1976), B. subtilis (Le-Hegarat & Anagnostopoulos, 1977; Tanaka & Koshikawa, 1977; Tanaka et. al., 1977); however none carried any identifiable phenotypic trait (cryptic). Cell-free lysates of these strains were obtained by lysozyme-triton or lysozyme-SDS procedures and plasmids detected in caesium chloride-ethidium bromide density gradients. Therefore a similar plasmid screening method was used for screening several Bacilli species and the RS strains of thermophilic bacilli.

The first plasmids isolated were from the Gram-negative organism T. aquaticus; pTA-B (strain B) and pTA-X (strain X1) and at a later date 4 plasmids were isolated from different Thermus strains (Hishinuma et. al., 1978). The plasmids from the Thermus strains are unlikely to replicate in Bacilli, since no plasmid from a Gram-negative bacterium has been shown to replicate in Bacilli (Broda, 1979), and for this reason pTA-B and pTA-X were not characterised.

Only one plasmid was identified when screening the RS strains of thermophilic bacilli; plasmid pT93 from the bacteriocin producer RS93, and it was noted that the degree of lysis of RS93 cells was not sufficient to allow easy isolation of the plasmid. The lysis of RS93, and in general thermophilic bacilli, was improved by altering the growth conditions of the cells and lysozyme-EDTA treatment. The improved method was used to screen the TB strains of antibiotic resistant and heavy metal-ion tolerant thermophilic bacilli, and five plasmid-bearing strains were isolated. These strains were characterised and as for the RS strains could not be classified as characteristic strains of B. coagulans nor B. stearothermophilus, but appeared more related to B. stearothermophilus than B. coagulans. It was found that two of the Sm^r plasmid-bearing TB strains (TB118, TB150) were identical in all respects except for colony morphology as were two of the Tc^r plasmid-bearing strains (TB124, TB144).

Using the improved lysis procedure for thermophilic bacilli it was still not possible to obtain sufficient quantities of pT93 from RS93, probably due to the low copy number, to allow its full characterisation. Since no transformation of B. stearothermophilus by plasmid DNA has been reported it was not possible to transfer the plasmid to another thermophilic bacillus in order to determine whether it confers bacteriocin production. This plasmid has little immediate value as a possible vector

for genetic manipulation in Bacilli. However, it was possible to obtain a larger quantity of plasmid DNA from the other plasmid-bearing strains and these were first characterised with restriction endonucleases.

The two plasmids (pAB118A, pAB118B) isolated from the streptomycin resistant strains TB118 and TB150 were shown to contain several sites for restriction endonucleases that could be used for inserting foreign DNA, namely single sites for BamHI, KpnI, SalI, XbaI and XmaI (Table 4.2). However, it could not be demonstrated that either conferred streptomycin resistance to B. subtilis (Table 4.5). Several problems associated with transforming plasmids into competent B. subtilis cells became evident. B. subtilis has a high natural tolerance to streptomycin (up to 25 $\mu g.ml^{-1}$) and a quite high spontaneous mutation frequency (Table 4.5) making selection difficult. Furthermore, oligomeric forms of a plasmid are required for transformation of competent B. subtilis cells (Canosi et. al., 1978). It is possible therefore that the plasmid preparation from TB118 and TB150 did not contain oligomeric forms of the plasmids. This would seem unlikely since plasmid DNA from tetracycline resistant strains TB124, TB144 and TB128 all could transform competent B. subtilis cells to Tc^r (Table 4.5). Transformation of plasmid DNA from TB118 and TB150 into B. subtilis protoplasts, which have no requirement for oligomeric forms of plasmid DNA, failed to show conversion to a Sm^r phenotype. Thus the two plasmids in these strains, pAB118A and pAB118B, would appear to be cryptic plasmids, their function remaining a mystery.

Of the two plasmids shown to confer Tc^r to B. subtilis, pAB124 (from TB124, TB144) and pAB128 (from TB128), the former would appear to have most potential as a vector for genetic manipulation in bacilli. pAB124 contains one restriction endonuclease cleavage site for XbaI, suitable for inserting foreign DNA while pAB128 does not contain any single sites for restriction endonucleases producing cohesive termini (Table 4.2). Also the copy number (Chapter 4) of pAB124 is higher than for pAB128, 6-9 as opposed to 3-5, making it easier to extract plasmid DNA from pAB124 containing strains. One complication in the isolation of pAB124 was the poor separation of the plasmid DNA band from the chromosomal DNA band in caesium chloride-ethidium bromide density gradients. However, this problem was overcome by incorporating an alkali denaturation step in the extraction procedure thus reducing the chromosomal DNA present (Fig 4.1). This poor separation of pAB124 plasmid DNA and chromosomal DNA in density gradients is rather unusual and curious. Ethidium bromide is an intercalative dye and at a specific concentration unwinds plasmid supercoils (CCC) so that the number of superhelical turns reduces to zero. Higher levels of dye then result in formation of

superhelices of the opposite sign or handedness (Vinograd et. al., 1963). These new supercoiled (CCC) plasmid molecules contain a higher level of mechanical stress in the duplex and have a more ordered conformation. These effects increase the free energy of formation of the DNA-dye complex. The maximum amount of dye that can be bound by these CCC molecules is therefore smaller than by linear or open circular DNA molecules (Radloff et. al., 1967). Since open circles and linear molecules bind more dye, in caesium chloride solutions, Cs^+ ions are displaced and the DNA becomes less dense (Broda, 1979). Because of this difference in density, CCC molecules can be separated from open circles and linear molecules on caesium chloride-ethidium bromide equilibrium density gradients. It would therefore seem that with pAB124 the CCC species in opposite sign, or handed-ness does not contain such a high level of mechanical stress or ordered conformation as most plasmids, and therefore binds almost the same amount of dye as linear and open circular DNA molecules. The resulting poor separation in density gradients illustrates the value of incorporating gel-electrophoretic analysis of cell-free lysates in a plasmid-screening protocol. Since no plasmid satellite band was observed with pAB124 in caesium chloride-ethidium bromide density gradients it would have been assumed that strains TB124/TB144 did not contain a plasmid if this was the sole means of detection. However, agarose-gel electrophoresis of a sample of cleared lysate of TB124 and TB144 clearly indicated the presence of a plasmid.

The two Tc^r plasmids pAB124 and pAB128 produced similar restriction endonuclease fragment patterns when digested with several enzymes. It was shown that the two plasmids did have some sequence homology as determined by DNA-DNA hybridisation on nitrocellulose filters (Plate 4.5). It is possible therefore that pAB128 (2.5 Md) is a deletion variant of pAB124 (2.9 Md). However, time did not permit further examination of this possibility; in particular by heteroduplex analysis.

