Two Replication Determinants of an Antibiotic-resistance Plasmid, pTB19, from a Thermophilic Bacillus

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Two different replication determinants were found on an antibiotic resistance plasmid, pTB19, from a thermophilic bacillus. One replication determinant (designated RepA) was functional only in *Bacillus subtilis*, whereas the other (designated RepB) functioned in both *B. subtilis* and *Bacillus stearothermophilus*. A deletion plasmid, pTB90, carrying the RepB derived from pTB19 coincidentally contained the specific 1.0 MDal *Eco*RI fragment of a cryptic plasmid pBSO2 from *B. stearothermophilus*. The presence of this 1.0 MDal *Eco*RI fragment in various deletion plasmids from pTB90 increased transformation frequencies for *B. stearothermophilus* 10^3 to 10^4 times and lowered plasmid copy numbers in the host strain to about one-tenth of those found for plasmids lacking this fragment.

INTRODUCTION

We have isolated the drug-resistance plasmid pTB19 (17.2 MDal) that specifies resistance to both kanamycin and tetracycline from a thermophilic bacillus and constructed some deletion plasmids from it (Imanaka *et al.*, 1981*a*, 1982). Among these deletants, pTB90, like the original plasmid pTB19, could transform both *Bacillus subtilis* and *Bacillus stearothermophilus*, whereas pTB51, pTB52 and pTB53 could replicate only in *B. subtilis* (Imanaka *et al.*, 1982). The success or failure of transformation in both strains depended clearly on the species of plasmid DNA. This observation stimulated further study on the difference or identity of the regulation mechanisms of plasmid DNA replication between mesophiles and thermophiles.

In this paper we describe the finding of two replication determinants on pTB19, and a specific DNA fragment (1.0 MDal *Eco*RI fragment of pTB90) which could affect one of the two replication determinants in *B. stearothermophilus* was characterized.

METHODS

Media and materials. All the media used in this work have been described previously (Imanaka *et al.*, 1981*a*, 1982). Unless otherwise specified, all chemicals used in this work came from the same sources as described in the previous papers (Imanaka *et al.*, 1981*a*, 1982).

Bacterial strains and plasmids. These are listed in Table 1. The restriction endonuclease cleavage maps of these plasmids are shown in Fig. 1. *Bacillus stearothermophilus* CU21, which is a cured strain from the streptomycin-resistant strain S1 carrying a cryptic plasmid pBSO1, was used as the recipient in transformation (Imanaka *et al.*, 1982). However, during the process of transformation some colonies of this strain CU21 fortuitously carrying another cryptic plasmid were observed. This plasmid, designated pBSO2, was larger than pBSO1; it is described later in this paper.

Preparation of plasmid and chromosomal DNA. Plasmid DNA was prepared by either the rapid alkaline extraction method or the CsCl-ethidium bromide equilibrium density-gradient centrifugation, as described by Imanaka *et al.* (1982). Chromosomal DNA was prepared as described by Imanaka *et al.* (1981*b*).

Abbreviations: Km, kanamycin; Tc, tetracycline.

Table 1. *Bacterial strains and plasmids*

Strain	Characteristics*	Reference	
<i>B. stearothermophilus</i> CU21	Str ^r	Imanaka <i>et al.</i> (1982)	
<i>B. subtilis</i> MI113	<i>arg-15 trpC2</i> r _M ⁻ m _M ⁻	Imanaka <i>et al.</i> (1981 <i>a</i>)	
Plasmid	Mol. wt (MDal)	Characteristics	Reference
pTB19	17.2	Km ^r Tc ^r	Imanaka <i>et al.</i> (1981 <i>a</i>)
pTB51	8.4	Km ^r	Imanaka <i>et al.</i> (1981 <i>a</i>)
pTB52	7.0	Tc ^r	Imanaka <i>et al.</i> (1981 <i>a</i>)
pTB53	11.2	Km ^r Tc ^r	Imanaka <i>et al.</i> (1981 <i>a</i>)
pTB90	6.7	Km ^r Tc ^r	Imanaka <i>et al.</i> (1982)
pBSO1	18.3	—	Imanaka <i>et al.</i> (1982)
pBSO2	19.5	—	This work
pUB110	3.0	Km ^r	Imanaka <i>et al.</i> (1981 <i>b</i>)
pUB111	4.0	Km ^r	This work

* Str^r, streptomycin resistant.

Fig. 1. Restriction endonuclease cleavage maps of plasmids. Cleavage sites of *Bam*HI, *Bgl*II, *Bst*EII, *Pst*I, *Hind*III, and *Xba*I are indicated by Ba, Bg, Bs, P, H and X, respectively. Cleavage sites of *Eco*RI are indicated by the bars inside the circles.

