Characterization of *Bacillus stearothermophilus* Plasmid pAB124 and Construction of Deletion Variants

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A restriction endonuclease cleavage map of the tetracycline resistance plasmid pAB124, originally isolated from *Bacillus stearothermophilus*, was constructed using ten enzymes. Tetracycline resistance was associated with a 1.95 megadalton (Md) region of pAB124 lying between two *EcoRI* sites, and this region was circularized to produce a viable tetracycline resistance plasmid (pAB224), with two *EcoRI* fragments of pAB124 deleted amounting to 0.95 Md. A second plasmid (pAB524) with one *EcoRI* fragment (0.6 Md) of pAB124 deleted was also constructed. Restriction endonuclease cleavage maps of pAB224 and pAB524 were constructed.

INTRODUCTION

Several plasmids have been isolated from Gram-positive spore-forming bacilli, e.g. *Bacillus pumilus* (Lovett & Bramucci, 1975), *B. megaterium* (Carlton & Helinski, 1969) and *B. subtilis* (Tanaka *et al.*, 1977; Le Hegarat & Anagnostopoulos, 1977), although none of these has been shown to be associated with any phenotypic trait. More recently, a plasmid from *B. cereus* coding for tetracycline resistance and one from *B. subtilis* producing a bacteriocin have been isolated (Bernhard *et al.*, 1978).

The presence of a plasmid in a bacteriocin (thermocin)-producing strain of *B. stearothermophilus* has recently been reported (Sharp *et al.*, 1979), and we have described the isolation of four plasmids from antibiotic-resistant thermophilic bacilli (Bingham *et al.*, 1979), one of which (pAB124) was shown to confer tetracycline resistance on *B. subtilis*. This paper describes the characterization of pAB124 and the construction of deletion variants of this plasmid.

METHODS

Bacterial strains. Thermophilic *Bacillus* strain TB124, isolated as previously described (Bingham *et al.*, 1979), was classified as *Bacillus stearothermophilus* using standard identification procedures (Cowan & Steel, 1974). *Bacillus subtilis* harbouring plasmid pAB124 was prepared by direct plasmid transformation. *Bacillus subtilis* IG20 (*hsr hsm trp*) was kindly provided by A. Docherty, Department of Bacteriology, Bristol University.

Culture media. For plasmid isolation all strains were grown on TYS medium (Bingham *et al.*, 1979) supplemented with tetracycline at 25 µg ml⁻¹. The following minimal medium (SMS) was used for *B. subtilis* (g l⁻¹): (NH₄)₂SO₄, 2; K₂HPO₄, 14; KH₂PO₄, 6; sodium citrate, 1; MgSO₄·7H₂O, 0.2; glucose, 5.

Isolation of plasmid DNA. This was carried out with 400 ml cultures using the procedure previously described (Bingham *et al.*, 1979). Plasmid pAB124 was originally isolated from *B. stearothermophilus* TB124 and transformed into *B. subtilis* (Bingham *et al.*, 1979). All experiments described in this paper used pAB124 isolated from *B. subtilis*. Plasmid pUB1654 was a gift from A. Docherty.

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Small-scale plasmid preparation. This was used for extracting recombinant DNA from large numbers of clones. Cultures (15 ml) in TYS medium containing the selective antibiotic(s) were grown overnight at 37 °C without shaking. Cells were harvested at 10000 *g*, washed once in 5 ml TES buffer (30 mM-Tris/HCl pH 8.0, 5 mM-Na₂EDTA, 50 mM-NaCl) and resuspended in 100 µl TES. EDTA (25 µl, 0.25 M) and lysozyme (25 µl, 15 mg ml⁻¹) were added and the suspension was shaken gently on ice for 10 min. The suspension was then placed in a 37 °C water bath for 5 min and 25 µl sodium dodecyl sulphate (10 %, w/v) was added, to bring about lysis, followed by 50 µl NaCl (5 M); this mixture was left at 0 °C for 2.5 to 3 h. A cleared lysate was obtained by centrifugation at 45000 *g* for 45 min, and this was then extracted twice with chloroform/3-methylbutan-1-ol (24:1, v/v), once with phenol (freshly distilled over N₂) equilibrated in TES buffer and three times with diethyl ether. The plasmid DNA obtained was suitable for restriction endonuclease analysis.