The stability of pAB124 is an important factor when assessing its potential as a vector for genetic manipulation experiments. Rapid explulsion of a plasmid when no selective pressure is applied would be a disadvantage. It has been shown that the E. coli vectors commonly in use, pBR322 and pBR325 are deleted from all the population after about 50 h in continuous culture (carbon limitation), Dr. S. Jones, pers. comm. In contrast, pAB124 and pAB224 were not lost after 300 h when grown under the same conditions (Miss E. Thompson & Dr. S. Jones, pers. comm.). The extreme stability of pAB124 was also shown by the difficulty experienced in curing TB124 or TB144 of the plasmid. This was eventually achieved and the plasmid-free strain obtained (B. stearothermophilus LO2)

has a Tc^S phenotype. It was not possible to cure TB118, TB150 of their plasmids due to the selection problems already mentioned. The plasmid pAB128 confers Tc^r but it was not possible to cure TB128 of this plasmid. The only Tc^S strains obtained still contained the plasmid, suggesting that a mutation had occurred preventing expression of the Tc^r genes of pAB128. Time did not permit further examination of this mutant strain.

The restriction endonuclease characterisation of pAB124 revealed an uneven distribution of cleavage sites. A 1.0 Md region contained no sites for any of the restriction endonucleases used for constructing the cleavage site map. This region lies between the HaeIII site, at map distance 0.53 Md, and the EcoRI site, at map distance 1.55 Md (Fig 5.5). Since pAB124 contained three EcoRI sites it was possible to clone the fragments in the Staphylococcal vector pUB1654 (Neo^r Sm^r) which contains one EcoRI site within the Sm^r gene . It was found that tetracycline resistance was associated with only one of the fragments (1.95 Md), Table 5.4. It was not possible to locate the tetracycline resistance gene within this region more precisely. An attempt to clone pUB110 at the restriction endonuclease cleavage sites within the 1.95 Md region failed to produce any recombinant plasmids of a Neo^r Tc^r or Neo^r Tc^S phenotype. It was later found (Canosi et. al., 1978) that this was because of the requirement of competent B. subtilis cells for oligomeric forms of the plasmid and the particular method of cloning used (tailing with terminal transferase) excludes the formation of dimers. Attempts to clone the BclI fragments of pAB124 into pUB110 suggested that the two BclI sites within the EcoRI 1.95 Md fragment (Fig 5.7) may be within the Tc^r genes Since the results (Chapter 5) suggested that all 3 BclI fragments of pAB124 were required for a Tc^r phenotype.

It was found that the 1.95 Md EcoRI fragment of pAB124 also contained the replicon since this fragment could circularise to produce a viable Tc^r plasmid, pAB224, of 1.95 Md (Table 5.5). This small plasmid has great potential as a vector for genetic-manipulation in bacilli since it contains one cleavage site for the restriction endonucleases EcoRI, BalI, BstEII, CauII, HpaI, HpaII, HhaI, PvuII and ThaI (Fig 5.8). The second deletion variant of pAB124 constructed, pAB524, has no particular advantage over pAB224 since it no longer contains one cleavage site for EcoRI, HpaII, HhaI, or ThaI (Fig 5.7). The failure to construct a plasmid composed of the EcoRI fragments A + B (1.95 Md, 0.60 Md) in a similar manner to pAB524 (Table 5.5) was surprising. This might be due to an incompatability problem associated with the 0.60 Md fragment which contains the XbaI site. When pUB110 (Neo^r) was cloned within this region at the XbaI site (Fig 6.6), expression of the Staphylococcal plasmid

surprisingly did not occur. Gryczan & Dubnau (1978) have reported that insertion of DNA at the XbaI site of pUB110 does not inactivate the Neo^r genes. Only when the two regions of the 0.6 Md fragment, now physically distant within the hybrid plasmid, were deleted to form pAB324 (Fig 6.7) did expression of the Neo^r genes occur.

The region of pAB124 deleted to produce pAB224 (0.95 Md) was shown not to be necessary for Tc^r. It was not possible to identify any phenotypic trait associated with this region when other antibiotic resistance and heavy metal ion tolerance was examined. The function of this region of pAB124 is therefore unknown.

As mentioned, the failure to obtain neomycin resistance with a hybrid plasmid of pUB110 (Neo^r) and pAB124 (Tc^r) was not expected. Chimeric plasmids with a variety of Staphylococcal plasmids cloned at the XbaI site of pUB110 do express the Neo^r genes, eg pE194 (Em^r), pSA0501 (Sm^r), pSA2100 ($Sm^r.Cm^r$), cloned at the XbaI site of pUB110 to produce pBD9, pBD6, pBD8 respectively (Gryczan & Dubnau, 1978). However, the level of kanamycin used ($\mu g.ml^{-1}$) by Gryczan & Dubnau was surprisingly low. *B. subtilis* wild-type cells are not completely inhibited by kanamycin at this level. Therefore the *B. subtilis* cells containing the chimeric plasmids of pUB110 may not need to efficiently express the Neo^r genes. Selection for the chimeric plasmid of pUB110 and pAB124 (Chapter 6) was carried out at high levels of neomycin (12 $\mu g.ml^{-1}$, 25 $\mu g.ml^{-1}$), and it remains a possibility that expression of the Neo^r genes of pUB110 at a high level of antibiotic, within a chimeric plasmid constructed at the XbaI site is not possible without deletion or insertion.

The *in vivo* construction of pAB324 (Chapter 6) and pUB1654 (A. Docherty, unpublished) tends to confirm this. Examination of pAB324 showed that at least one major deletion and one major insertion had occurred to the original chimera of pUB110 and pAB124 (Fig 6.7) that did not express Neo^r. The deletion had occurred around one XbaI site resulting in deletion of part of both pUB110 and pAB124. The same region of pUB110 was deleted during the construction of pUB1654 (a chimera of pUB110 + pSA0501 at their XbaI sites, A. Docherty unpublished. Fig 5.6) where high levels of neomycin was used (15 $\mu g.ml^{-1}$). It seems unlikely that deletion of pAB124 is needed for Neo^r expression in chimeric plasmids since pAB124 is not cloned in close proximity to the Neo^r gene (Fig 6.6) and so is unlikely to be promoting Neo^r genes. Therefore deletion of part of the region of pUB110 around the EcoRI and XbaI sites seems to be required for Neo^r expression in the chimeric plasmid.