Transformation. Transformation of *B. stearothersophilus* protoplasts with plasmid DNA was done as described by Imanaka *et al.* (1982). Transformants were selected on regeneration agar containing either kanamycin (Km; 25 µg ml⁻¹) or tetracycline (Tc; 5 µg ml⁻¹) and stored on L agar containing Km (5 µg ml⁻¹) or Tc (5 µg ml⁻¹) at 4 °C.

Transformation of competent cells of *B. subtilis* MI113 with plasmid DNA was carried out as described by Imanaka *et al.* (1981a). Transformants were selected on L agar containing Km (5 µg ml⁻¹) or Tc (25 µg ml⁻¹) and stored on the same plates at room temperature.

Assessment of the plasmid copy number. The plasmid copy number was assessed by the procedure of Kieser *et al.* (1982) with some modifications. Cells grown in L broth were harvested by centrifugation (12000 g, 5 min), and washed with TE buffer consisting of 50 mM-Tris/HCl (pH 8.5) and 1 mM-Na₂EDTA. Cells were resuspended in a

solution of 15% (w/v) sucrose, 50 mM-Tris (pH 8.5), 50 mM- Na_2EDTA , and 1 mg lysozyme ml^{-1} for *B. stearothermophilus* (5 mg lysozyme ml^{-1} for *B. subtilis*). The mixture was incubated for 30 min at 0 °C in the case of *B. stearothermophilus* (37 °C for *B. subtilis*). Lysis was brought about at room temperature by the addition of one volume of a solution containing 1% (w/v) Sarkosyl, 50 mM-Tris (pH 8.5), and 50 mM- Na_2EDTA , and the mixture was heated at 70 °C for 5 min and, after cooling, treated once with phenol, followed by treatment with chloroform. DNA in the aqueous supernatant was concentrated by precipitation with ethanol and then serially diluted in a solution that contained 14% (w/v) sucrose, 10 mM- Na_2EDTA , 100 μg RNAase ml^{-1} and 0.1% (w/v) bromophenol blue.

After incubation at 37 °C for 1 h, samples for each dilution were loaded onto gels for electrophoresis. The gels were stained for 1 h in ethidium bromide solution (1.0 μg ml^{-1}), irradiated with short wavelength UV to nick closed-circular DNA, and stained for another 1 h in the ethidium bromide solution. The gels were destained by rinsing with water and photographed with Polaroid type 665 film. The negatives were scanned with a densitometer (PC-910, Shimadzu Co., Kyoto, Japan) to measure the peak areas of chromosome and plasmid. The ratio of plasmid to chromosomal DNA was assessed from that of peak areas at a definite dilution, having confirmed that intensities of both peaks on densitometer were proportional to the dilution ratio of the samples. The molecular weights of the chromosomes of *B. subtilis* and *B. stearothermophilus* were taken as 2×10^9 (Imanaka *et al.*, 1981a).

Nick translation and Southern hybridization. Nick-translated DNA was prepared by using nick translation kit PB 5025 (Amersham). The labelled DNA was freed from low molecular weight materials by passing through a Sephadex G50 column (Pharmacia). Blotting hybridization experiments were carried out by the method of Maniatis *et al.* (1982).

Other procedures. Other procedures for digestion of plasmid DNA with restriction endonucleases, ligation of DNA and agarose gel electrophoresis are all described elsewhere (Imanaka *et al.*, 1981a, 1982).

RESULTS

Description of the host bacterium

A streptomycin-resistant mutant strain, S1, which was spontaneously obtained from *B. stearothermophilus* IFO 12550 (ATCC 12980) carried the plasmid pBSO1 (18.3 MDal), which could not be detected as a plasmid in the cured strain CU21 (Imanaka *et al.*, 1982). However, some of the single colonies of CU21 were found during successive transfers on agar plates to have carried another plasmid pBSO2 (19.5 MDal). Plasmid pBSO2 was compared with pBSO1 for restriction endonuclease cleavage sites. Both pBSO1 and pBSO2 prepared by CsCl-ethidium bromide equilibrium density-gradient centrifugation were cleaved with *EcoRI*, *HindIII*, *BamHI* and *SalI* at 8, 7, 4 and 2 sites, respectively. The digestion patterns of pBSO2, one of which is shown in Figs 2 and 3 (lane C), were very similar to those of pBSO1. It was most likely that the plasmid pBSO2 might have been integrated into the chromosome of *B. stearothermophilus* CU21. This possibility was examined by Southern transfer, using pBSO2 as a probe. The chromosomal DNA prepared by CsCl-ethidium bromide equilibrium density-gradient centrifugation from *B. stearothermophilus* CU21, in which the plasmid was not detected, was digested with *EcoRI* and was subjected to agarose gel electrophoresis. The DNA bands were transferred to a nitrocellulose filter, heated and hybridized with ^{32}P -labelled pBSO2. It was shown that pBSO2 hybridized to the chromosomal DNA of *B. stearothermophilus* CU21 (Fig. 3, lanes D, d and d'). It was concluded that the plasmid pBSO2 was integrated into the chromosomal DNA of *B. stearothermophilus* CU21. This kind of integration of a plasmid into the host chromosome has already been reported for *B. subtilis* (pE194) (Hofemeister *et al.*, 1983). Needless to say, *B. stearothermophilus* CU21, in which no plasmid was detected, was used as the recipient in the transformation experiments.