Plasmid transformation. *Bacillus subtilis* IG20 was grown overnight at 37 °C in 200 ml SMS medium supplemented with 0.5 ml tryptophan (2 mg ml⁻¹) and then diluted with an equal volume of similar medium (prewarmed). The absorbance (540 nm) was monitored at 30 min intervals, and at a value equivalent to maximum competence (30 to 60 min after cessation of exponential growth) 1 ml samples of the culture were added to plasmid DNA (0.5 to 5 µg) in 50 µl polyethylene glycol 1000 (50 %, w/v) and incubated for 1 h at 37 °C with vigorous aeration. Prewarmed TYS medium (5 ml) was then added and incubation was continued for a further hour prior to plating on selective media. Selective concentrations of antibiotics used were 25 µg ml⁻¹ for tetracycline and neomycin and 75 µg ml⁻¹ for streptomycin. It was not necessary to induce competence by dilution of the culture into a starvation medium (Stacey, 1968).

Restriction endonucleases. *EcoRI* and *CauII* were purified by procedures developed in this laboratory and digestions were carried out in buffers previously described (Bingham *et al.*, 1979). *BstEII*, *HaeIII*, *HhaI*, *HindII*, *HpaI*, *HpaII*, *SalI*, *XbaI* and T4-DNA ligase were purchased from Uniscience Ltd, Cambridge, and digestions carried out in the buffers recommended by the manufacturer. Reaction mixtures contained 0.5 to 1 µg plasmid DNA in a final volume of 20 to 25 µl, and reactions were terminated by adding Na₂EDTA to a final concentration of 10 mM (*CauII*, *BstEII*, *SalI*) or heating at 66 °C for 10 min. For double digestions involving enzymes with different buffer requirements, plasmid DNA was first digested with the enzyme requiring the buffer of lower ionic strength, and then the buffer was adjusted with 10× concentrated components prior to the addition of the second enzyme.

Agarose gels (0.8 %, w/v) were used as previously described (Bingham *et al.*, 1979), with an *EcoRI* plus *HindIII* λ CI857 digest providing fragments of standard sizes: 13.4, 3.35, 3.2, 2.8, 2.32, 1.39, 1.27, 1.05, 0.89, 0.59, 0.47 and 0.31 megadaltons (Md) (Murray & Murray, 1975). The sizes of unknown fragments were determined graphically from a 10× enlargement of a photograph (35 mm) and were taken as an average of three estimations.

Cloning of pAB124 *EcoRI* fragments. Plasmids pAB124 (1 µg) and pUB1654 (1 µg) were digested to completion with *EcoRI* and then the enzyme was denatured by heating at 66 °C for 10 min. The fragments were precipitated by adding 3 M-sodium acetate to a final concentration of 0.3 M and 2 vol. cold (−20 °C) ethanol. After 10 min in a solid CO₂/ethanol bath the precipitate was collected by centrifugation. After removal of all the ethanol the precipitate was resuspended in T4 ligase buffer (66 mM-Tris/HCl pH 7.6, 6.6 mM-MgCl₂, 10 mM-dithiothreitol, 0.4 mM-ATP) and incubated overnight at 4 °C with 0.05 units T4-DNA ligase.

RESULTS

Restriction endonuclease site mapping of pAB124

The fragment sizes obtained by digestion of pAB124 DNA (isolated from *B. subtilis*) with *BstEII*, *CauII*, *EcoRI*, *HaeIII*, *HindII*, *HhaI*, *HpaI*, *HpaII*, *SalI* and *XbaI* are given in Table 1. These sizes were determined graphically using a lambda DNA digested with *HindIII* plus *EcoRI* as standards subjected to electrophoresis on the same gels as the unknown fragments. The average value for the total size of pAB124 was close to 2.9 Md. A series of double digestion experiments was carried out in order to construct a cleavage map of pAB124 (Table 2).

The positions of the four single sites (*BstEII*, *CauII*, *HpaI*, *XbaI*) relative to each other were determined taking *CauII* as the reference point. An *HpaI* plus *CauII* digest and a *BstEII* plus *CauII* digest both gave a small fragment of 0.35 Md; thus the *BstEII* and *HpaI* sites are the same distance from *CauII* site either on the same side, i.e. very close together, or on opposite sides 0.7 Md apart. The latter position was confirmed by a *BstEII* plus *HpaI* double digestion. The *CauII* plus *XbaI* digestion generated fragments of 1.85 and 1.05 Md. It was assumed that the *XbaI* site was 1.85 Md to the right of *CauII* and the *HpaI*

Table 1. *Fragments produced by restriction endonuclease digestion of pAB124*
Digestions and agarose-gel electrophoresis were carried out as described in Methods using plasmid DNA isolated from *B. subtilis*.