The function of the insert in pAB324 from the *B. subtilis* chromosome detected by DNA-DNA hybridisation (Plate 6.1) is unknown. It is unlikely to be associated with the Neo^r on Tc^r genes since its absence

in the original chimera of pUB110 and pAB124 (Fig 6.6) did not impair Tc^r and it is physically distant from the Neo^r genes (Fig 6.6), separated by a region of DNA not necessary for neomycin resistance (Gryczan & Dubnau, 1978) making it unlikely to be promoting Neo^r genes. The insertion of this region of DNA from the chromosome may have occurred during the uptake of the plasmid by competent cells or by integration with the chromosome, and it is maintained even in strains not deficient in recombination (eg, IG20).

The deletions of part of the chimera of pAB124: pUB110 (Fig 6.7) to form pAB324 has resulted in the deletion, or inactivation, of the pAB124 replicon. The parent chimera was present at 6-9 copies per cell corresponding to pAB124 while pAB324 was present at 20-30 copies per cell corresponding to pUB110. Therefore pAB324 may not be able to replicate in B. stearothermophilus and have little value as a vector for gene-cloning in thermophilic bacilli. It is however a very useful vector for B. subtilis, having one site for the restriction endonucleases BamHI, BglII, HindIII, XbaI and XhoI, especially since the construction of pAB624 and pAB724 (Fig 6.8) indicated that insertion of foreign DNA at the BglII site inactivates the Neo^r genes. This has recently been confirmed (Gryczan et. al., 1980).

The ability to clone DNA at the XbaI site of pAB124 was demonstrated by the construction of pAR2 (Fig 6.11). This contains the E. coli plasmid pROG29 (Ap^r Tc^s $leuB^+$) cloned in the XbaI site of pAB124 (Tc^r) and has an Ap^r Tc^r $leuB^+$ phenotype in E. coli but only a Tc^r phenotype in B. subtilis. It has never been shown that an E. coli plasmid can replicate independently in B. subtilis or that the ampicillin resistance gene of pROG29 (a recombinant plasmid of pBR322) can be expressed in B. subtilis (Goebel et. al., 1979). Therefore it would appear that the pAB124 replicon is responsible for replication of pAR2 in B. subtilis and by inference pBR322 (pROG29) replicon responsible for replication in E. coli, since no B. subtilis plasmid can replicate in E. coli (Goebel et. al., 1979). It was not possible to demonstrate complementation of the leuC7 mutation in B. subtilis by the yeast leu2 genes of pAR2. The failure to obtain expression of yeast leu2 genes in B. subtilis may be due to the inability of the yeast promotor to promote expression in B. subtilis or that the leu2 promotor has undergone a mutation allowing expression in E. coli but not B. subtilis, or may be due to the effect of inserting DNA in the yeast 2 μ DNA region of pROG29 (Fig 6.9). There have been no reports of cloning and expression of yeast genes in B. subtilis.

The Tc^r phenotype of pAR2 in E. coli may be due to the Tc^r genes of pAB124; the promotor of the Tc^r genes of pBR322 was inactivated by the

construction of pROG29. However, it is possible that pAB124 can promote expression of the pBR322 Tc^r genes rather than being responsible for tetracycline resistance itself. Time did not permit further examination of this problem by eg inserting a gene at the BamHI or SalI sites to inactivate the pBR322 based tetracycline resistance genes.

Several unsuccessful attempts were made to clone chromosomal genes in pAB124 and pAB224 by 'shotgun' experiments into competent B. subtilis cells. There has only been one report of a successful 'shotgun' experiment in B. subtilis where the trp genes were cloned in pUB110 (Keggins et. al., 1978) and five laboratories have reported failure to clone chromosomal genes in Staphylococcal vectors in B. subtilis (Gryczan et. al., 1980b).

An explanation for this was provided by Canosi et. al., (1978) who demonstrated that monomeric CCC plasmid DNA is inactive in transforming competent B. subtilis cells. In addition Gryczan et. al., (1980b) showed that although plasmid dimers have detectable transforming activity they are about 100 fold less active on a mass basis than higher multimers. Since plasmid multimers are required to transform competent cells of B. subtilis an unlikely sequence of events is required to produce an active recombinant plasmid from a 'shotgun' experiment. If the ratio of foreign to vector DNA is elevated in order to increase the proportion of recombinant molecules generated, then the yield of transformants will decrease rapidly due to competition between vector-vector and vector-foreign DNA ligation, both of which are required.

An alternative to competence transformation has been reported (Chang & Cohen, 1979) where monomeric plasmid DNA was successfully transformed into B. subtilis protoplasts at high frequency (10^7.µg DNA^{-1}). It was found that pAB124 would also transform protoplasts at high frequency (10^6.μg^{-1}). However, it was noted that plasmids produced by in vitro endonuclease digestion followed by ligation transformed B. subtilis protoplasts at a much lower frequency (10^5.µg DNA^{-1}) ; a 100-fold reduction (Chang & Cohen, 1979). This will reduce the possibility of transforming recombinant molecules from a 'shotgun' experiment into B. subtilis protoplasts. An additional disadvantage of this method is the fact that regeneration of protoplasts to the bacillary form requires a very complex medium (Gabor & Hotchkiss, 1979) making selection for chromosomal determinants impossible directly, and therefore requiring a replica-plating step.

The successful cloning of chromosomal determinants has recently been reported using a modification of competence transformation (Gryczan et. al., 1980b). It was found that if plasmid DNA was linearised with a

restriction endonuclease no transformation of competent B. subtilis cells was detected. If, however, the recipient cells carry a homologous plasmid, or one with substantial homology, and if the restriction cut occurs within the homologous moiety, then the same plasmid transforms efficiently (Contente & Dubnau, 1976). This 'rescue' of donor plasmid is dependent on recombination ie on the recE gene (Gryczan et. al., 1980b). This 'rescue' process was found to work very well for recombinant molecules produced by a 'shotgun' experiment with chromosomal genes (Gryczan et. al., 1980b). However, since the process is dependent on recombination a disadvantage of the method is that donor DNA containing significant sequence homology to the recipient cells chromosome can not be used, ie, B. subtilis genes can not be cloned into a B. subtilis host. The work of Gryczan et. al., (1980b) was limited to cloning B. licheniformis chromosomal genes. This work has only very recently been published and time did not permit the investigation of the possible application of plasmid 'rescue' to cloning in pAB124, pAB224, pAB324 and pAB524. However, the investigation of this phenonemum in the future would be a worthwhile extension of this project.

One of the main aims of this project was to develop transformation in thermophilic bacilli. Transformation of B. stearothermophilus with bacteriophage DNA has been reported (Welker, 1978) and a competence-inducing factor isolated (Streips & Young, 1971). However, this transfection appears to be difficult to reproduce (N.E Welker, pers. comm.).

