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 4.9×10^6 (7.0 kilobases) and pAB118B of molecular weight 3.0×10^6 (4.3 kilobases). Two of the tetracycline-resistant strains each contained a plasmid (pAB124) of molecular weight 2.9×10^6 (4.14 kilobases), while a third harboured a small plasmid (pAB128) of molecular weight 2.5×10^6 (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; K₂HPO₄, 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-Na₂EDTA, 50 mM-NaCl) and suspended in 3.0 ml TES buffer containing 25% (w/v) sucrose; 0.5 ml 0.2 M-Na₂EDTA 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 45 000 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 45 000 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 45 000 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 12 000 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 45 000 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 150 000 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-Na₂EDTA, pH 7.5] for 3 h at 90 000 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 N₂) equilibrated in 100 mM-Tris/HCl pH 8.0, 10 mM-Na₂EDTA, 50 mM-NaCl and mixed gently for 5 min. The phenol phase was removed after centrifuging at 10 000 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 40 000 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-Na₂EDTA. 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 *Hinf*I 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-MgCl₂, 0.5 mM-dithiothreitol (DTT) and 0.5 mg gelatine ml⁻¹ (*Bam*HI, *Bcl*I, *Bgl*II, *Bgl*II, *Cau*I, *Cau*II, *Kpr*I, *Psr*I, *Sal*I, *Sst*I, *Xba*I and *Xma*I); B, 20 mM-Tris/HCl pH 7.4, 50 mM-NaCl, 20 mM-MgCl₂, 0.5 mM-DTT and 0.5 mg gelatine ml⁻¹ (*Hae*III, *Hha*I, *Hind*II, *Hind*III, *Hinf*I and *Hpa*I); and C, 100 mM-Tris/HCl pH 7.4, 50 mM-NaCl, 20 mM-MgCl₂, 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-Na₂EDTA, 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 transilluminator, 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. Pre-warmed TY5 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 $\mu\text{g ml}^{-1}$) 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 $\mu\text{g ml}^{-1}$. 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.

Enzyme (sequence)*	No. of cleavage sites detected in plasmid			
	pAB118A (7.0 kb)	pAB118B (4.3 kb)	pAB124 (4.14 kb)	pAB128 (3.57 kb)
<i>Bam</i> HI (G/GATCC)	1	1	0	0
<i>Bcl</i> I (T/GATCA)	4	2	3	4
<i>Bgl</i> II	3	0	0	1
<i>Bgl</i> II (A/GATCT)	0	0	0	0
<i>Cau</i> I (GG ^A _T CC)	7	4	4	5
<i>Cau</i> II	3	2	1	1
<i>Eco</i> RI (G/AATTC)	2	0	4	3
<i>Hae</i> III (GG/CC)	6	3	4	3
<i>Hha</i> I (GCG/C)	6	5	2	3
<i>Hind</i> II (GTPy/PuAC)	4	2	2	2
<i>Hind</i> III (A/AGCTT)	3	0	0	0
<i>Hinf</i> I (G/ANTC)	10	6	6	6
<i>Hpa</i> I (GTT/AAC)	2	0	1	1
<i>Kpn</i> I (GGTAC/C)	1	1	0	0
<i>Pst</i> I (CTGCA/G)	4	2	0	0
<i>Sal</i> I (G/TCGAC)	1	1	0	0
<i>Sst</i> I (GAGCT/C)	0	0	0	0
<i>Xba</i> I (T/CTAGA)	1	1	1	0
<i>Xma</i> 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 (1.1×10^{-5} at $100 \mu\text{g ml}^{-1}$); 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 2.8×10^8 bacteria in minimal medium (Stacey, 1968).

Plasmid [source]	Antibiotic ($\mu\text{g ml}^{-1}$)	No. of trans-formants ($\mu\text{g DNA}$) ⁻¹	$10^5 \times$ Trans-formation 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 <i>B. subtilis</i>]	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*II 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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