Enzyme	Fragment size (Md)			Sum of fragment sizes (Md)
	A	B	C	
<i>Bst</i> EII	2.90			2.90
<i>Cau</i> II	2.90			2.90
<i>Hpa</i> I	2.90			2.90
<i>Xba</i> I	2.90			2.90
<i>Hha</i> I	2.23	0.70		2.93
<i>Hind</i> II	2.30	0.62		2.92
<i>Hae</i> III	1.50	1.18	0.25	2.93
<i>Hpa</i> II	1.80	0.70	0.42	2.92
<i>Tha</i> I	1.84	0.65	0.40	2.89
<i>Eco</i> RI	1.95	0.61	0.33	2.89

Table 2. *Fragments produced by digestion of pAB124 with two restriction endonucleases*
Double digestions and agarose-gel electrophoresis were carried out as described in Methods using plasmid DNA isolated from *B. subtilis*.

Enzyme pair	Fragment size (Md)				Sum of fragment sizes (Md)
	A	B	C	D	
<i>Bst</i> EII + <i>Cau</i> II	2.55	0.35			2.90
<i>Bst</i> EII + <i>Hpa</i> I	2.23	0.70			2.93
<i>Bst</i> EII + <i>Xba</i> I	2.20	0.70			2.90
<i>Cau</i> II + <i>Hpa</i> I	2.57	0.35			2.92
<i>Cau</i> II + <i>Xba</i> I	1.85	1.05			2.90
<i>Hpa</i> I + <i>Xba</i> I	1.48	1.40			2.88
<i>Hha</i> I + <i>Cau</i> II	2.20	0.44	0.25		2.89
<i>Hha</i> I + <i>Xba</i> I	1.59	0.71	0.60		2.90
<i>Hha</i> I + <i>Hpa</i> I	2.10	0.70	0.10		2.90
<i>Hind</i> II + <i>Cau</i> II	2.30	0.33	0.28		2.91
<i>Hind</i> II + <i>Xba</i> I	1.50	0.78	0.62		2.90
<i>Hind</i> II + <i>Hpa</i> I	2.30	0.61	—		2.91
<i>Hae</i> III + <i>Cau</i> II	1.24	1.18	0.25	0.24	2.91
<i>Hae</i> III + <i>Bst</i> EII	1.18	0.89	0.59	0.25	2.91
<i>Hae</i> III + <i>Xba</i> I	1.30	1.18	0.25	0.18	2.91
<i>Hae</i> III + <i>Hpa</i> I	1.51	1.19	0.16	0.10	2.96
<i>Hpa</i> II + <i>Cau</i> II	1.80	0.70	0.42	—	2.92
<i>Hpa</i> II + <i>Bst</i> EII	1.80	0.42	0.35	0.32	2.89
<i>Hpa</i> II + <i>Xba</i> I	1.80	0.70	0.38	(0.05)†	2.93
<i>Hpa</i> II + <i>Hpa</i> I	1.45	0.72	0.42	0.35	2.94
<i>Tha</i> I + <i>Cau</i> II	1.80	0.42	0.40	0.23	2.85
<i>Tha</i> I + <i>Bst</i> EII	1.83	0.62	0.40	(0.05)†	2.90
<i>Tha</i> I + <i>Xba</i> I	1.60	0.65	0.40	0.26	2.91
<i>Tha</i> I + <i>Hpa</i> I	1.75	0.65	0.40	0.10	2.90
<i>Eco</i> RI + <i>Cau</i> II	1.55	0.60	0.40	0.33	2.88
<i>Eco</i> RI + <i>Bst</i> EII	1.90	0.62	0.33	(0.05)†	2.90
<i>Eco</i> RI + <i>Xba</i> I	1.95	0.33*	0.29	—	2.88
<i>Eco</i> RI + <i>Hpa</i> I	1.20	0.75	0.60	0.32	2.87

* This fragment was overabundant and assumed to be a 'double'.

† Fragment not detected, but its presence can be predicted.

and *Bst*EII sites were positioned using this orientation. If *Hpa*I lies 0.35 Md to the right of the *Cau*II site a double digestion with *Hpa*I plus *Xba*I would generate 1.48 and 1.40 Md fragments, whereas if it lies 0.35 Md to the left, 0.8 and 2.1 Md fragments would be generated. Since fragments of the former sizes were obtained, the *Hpa*I site must lie to the right of the

Fig. 1. Relative positions of fragments produced by digestion of pAB124 with several restriction endonucleases.