The application of three established transformation procedures to thermophilic bacilli were investigated. These were transformation of (1) competent B. subtilis cells (Stacey, 1968), (2) B. subtilis protoplasts (Chang & Cohen, 1979) and (3) $CaCl_2$ treatment (E. coli), Cohen et. al., 1972.

It was not possible to demonstrate transformation of B. stearothermophilus NCA1503 (met^- ilv^- bio^- nic^- thi^-) or B. stearothermophilus RS93 (his^- pro^- arg^-) with pAB124 from TB124 by competence induction. This may have been due to the difficulty experienced in 'starving' the strains for their auxotrophic requirements by limitation. The mutations of both strains appear very 'leaky', it is quite easy to obtain prototrophic strains of RS93 and NCA1503 and the cultures never appear to be completely limited of auxotrophic requirements (Fig 7.1). The application of the E. coli transformation protocol ($CaCl_2$ treatment) to the two strains was unsuccessful. Both strains showed a high level of autolysis after $CaCl_2$ treatment.

The preparation of B. stearothermophilus NCA1503-4R protoplasts has been reported (Abram, 1965; Wisdom & Welker, 1973), although the conditions used where rather unusual. The level of lysozyme used was rather low,

5-30 $\mu g.ml^{-1}$, and no osmotic stabiliser, eg sucrose, was used. Of the four strains used in this investigation (B. stearothermophilus strains NCA1503, RS93, LO2 and B. caldovelox) only B. caldovelox was sufficiently sensitive to lysozyme to allow efficient protoplast formation at 5-30 $\mu g.ml^{-1}$ lysozyme; and all four strains required an osmotic stabiliser to prevent osmotic rupture of their protoplasts. It was found that the osmotic pressure in the cells of B. stearothermophilus strains NCA1503, LO2 and B. caldovelox was greater than that of B. subtilis since the level of sucrose in SMM buffer had to be increased from 0.5 M (B. subtilis) to 0.6 M.

Although it was easy to produce protoplasts of B. caldovelox, difficulty in regenerating them to the bacillary form was found. The regeneration medium of Chang & Cohen (1979) for B. subtilis, which is succinate based, would not even support growth of B. caldovelox cells. Therefore a variety of different chemicals as osmotic stabilisers were examined for their inhibitory effect on B. caldovelox (Fig 7.2). Only two, glucose and glycerol, were found that did not markedly inhibit growth of B. caldovelox at the concentration that might be expected to osmotically protect protoplasts. However, glycerol could not be used since B. caldovelox protoplasts were very sensitive to glycerol, lysis occurring rapidly suggesting that the membranes were sensitive to glycerol. Whilst the incorporation of glucose (0.6 M) as a replacement for succinate in the regeneration medium of Chang & Cohen (1979) failed to promote significant regeneration of B. caldovelox protoplasts to the bacillary form.

It was found by Clive & Landman (1970) that the presence of a solid barrier in close proximity to the organism is required to prime cell wall synthesis of B. subtilis protoplasts. Hard-agar (Landman & Halle, 1963) exogenous cell walls (Clive & Landman, 1970) and membrane filters (Landman, 1968; Clive & Landman, 1970) have been successfully used. In addition, regeneration can be promoted by gelatin (Miller et. al., 1968; Landman & Forman, 1969, De Castro-Costa & Landman, 1977; Gabor & Hotchkiss, 1979) or bovine serum albumin (Chang & Cohen, 1979). These proteins are thought to inhibit the activity of a reversion inhibitory factor induced by removal of the cell wall (De Castro-Costa & Landman, 1977).

It was not possible to obtain regeneration of B. caldovelox protoplasts by inducing with gelatin, bovine serum albumin, hard agar or exogenous cell walls of B. caldovelox. With B. stearothermophilus LO2 however, which is not inhibited by high levels of osmotic stabilisers to the same extent as the other strains (Fig 7.2) significant regeneration of protoplasts was obtained using the regeneration medium of Chang &

Cohen (1979) containing succinate or glutamate as the osmotic stabiliser (Table 7.2). The protoplasts of strain LO2 showed the same type of sensitivity to sorbitol as B. caldovelox protoplasts did to glycerol. Using the optimum conditions for regeneration of strain LO2 protoplasts, it was possible to transform pAB124 isolated from TB124 into strain LO2 using a slightly modified version of the method of Chang & Cohen (1979). Transformants resistant to 25 $\mu g.ml^{-1}$ tetracycline were obtained and pAB124 plasmid was successfully isolated from these transformants.

When preparing protoplasts of strain LO2 it was noticed that although all cells treated with lysozyme were osmotically sensitive (TSBA plates were sterile) a quite high number of rods were visualised under the microscope. Increasing the amount of lysozyme used did reduce the amount of rods present, and increase the number of transformants. However, high levels of lysozyme (above 5 $mg.ml^{-1}$) was found not to improve protoplast formation. The high proportion of rods present in a lysozyme treated suspension of strain LO2 suggested a poor efficiency of protoplast formation. In such a preparation there are four types of organism; osmotically insensitive bacilli, osmotically sensitive rods, spheres with adherent wall residues (quasi-spheroplasts) and protoplasts (Tichy & Landman, 1969). With the conditions used for strain LO2 osmotically insensitive bacilli appear largely absent, with about 1-5% osmotically sensitive rods and the remainder as spheres. The proportion of protoplasts to quasi-spheroplasts is unknown and it is not possible to determine which of them is responsible for transformation of pAB124, since quasi-spheroplasts of B. subtilis are known to be transformable (Tichy & Landman, 1969) as are protoplasts (Chang & Cohen, 1969).

It was not possible to improve protoplast formation of strain LO2 by pre-treatment with ampicillin (Pouwels et. al., 1963) since LO2 appears to have an inducible β-lactamase (Fig 7.3). The best transformation achieved with B. stearothermophilus LO2 using optimum conditions for protoplast formation was 1.2×10^5 transformants/μg pAB124 with 90% regeneration.

The evidence (Table 7.6) suggests that strain LO2 contains a restriction system, pAB124 or pAB224 from B. subtilis did not transform as well as plasmid DNA isolated from LO2 (Table 7.6). If this strain is to be used as a recipient for gene-cloning then it would be desirable to render it deficient in restriction. In addition, deficiency in recombination, polysaccharide production and sporogenisis would also be desirable. Therefore, the development of this strain would be a logical extension to this work.

The observation that pAB324 could not be transformed into strain LO2 confirms an hypothesis made at the outset of this project. That is,

plasmids from a mesophilic bacillus or other Gram-positive bacterium could not be expected to replicate or express their genetic determinants at elevated temperatures in B. stearothermophilus. pAB324 having lost the thermostable replicon of pAB124 during its construction failed to transform B. stearothermophilus LO2.