Comparison of pTB90 with pTB19

Plasmid pTB19 (17.2 MDal), encoding resistance to both kanamycin and tetracycline, which was originally isolated from a thermophilic bacillus, could transform both *B. subtilis* and *B. stearothermophilus* (Imanaka *et al.*, 1981a, 1982). Digestion of pTB19 with *EcoRI*, followed by ligation, yielded deletion plasmids pTB51 (Km^r), pTB52 (Tc^r), and pTB53 ($\text{Km}^r \text{Tc}^r$) in *B. subtilis* (Fig. 1). By restriction enzyme analyses, determinants of Km^r , Tc^r and DNA replication were known to be associated with *EcoRI* fragments R1b, R3, and R1a, respectively

Fig. 2. Restriction endonuclease analyses of plasmids. A, λ CI857S7 digested with *Hind*III (as control); B, pBSO1 digested with *Eco*RI; C, pBSO2 digested with *Eco*RI.

(Imanaka *et al.*, 1981*a*). However, none of the deletion plasmids could transform *B. stearothermophilus* (Imanaka *et al.*, 1982). On the other hand, another deletion plasmid, pTB90, which was constructed from an *Eco*RI digest of pTB19, could transform both *B. subtilis* and *B. stearothermophilus*. pTB90 was composed of three *Eco*RI fragments (2.9, 2.8 and 1.0 MDal) and only the Tc^r determinant clearly corresponded to the 2.8 MDal *Eco*RI fragment (i.e. the R3 fragment of pTB19) but the other *Eco*RI fragments (2.9 and 1.0 MDal) were not detected in the original plasmid pTB19 (Imanaka *et al.*, 1982). In order to examine further the correspondence between pTB19 and pTB90, Southern hybridization was done using pTB90 as a probe.

An *Eco*RI digest of pTB19 was subjected to agarose gel electrophoresis and was transferred to a nitrocellulose filter, followed by hybridization with ³²P-labelled pTB90. pTB90 hybridized to the *Eco*RI fragments R1a and/or R1b, R2, and R3 of pTB19, respectively (Fig. 4, lanes A and a). On the other hand, pTB90 only hybridized to the *Eco*RI fragment R3 of pTB52, which was composed of R1a and R3 (Fig. 4, lanes B and b). Thus, pTB90 hybridized to the *Eco*RI fragments R1b, R2, and R3 of pTB19.

Restriction endonuclease *Xba*I could not cleave the DNA of pTB19 (Imanaka *et al.*, 1981*a*), whereas the 1.0 MDal *Eco*RI fragment of pTB90 was cleaved with *Xba*I at a single site (Fig. 1). This indicates that the 1.0 MDal fragment of pTB90 is not from pTB19. To search for the origin of the 1.0 MDal fragment, pTB19 and pTB90 were subjected to hybridization with the ³²P-labelled pBSO2. pBSO2 only hybridized to the 1.0 MDal fragment of pTB90 and did not hybridize to pTB19 at all (Fig. 3, lanes A, a, B, b, and b'). This was confirmed by the hybridization of ³²P-labelled pTB90 with the 1.0 MDal *Eco*RI fragment of pBSO2 (Fig. 4, lanes C and c). Accordingly, the 2.8 and 1.0 MDal *Eco*RI fragments of pTB90 were from R3 of pTB19 and the 1.0 MDal *Eco*RI fragment of pBSO2, respectively. In consequence, it is inferred that the 2.9 MDal *Eco*RI fragment of pTB90 was from parts of the R1b and R2 fragments of pTB19.

Fig. 3. Hybridization and blotting experiments. *EcoRI* digests of plasmids were electrophoresed on agarose gel (A to E), transferred to a nitrocellulose membrane by blotting, and hybridized to ^{32}P -labelled pBSO2 DNA (a to e; b' to d' for a longer exposure). A, a, pTB19; B, b, b', pTB90; C, c, c', pBSO2; D, d, d', chromosomal DNA of the strain CU21; E, e, λ cI857S7 digested with *HindIII*.