CauII site with the *BstEII* site 0.35 Md to the left. The positions of the four sites (Fig. 1) were consistent with the results of all the double digestions carried out.

Using these four single sites as reference points the fragments generated by *HhaI*, *HindII*, *HpaII*, *HaeIII*, *ThaI* and *EcoRI* (Table 1) were ordered using the established techniques of double-digestion mapping. For example, the *EcoRI* fragments were ordered easily as only one position of *EcoRI* fragment A was consistent with it containing the *BstEII*, *CauII* and *HpaI* sites but not the *XbaI* site which is present in *EcoRI* fragment B. A *CauII* plus *EcoRI* digestion generated 1.55 and 0.4 Md fragments from *EcoRI* A; the 1.55 Md fragment could only lie to the right of the *CauII* site with the 0.4 Md fragment to the left (Fig. 1). The *EcoRI* B fragment must lie to the right of *EcoRI* A over the *XbaI* site in the position shown (Fig. 1) since an *EcoRI* plus *XbaI* digest yielded 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.

The fragments generated by digestion of pAB124 with *HhaI*, *HindII*, *HaeIII*, *HpaII* and *ThaI* were ordered in a similar manner and their relative positions are shown in Fig. 1.

Cloning of EcoRI fragments of pAB124 carrying the tetracycline resistance gene

Staphylococcal plasmid pUB1654 conferring streptomycin and neomycin resistance was used. This has a single *EcoRI* site lying within the streptomycin resistance gene (A. Docherty, personal communication). It would therefore be expected that insertion of pAB124 fragments at this site would inactivate streptomycin resistance. Transformation of the ligated fragments generated about 35% of clones that were *Nm^r Str^r Tc^r*. DNA was extracted from several of these clones and was analysed with *EcoRI* (Table 3). The minimum requirement for tetracycline resistance was shown to be the *EcoRI* fragment A (1.95 Md) of pAB124 present alone in 50% of the recombinant molecules examined.

Construction of small tetracycline resistance plasmids from cloned EcoRI fragments of pAB124

Plasmid DNA from each type of clone, representing all combinations of pAB124 *EcoRI* fragments (Table 3), was digested to completion with *EcoRI* and then ligated with T4-DNA ligase. The DNA was then transformed into *B. subtilis* IG20 and *Tc^r Nm^r Str^r* clones were identified. Two types of plasmid were isolated from the clones examined (Table 4): pAB224

Table 3. *Restriction endonuclease analysis of recombinant plasmids of pAB124 and pUB1654*

Plasmid DNA was extracted from clones of Nm^r Str^r Tc^r phenotype and digested with *Eco*RI as described in Methods.

Clones identified		<i>Eco</i> RI digestion		Type of recombinant
No.	Plasmid size (Md)	Fragment size (Md)	pAB124 fragment(s) inserted	
10	6.0	4.0*	—	I
		1.95	A	
3	6.55	4.0*	—	II
		1.95	A	
		0.60	B	III
2	6.30	4.0*	—	
		1.95	A	
		0.32	C	
4	6.90	4.0*	—	IV
		1.95	A	
		0.60	B	
		0.32	C	

* This fragment corresponds to pUB1654.

Table 4. *Eco*RI analysis of tetracycline resistance plasmids derived from pAB124:pUB1654 recombinant molecules

Plasmid DNA was extracted from Tc^r Nm^s Str^s clones and digested with *Eco*RI as described in Methods.

DNA source*		Tetracycline resistance plasmids			
Type of recombinant	pAB124 <i>Eco</i> RI fragment(s) present	No. of clones†	Size (Md)	<i>Eco</i> RI fragments (Md)	pAB124 <i>Eco</i> RI fragment(s) present
I	A	10	1.95	1.95	A (pAB224)
II	A+B	10	1.95	1.95	A (pAB224)
III	A+C	7	1.95	1.95	A (pAB224)
		3	2.3	1.95, 0.32	A+C (pAB524)
IV	A+B+C	8	1.95	1.95	A (pAB224)
		2	2.3	1.95, 0.32	A+C (pAB524)

* Recombinant plasmids of pAB124 and pUB1654 (see Table 3).

† Ten clones from each transformation were examined.

which was the recircularized *Eco*RI fragment A of pAB124 (1.95 Md) and pAB524 containing *Eco*RI fragments A plus C (2.3 Md). No plasmids containing *Eco*RI fragments A plus B or reconstructed pAB124 were detected.