In conclusion, several plasmids were isolated from thermophilic bacilli and characterised. One plasmid, pAB124 and its derivatives were shown to transform B. stearothermophilus protoplasts to Tc^r at high frequency and provides a basis of a host-vector system for genetic manipulation in thermophilic bacilli. Plasmid pAB124 satisfies all the requirements of a vector stated in Chapter one. It contains a selective marker (Tc^r), can accept foreign DNA (at the XbaI site) without inactivating essential functions and can infect two host bacteria, B. subtilis and B. stearothermophilus LO2. Several improvements to B. stearothermophilus LO2 must be made (r^-m^- rec^- spo^-.polysacc$^-$) before the full potential of this strain as a host for genetic-manipulation can be realised.

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Isolation and Partial Characterization of Four Plasmids from Antibiotic-resistant Thermophilic Bacilli

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Twenty-nine antibiotic-resistant isolates of thermophilic bacilli were examined for the presence of covalently closed circular duplex DNA molecules by agarose-gel electrophoresis and caesium chloride–ethidium bromide density gradient centrifugation. Five of the 29 strains tested contained covalently closed circular molecules. Two of the streptomycin-resistant strains contained the same two plasmids: pAB118A of molecular weight $4{\cdot}9 \times 10^6$ (7·0 kilobases) and pAB118B of molecular weight $3{\cdot}0 \times 10^6$ (4·3 kilobases). Two of the tetracycline-resistant strains each contained a plasmid (pAB124) of molecular weight $2{\cdot}9 \times 10^6$ (4·14 kilobases), while a third harboured a small plasmid (pAB128) of molecular weight $2{\cdot}5 \times 10^6$ (3·57 kilobases). These plasmids were digested with 19 different restriction endonucleases and the numbers of cleavage sites were determined. Transformation of *Bacillus subtilis* 168 (Trp^-) with purified plasmid DNA indicated that pAB124 conferred tetracycline resistance on the host.

INTRODUCTION

The presence of extrachromosomal genetic elements (plasmids) is well documented for members of the Enterobacteriaceae, *Staphylococcus aureus* and several species of *Pseudomonas* (Novick, 1969; Clowes, 1972; Helinski, 1976). The plasmids examined in most detail are characteristically small, covalently closed circular duplex DNA molecules conferring on the host known genetic functions such as the ability to conjugate, resistance to antibiotics and/or inorganic ions and the production of bacteriocins (Novick, 1969). Recently, Bernhard *et al.* (1978) reported the isolation of two plasmids from *Bacillus cereus* and *B. subtilis*, one coding for tetracycline resistance and the other for bacteriocin production; also, Lovett *et al.* (1976) reported the transfer of a *B. pumilus* plasmid into *B. subtilis* and subsequent expression of a plasmid-associated bacteriocin in the new host.

The recent development of *in vitro* enzymological techniques for the analysis, construction and transfer of recombinant DNA molecules to an *Escherichia coli* host has allowed a detailed analysis of the nature of the gene (Murray, 1976). At the same time, however, concern has been expressed over the potential biohazards associated with *E. coli*, despite the obvious potential benefits of the techniques of genetic manipulation. This has stimulated research into developing other systems for genetic manipulation experiments, e.g. using *B. subtilis*.

The use of the thermophile *B. stearothermophilus* could provide an alternative system since this organism has no known association with man and strains are available that are 'crippled' and will not grow below 42 °C (A. H. A. Bingham, R. J. Sharp & A. Atkinson; unpublished results). Furthermore, the transfer of genes from a mesophilic organism to a thermophilic

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Bacillus species would allow a study of gene expression at elevated temperatures and would provide an opportunity to amplify the production of *B. stearothermophilus* enzymes of current commercial and industrial interest.

To establish a system for genetic manipulation with *B. stearothermophilus*, the ultimate aim of our research, a vector stable at temperatures up to 65 °C is required. Plasmids have been isolated from several species of the genus *Thermus*, a Gram-negative extreme thermophile (Hishinuma *et al.*, 1978), although their functions are unknown. No plasmids have previously been isolated from Gram-positive thermophilic bacilli and so we first started to look for plasmids in strains in our existing collection of thermophilic bacilli. We were unable to detect any covalently closed circular molecules in preparations from over 50 different strains tested and sought an alternative screening programme.

This paper describes the detection and isolation of four plasmids from antibiotic-resistant thermophilic bacilli.

METHODS

Isolation of bacterial strains. Antibiotic-resistant strains of thermophilic bacilli were isolated at 55 °C from samples of river sludge and silage on TSB medium (pH 7·3) containing (g l^{-1}): Tryptone (Oxoid), 17; Soya Peptone (Oxoid), 3; NaCl, 5; K_2HPO_4, 2·5; glucose, 2·5; agar (Oxoid), 15; and one of the following antibiotics (all from Sigma): ampicillin, chloramphenicol, kanamycin or tetracycline hydrochloride (each at 25 μg ml^{-1}) or streptomycin sulphate (100 μg ml^{-1}). All strains were maintained on TSB containing the appropriate antibiotic.

Culture medium and conditions. For plasmid isolation, the strains of thermophilic bacilli were inoculated from TSB plates into TYS medium containing (g l^{-1}): Bacto-Tryptone (Difco), 20; yeast extract (Oxoid), 10; NaCl, 10; and the appropriate antibiotic at the concentrations indicated above. Cultures were grown with vigorous aeration at 50 °C for 8 to 10 h.

DNA extraction. The procedures normally used for preparing cleared lysates of *E. coli* were found to be ineffective for thermophilic bacilli since only partial lysis was achieved and so insufficient quantities of covalently closed circular DNA were obtained for easy detection. The following protocol was found to give satisfactory results for over 70 strains tested. Bacteria from 200 ml cultures were harvested, washed in 100 ml TES buffer (30 mM-Tris/HCl pH 8·0, 5 mM-Na_2EDTA, 50 mM-NaCl) and suspended in 3·0 ml TES buffer containing 25% (w/v) sucrose; 0·5 ml 0·2 M-Na_2EDTA pH 8·0 and 1·0 ml lysozyme (10 mg ml^{-1} in TES buffer) were then added. The mixture was vortexed and kept on ice for 10 min, before transferring to a 37 °C water bath for a further 10 min. Lysis was accomplished by adding sodium dodecyl sulphate (SDS) (0·5 ml; 10%, w/v) followed by 1·0 ml 5 M-NaCl and the mixture was left at 4 °C overnight. A cleared lysate was obtained by centrifuging at 45000 *g* for 1 h. The cleared lysates were extracted twice with an equal volume of chloroform/3-methylbutan-1-ol (24:1, by vol.), centrifuged at 6000 *g* for 5 min and the upper aqueous phase was carefully removed from the denatured protein at the interface. The precipitate of SDS in the aqueous phase was removed by centrifuging at 45000 *g* for 10 min. The volumes given above are for plasmid screening experiments; for preparative purposes 20 times greater volumes of cultures and reagents were used.

Alkali denaturation of chromosomal DNA. This was used for preparative purification of plasmids (see Results) and was carried out as follows. The pH of chloroform-extracted, cleared lysates was slowly adjusted (taking about 3 min) to 12·2±0·1 with 50 μl additions of 3 M-NaOH while mixing gently with a magnetic stirrer. After gentle stirring for 10 min at pH 12·2, the pH was reduced (taking at least 3 min) to 8·5 with 100 μl additions of 2 M-Tris/HCl pH 7·0. After 5 min on ice, the lysate was extracted twice with chloroform/3-methylbutan-1-ol (24:1, by vol.) and centrifuged at 3000 *g* for 5 min to remove precipitated single-stranded chromosomal DNA at the interface. The supercoiled, closed circular duplex DNA molecules remained in the aqueous phase and any precipitated single-stranded DNA was removed by centrifugation at 45000 *g* for 10 min. This process also removed the open circular species of plasmid DNA.

Concentration by polyethylene glycol. Polyethylene glycol (PEG) 6000 was added to a final concentration of 10% (w/v) to the cleared lysates after alkali denaturation; the mixture was stirred gently until the PEG had dissolved and then left for 3 h at 0 °C. The precipitated DNA was removed by centrifuging at 12000 *g* for 15 min, resuspended in a small volume of TES buffer and extracted three times with an equal volume of chloroform/3-methylbutan-1-ol (24:1, by vol.); centrifuging at 6000 *g* for 5 min removed the PEG at the interface and any remaining PEG precipitate in the aqueous phase was removed by centrifuging at 45000 *g* for 10 min.

Isopycnic centrifugation. When screening for plasmid DNA, 3·8 g caesium chloride (BDH) was dissolved

in a mixture of 2·0 ml chloroform-extracted cleared lysate, 0·5 ml ethidium bromide (1 mg ml^{-1} in 25 mM-Tris/HCl pH 8·0) and 1·5 ml TES buffer. Centrifugation was carried out in 10 ml polycarbonate tubes in a 10 × 10 ml Titanium rotor at 150000 *g* for 24 h at 10 °C in a MSE Superspeed 65 ultracentrifuge. Satellite DNA bands were visualized by fluorescence under ultraviolet light and collected via a density gradient scanner (model 2580) coupled to a Gilford 250 spectrophotometer (Gilford Instruments, Oberlin, Ohio, U.S.A.). Absorbance at 260 nm was recorded and fractions (100 ml) were collected with a Redirac 2112 fraction collector (LKB).

The procedure for analytical gradients was very similar to the above. The amount of sample was reduced to 0·5 to 1·0 ml and the amount of TES buffer was correspondingly increased to give a total gradient volume of 4 ml added to 4·0 g caesium chloride.

Ethidium bromide was removed from samples by extraction with Dowex 50W-X8 (Tris) beads (BDH).

Sucrose gradient centrifugation. Plasmid DNA was centrifuged on linear sucrose gradients [5 to 20% (w/v) in 20 mM-Tris/HCl, 1 M-NaCl, 20 mM-Na_2EDTA, pH 7·5] for 3 h at 90000 *g*. Gradients were collected with a Gilford density gradient scanner as described above.

Phenol extraction and ethanol precipitation of DNA. Plasmid DNA was treated twice with an equal volume of buffer-saturated phenol (freshly distilled over N_2) equilibrated in 100 mM-Tris/HCl pH 8·0, 10 mM-Na_2EDTA, 50 mM-NaCl and mixed gently for 5 min. The phenol phase was removed after centrifuging at 10000 *g* for 5 min and the aqueous phase was extracted twice with chloroform/3-methylbutan-1-ol (24:1, by vol.) and three times with diethyl ether. The aqueous phase was adjusted to 0·3 M-sodium acetate with a 3 M solution and two volumes of cold (−20 °C) ethanol were added. After 17 h at −20 °C the precipitated plasmid DNA was removed by centrifuging at 40000 *g* for 20 min (−5 °C) and then suspended in 500 µl of 10 mM-Tris/HCl pH 8·0, 10 mM-NaCl, 0·5 mM-Na_2EDTA. After two extractions with chloroform/3-methylbutan-1-ol (24:1, by vol.), the DNA solution was dialysed for 24 h against two changes of the same buffer.

Restriction endonuclease digestions. Enzymes were purified by procedures developed in this laboratory (Bingham & Atkinson, 1978), except *Sst*I, *Hae*III and *Hin*fI which were a kind gift from Dr P. W. J. Rigby, Imperial College, London, and *Bam*HI which was a gift from Dr R. Flavell, Plant Breeding Institute, Cambridge. The nomenclature of Smith & Nathans (1973) is used for restriction endonucleases. Digestions were carried out in three basic buffers: A, 10 mM-Tris/HCl pH 7·4, 10 mM-NaCl, 10 mM-$MgCl_2$, 0·5 mM-dithiothreitol (DTT) and 0·5 mg gelatine ml^{-1} (*Bam*HI, *Bcl*I, *Bgl*I, *Bgl*II, *Cau*I, *Cau*II, *Kpr*I, *Pst*I, *Sal*I, *Sst*I, *Xba*I and *Xma*I); B, 20 mM-Tris/HCl pH 7·4, 50 mM-NaCl, 20 mM-$MgCl_2$, 0·5 mM-DTT and 0·5 mg gelatine ml^{-1} (*Hae*III, *Hha*I, *Hin*dII, *Hin*dIII, *Hin*fI and *Hpa*I); and C, 100 mM-Tris/HCl pH 7·4, 50 mM-NaCl, 20 mM-$MgCl_2$, 0·5 mM-DTT and 0·5 mg gelatine ml^{-1} (*Eco*RI). Plasmid DNA (0·5 µg) was incubated for 30 min with 1 unit of endonuclease at 37 °C, except *Bcl*I, *Cau*I and *Cau*II which were incubated at 50 °C.

Plasmid DNA was removed from agarose gels by the freeze–squeeze method of Thuring *et al.* (1975) where it was necessary to separate a mixture of two different plasmids. A recovery of about 50% was usually achieved.