Plasmid pAB224 was shown to contain seven single sites (*Eco*RI, *Bst*EII, *Cau*II, *Hpa*II, *Hha*I, *Tha*I and *Hpa*I). The position of each site was determined by double digestions and was shown to be consistent with pAB224 being the recircularized *Eco*RI fragment A of pAB124 (Fig. 2). Therefore *Eco*RI A contains the tetracycline resistance genes and all the known essential functions of pAB124.

An *Eco*RI digest of pAB524 generated fragments of 1.95 Md and 0.32 Md. The larger fragment contained *Bst*EII, *Cau*II and *Hpa*I sites and double digestions confirmed that it was the *Eco*RI fragment A of pAB124; the smaller fragment contained *Hha*I and *Hpa*II sites and was assumed to be the *Eco*RI fragment C of pAB124. Two orientations of *Eco*RI C within pAB524 were possible and each would give different sized fragments when digested with *Hpa*II or *Hha*I. The observed sizes of the fragments obtained from five different examples of pAB524 indicated that the order of *Eco*RI fragments A and C was the same as that observed in pAB124 (Fig. 2).

DISCUSSION

Classification of the thermophilic bacilli harbouring pAB124 by standard procedures indicated that the strains were quite closely related to *B. stearothermophilus* but not identical to the neotype strain NCA 1503. The aim of this research is to develop a vector for cloning in *B. stearothermophilus* and pAB124 could provide the basis for such a vector. The expression of pAB124 in *B. subtilis* will also allow the use of this plasmid as a vector for gene cloning in this organism, and it is important to note that the plasmid is stable with no apparent alteration upon uptake and replication in *B. subtilis*, as determined by restriction endonuclease analysis. The transformation frequencies, however, are lower with plasmid DNA isolated from *B. stearothermophilus* than with that from *B. subtilis*; this may in part be due to the formation of concatemers in *B. subtilis* that transform at a higher frequency than the monomeric species (Canosi *et al.*, 1978). The most convenient restriction site for cloning in pAB124 is the single *Xba*I, since this enzyme produces a cohesive terminus and lies within the region of pAB124 not essential for tetracycline resistance or plasmid replication (Fig. 2). We have successfully inserted a staphylococcal neomycin resistance plasmid (pUB110) at the *Xba*I site without inactivating tetracycline resistance. Of the remaining single restriction sites in pAB124, it is not known whether *Bst*EII and *Cau*II produce a cohesive terminus and *Hpa*I is known to produce base-paired termini, thus making the use of these sites for cloning more difficult. In addition, these latter sites lie within the region of pAB124 that appears to be associated with tetracycline resistance.

The development of the staphylococcal vector pUB1654 (Nm^r Str^r; A. Docherty, personal communication) allowed us to examine which region of pAB124 contained the

tetracycline resistance genes. Only the large *Eco*RI fragment (A) was essential for the expression of the Tc^r phenotype and the survival of the plasmid as a replicon. The circularized *Eco*RI fragment A (pAB224) contained one site for each of seven restriction endonucleases (Fig. 2); three of these enzymes produce a cohesive terminus (*Eco*RI, *Hpa*II and *Hha*I), and therefore this plasmid is of potential use as a small (1.95 Md) vector for gene cloning. We have attempted to insert at the *Eco*RI site the *B. licheniformis* β -lactamase that is cloned between two *Eco*RI sites in a lambda vector (W.J. Brammar, personal communication) and also the β -lactamase of the staphylococcal plasmid pI258 (Novick *et al.*, 1979). In both cases we could not isolate recombinant molecules containing a β -lactamase gene. We are therefore continuing to examine the ability of pAB224 to express genes cloned at the *Eco*RI site.

The construction of pAB524 in a similar manner to pAB224 gives rise to a plasmid with two *Eco*RI, two *Hpa*II and two *Hha*I sites, but no *Xba*I site. A potentially useful deletion plasmid would be that formed by circularization of *Eco*RI fragments A plus B of pAB124, which would retain the single *Xba*I site and also contain one *Hha*I site. However, repeated ligations of *Eco*RI-digested recombinants of pUB1654 containing *Eco*RI fragments A plus B failed to generate this plasmid; only pAB224 (*Eco*RI A) and pUB1654 containing *Eco*RI fragment A of pAB124 were obtained. This was rather surprising since one would expect circularization of *Eco*RI fragments A plus B to be about as efficient as circularization of *Eco*RI fragments A plus C (pAB524; Table 4).

We intend to examine the ability of pAB124 and pAB224 to express chromosomal functions cloned at the *Xba*I and *Eco*RI sites, respectively, in order to evaluate their use as vectors for gene cloning in *Bacillus* species.

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