Agarose-gel electrophoresis. Slab gels (0·5 × 14 × 18 cm) of 0·8% (w/v) agarose (BioRad) in 90 mM-Tris, 90 mM-boric acid, 3 mM-Na_2EDTA, 0·5 µg ethidium bromide ml^{-1} were used. Electrophoresis was carried out for 5 h at 150 V (30 mA) and the DNA was visualized by fluorescence with an ultraviolet mineralight transluminator, 254 nm (Ultra-violet Products, Winchester). Photographs were taken on Ilford FP4 film with a Practica super TL camera and a Soligor Ø49 G orange filter.

Electron microscopy. Most plasmid preparations contained about 10% open circles and spreading of DNA for electron microscopy was performed as described by Davis *et al.* (1971).

Transformation. Competent cells of *B. subtilis* 168 (Trp^-) were produced as described by Stacey (1968). Plasmid DNA (5 µg) was added to 1 ml suspension of competent *B. subtilis*, containing polyethylene glycol 1000 (BDH) at a final concentration of 2·5%, and incubated at 37 °C for 1 h with vigorous aeration. Prewarmed TYS medium (5 ml) was then added and incubation was continued for a further hour prior to plating on selective plates. The degree of competence of the *B. subtilis* suspension was estimated using plasmid pUB110 (Neo^R), kindly provided by A. Docherty, Bristol University, which is known to transform this strain to neomycin resistance at 25 µg ml^{-1}.

RESULTS

Thermophilic bacilli

Eight streptomycin-, three ampicillin-, eight tetracycline-, six chloramphenicol- and four kanamycin-resistant strains were isolated at 55 °C from the samples of river sludge and silage. These strains differed morphologically both in colony characteristics and microscopically.

Plasmid screening

All 29 antibiotic-resistant strains of thermophilic bacilli isolated were examined by agarose-gel electrophoresis for the presence of plasmid DNA in the DNA extracted from cleared lysates. Plasmids were easily detected in lysates from five strains (Table 1) and isopycnic centrifugation confirmed the presence of plasmid DNA in three of them. For strains TB124 and TB144, the buoyant density of the plasmid DNA in caesium chloride–ethidium bromide density gradients did not appear to differ from that of chromosomal DNA. However, analysis of the 'chromosomal' band by agarose-gel electrophoresis confirmed the presence of plasmid DNA.

Large-scale preparation of plasmid DNA

The plasmids were isolated as described in Methods. Alkali denaturation of crude extracts after NaCl–SDS precipitation removed 95 to 99% of the chromosomal DNA and all the linear and open circle forms of the plasmids. This method proved very effective on a preparative scale, particularly with pAB124 from TB124 and TB144 which was difficult to separate from chromosomal DNA in caesium chloride–ethidium bromide gradients.

The low levels of plasmid DNA that could be isolated from these strains required concentration with PEG 6000 before caesium chloride–ethidium bromide density gradient centrifugation. Phenol extraction was used to remove protein and any remaining traces of PEG.

Strain TB128 grown on TSB or TYS medium had very low viability and produced large amounts of non-specific nucleases. Extensive nicking of supercoiled plasmid DNA occurred causing a high loss of plasmid during the purification. Supplementation of the culture medium with thymine (50 μg ml^{-1}) dramatically increased viability and decreased nuclease production.

Digestion with restriction endonucleases

Digestion of plasmid DNA from strains TB118 and TB150 with *Bcl*I generated six identically sized fragments indicating that these preparations contained the same plasmid, designated pAB118. Similarly, digestion of plasmid DNA from strains TB124 and TB144 with *Bcl*I generated three identical fragments; this plasmid was designated pAB124. The digestion of pAB118 with *Sal*I, *Kpn*I or *Xba*I in each case generated two bands of 7·0 kilobases (kb) and 4·3 kb. Since it is unlikely that three different enzymes would generate identically sized fragments, the preparation probably contained one plasmid of 7·0 kb and one of 4·3 kb each with a single site for *Sal*I, *Kpn*I and *Xba*I; these plasmids were designated pAB118A and pAB118B, respectively. The two plasmids were extracted from agarose gels (Thuring *et al.*, 1975) and digested with several restriction endonucleases.

The numbers of cleavage sites for each plasmid are summarized in Table 2.

Electron microscopy

Electron micrographs of open-circle forms of pAB118A/pAB118B, pAB124 and pAB128 are shown in Fig. 1. The presence of the two plasmids in strains TB118 and TB150 was confirmed by electron microscopy.

Transformation

Bacillus subtilis 168 (Trp$^-$) was transformed with each of the purified plasmid preparations with selection for streptomycin (pAB118) and tetracycline resistance (pAB124, pAB128). The results are summarized in Table 3 and clearly show that pAB124 conferred tetracycline resistance to *B. subtilis* at 25 μg ml^{-1}. The presence of the plasmids in the transformed *B. subtilis* clones was confirmed by agarose-gel electrophoresis and isopycnic centrifugation of cleared lysates prepared in the same manner as for the antibiotic-resistant thermophiles. No tetracycline-resistant *B. subtilis* clones were obtained when transforming with pAB128.

Table 1. *Screening of antibiotic-resistant thermophilic bacilli for plasmid DNA*

The maximum growth temperature was 65 °C for strains TB119 and TB123, and 60 °C for all other strains. DNA was extracted as described in Methods and cleared lysates were examined for the presence of plasmids by agarose-gel electrophoresis and isopycnic centrifugation.

Strain no.	Antibiotic selection	Detection of plasmid DNA*
TB113 to 117	Streptomycin	−
TB118	Streptomycin	+ (pAB118)
TB119	Streptomycin	−
TB150	Streptomycin	+ (pAB118)
TB120 to 122	Ampicillin	−
TB123	Tetracycline	−
TB124	Tetracycline	+ (pAB124)
TB125 to 127	Tetracycline	−
TB128	Tetracycline	+ (pAB128)
TB144	Tetracycline	+ (pAB124)
TB129 to 134	Chloramphenicol	−
TB139 to 142	Kanamycin	−

* −, No plasmid detected; +, plasmid detected. Plasmids are named according to the strain from which they were first isolated.

Table 2. *Restriction endonuclease cleavage sites in plasmid DNA*

Plasmid DNA, purified as far as extraction with phenol, was digested with restriction endonucleases as described in Methods. The resulting fragments were separated by agarose-gel electrophoresis.

	No. of cleavage sites detected in plasmid			
Enzyme (sequence)*	pAB118A (7·0 kb)	pAB118B (4·3 kb)	pAB124 (4·14 kb)	pAB128 (3·57 kb)
*Bam*HI (G/GATCC)	1	1	0	0
*Bcl*I (T/GATCA)	4	2	3	4
*Bgl*I	3	0	0	1
*Bgl*II (A/GATCT)	0	0	0	0
*Cau*I ($GG\frac{A}{T}CC$)	7	4	4	5
*Cau*II	3	2	1	1
*Eco*RI (G/AATTC)	2	0	4	3
*Hae*III (GG/CC)	6	3	4	3
*Hha*I (GCG/C)	6	5	2	3
*Hin*dII (GTPy/PuAC)	4	2	2	2
*Hin*dIII (A/AGCTT)	3	0	0	0
*Hin*fI (G/ANTC)	10	6	6	6
*Hpa*I (GTT/AAC)	2	0	1	1
*Kpn*I (GGTAC/C)	1	1	0	0
*Pst*I (CTGCA/G)	4	2	0	0
*Sal*I (G/TCGAC)	1	1	0	0
*Sst*I (GAGCT/C)	0	0	0	0
*Xba*I (T/CTAGA)	1	1	1	0
*Xma*I (C/CCGGG)	1	0	0	0

* Within the recognition sequence, Py denotes any pyrimidine, Pu any purine, N any nucleotide; / denotes the position of cleavage.

The strain of *B. subtilis* used for transformations has a high level of spontaneous mutation to streptomycin resistance ($1{\cdot}1 \times 10^{-5}$ at 100 μg ml^{-1}); however, when transforming with the two plasmids present in strains TB118 and TB150, the frequency of streptomycin resistance was not raised suggesting that neither of the two plasmids conferred streptomycin resistance to *B. subtilis*.

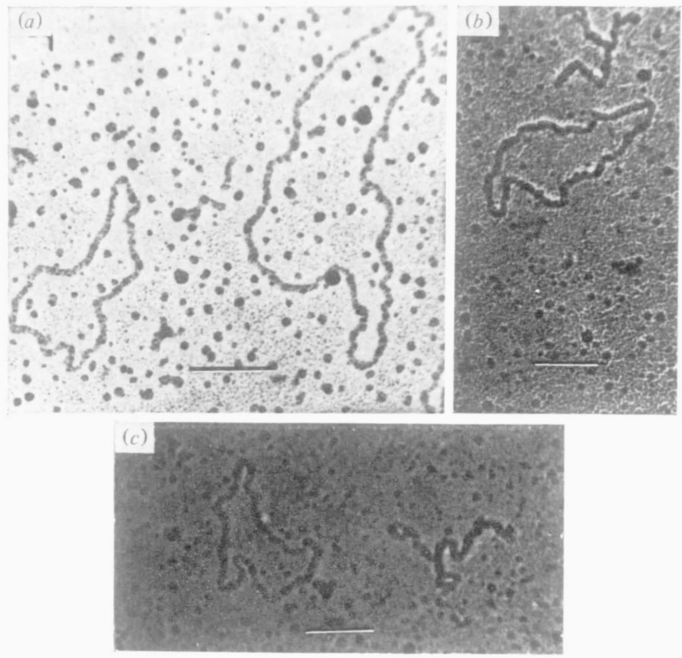

Fig. 1. Electron micrographs of open circular forms of plasmid DNA isolated from thermophilic bacilli: (*a*) pAB118A (7·0 kb) and pAB118B (4·3 kb) isolated from TB118; (*b*) pAB124 (4·14 kb) isolated from TB124; (*c*) pAB128 (3·57 kb) isolated from TB128 (Thy$^-$). Bar markers represent 0·2 μm.

Table 3. *Transformation of B. subtilis* 168 (Trp$^-$) *with plasmid DNA*

Transformation was done as described in Methods using $2{\cdot}8 \times 10^8$ bacteria in minimal medium (Stacey, 1968).

Plasmid [source]	Antibiotic (μg ml^{-1})	No. of transformants (μg DNA)$^{-1}$	$10^5 \times$ Transformation frequency
pAB124 [from mucoid strain TB124]	Tetracycline (25)	1000	3·6
	(12·5)	1950	6·9
pAB124 [from non-mucoid strain TB144]	Tetracycline (25)	1050	3·8
	(12·5)	1800	6·4
pAB128 [from strain TB128 (Thy$^-$)]	Tetracycline (25)	0	–
	(12·5)	0	–
pAB118 [from mucoid strain TB118]	Streptomycin (75)	0	–
	(100)	0	–
pAB118 [from non-mucoid strain TB150]	Streptomycin (75)	0	–
	(100)	0	–
pUB110 [from *B. subtilis*]	Neomycin (25)	3000	11
	(12·5)	3550	13

DISCUSSION

We isolated 29 antibiotic-resistant thermophilic bacilli and, after screening for closed circular duplex DNA molecules, isolated four different plasmids. The isolation of these strains resistant to five different antibiotics from one sample of river sludge and silage was rather surprising and suggests that antibiotic-resistant thermophiles are quite widespread in nature.

The plasmids isolated from TB118 and TB150 (Str^R) and TB124 and TB144 (Tet^R) were shown by restriction endonuclease digestion to be identical with each other although each

pair of strains differed morphologically. Strains TB118 and TB124 grown on TSB medium produced very large, flat, budding and mucoid colonies, whereas TB150 and TB144 produced small, round, non-budding and non-mucoid colonies on this medium. Their fermentation patterns against 15 different carbohydrate sources indicated that the difference between the two pairs of strains was only morphological.

The smaller plasmids pAB118B, pAB124 and pAB128 might make suitable vectors for cloning purposes since pAB118B has one site for *Bam*HI, *Kpn*I, *Sal*I and *Xba*I, pAB124 has one site for *Xba*I and *Cau*II, and pAB128 has one site for *Bgl*I and *Cau*II. However, the recognition sequence of *Cau*II has not been determined and it is not known whether this enzyme produces a cohesive terminus.

The successful transformation of *B. subtilis* with pAB124 and the expression of the plasmid's tetracycline resistance gene will enable the further development of this plasmid as a vector for genetic manipulation in thermophilic bacilli and *B. subtilis*. Recently, several plasmids coding for antibiotic resistance functions have been isolated from *Staphylococcus aureus* (Lofdahl *et al.*, 1978*a*; Wilson & Baldwin, 1978) and are expressed in *B. subtilis* (Ehrlich, 1977; Gryczan *et al.*, 1978). The possibility of the use of these plasmids for molecular cloning in *B. subtilis* has been reported (Keggins *et al.*, 1978; Ehrlich, 1978; Gryczan & Dubnau, 1978; Lofdahl *et al.*, 1978*b*), and their application to *B. stearothermophilus* in conjunction with pAB124 is now being investigated.

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