Fig. 4. Hybridization and blotting experiments (continued). *EcoRI* digests of plasmids were electrophoresed on agarose gel (A to E), transferred to a nitrocellulose membrane by blotting, and hybridized to ^{32}P -labelled pTB90 DNA (a to e). A, a, pTB19; B, b, pTB52; C, c, pBSO2; D, d, pTB90; E, e, λ cI857S7 digested with *HindIII*. R1 to R4 on the left-hand side are *EcoRI* fragments of pTB19 (Imanaka *et al.*, 1982); the numbers on the right-hand side are molecular sizes (MDal) of relevant DNA fragments.

Replication determinant of pTB90

The determinant of pTB19 DNA replication is known to be associated with the *EcoRI* fragment R1a of this plasmid (Imanaka *et al.*, 1981*a*). However, its derivative pTB90 did not contain any part of R1a (see above). This fact clearly indicates that pTB90 should have another replication determinant different from that existing on R1a. Indeed, the largest *EcoRI* fragment (2.9 MDal) of pTB90 could replicate autonomously (see later) and this fragment was from the R1b and R2 fragments of pTB19. Since the R1b fragment contains the Km^r determinant, the replication determinant of pTB90 should be associated with the R2 fragment. Since the R2 and R1b fragments are adjacent on pTB19 (Fig. 1), the 2.9 MDal *EcoRI* fragment of pTB90 might have emerged by the excision of this region from pTB19.

The deletion plasmid from pTB19 was obtained as follows. After cultivating *B. stearothermophilus* harbouring pTB19 in the presence of Km , plasmid DNA was extracted from this culture and was used to transform *B. subtilis*. Transformants were selected on L agar containing Km . Plasmid DNAs isolated from Km^r Tc^s transformants were digested with *EcoRI* and examined by agarose gel electrophoresis. A single band which corresponded to 2.9 MDal was found for all of the DNAs tested. Single and double digestions of this plasmid with *EcoRI*, *BglII* and *HincII* exhibited the same digestion pattern to that obtained from the 2.9 MDal *EcoRI* fragment of pTB90 (data not shown). The deletion plasmid was therefore considered to be identical with the 2.9 MDal *EcoRI* fragment of pTB90, and was designated as pTB913.

These results revealed that another DNA replication determinant was on the R2 fragment of pTB19 and that this plasmid has two replication determinants, i.e. the one on fragment R1a and another on R2. The first and the second determinants for the replication of pTB19 were designated RepA and RepB, respectively. It was shown that pTB90 contained RepB of pTB19 but not RepA. It is inferred that pTB90 was constructed according to the scheme shown in Fig. 5. The DNA sample of pTB19 when prepared from *B. stearothermophilus* CU21 may have contained a small amount of both pTB913 and pBSO2. pTB90 was obtained at relatively low efficiency from the ligation mixture of the *EcoRI* digest of the DNA sample. The scheme of Fig. 5 would account for the observation that pTB90 contained 2.9 MDal and 1.0 MDal *EcoRI* fragments which were not detected in the original plasmid pTB19.

Construction of deletion plasmids

Deletion plasmids pTB51, pTB52, and pTB53 derived from pTB19 could replicate only in *B. subtilis*, whereas another deletion plasmid derived from pTB19, viz. pTB90, could replicate in both *B. subtilis* and *B. stearothermophilus* (Imanaka *et al.*, 1982). However, pTB90 contained the specific fragment (1.0 MDal *EcoRI* fragment) which came from pBSO2 but which was not present in pTB51, pTB52 or pTB53. Thus the 1.0 MDal fragment of pTB90 played a significant role in the transformation of *B. stearothermophilus*, i.e. in the replication or maintenance of the plasmid in the strain.

To examine the role of this 1.0 MDal fragment in *B. stearothermophilus*, various deletion plasmids of pTB90 were constructed. After digestion of pTB90 with *EcoRI* and ligation, *B. subtilis* MI113 cells were transformed with the ligation mixture. Two kinds of transformants (Km^r Tc^s and Km^r Tc^r) were obtained. No Km^s Tc^r transformants were obtained. Plasmid DNA species isolated from Km^r Tc^s transformants were digested with *EcoRI* and analysed by agarose gel electrophoresis. A small plasmid (pTB913, 2.9 MDal) and a large plasmid (2.9 + 1.0 MDal) were found, and the latter was designated as pTB919. Two different deletion plasmids that were composed of the two *EcoRI* fragments (2.9 and 2.8 MDal) could be obtained from Km^r Tc^r transformants, and were designated as pTB914 and pTB916. The direction of the two *EcoRI* fragments of these plasmids was opposite (Fig. 6). Another deletion plasmid (Tc^r) designated as pTB902 was obtained from pTB916, but not from pTB914. Furthermore, pTB913 and pTB902 were digested with *EcoRI* and ligated, respectively, with the 1.0 MDal *EcoRI* fragment. These ligation mixtures were used to transform *B. subtilis*. The recombinant plasmids (pTB913 + 1.0 MDal, pTB902 + 1.0 MDal) were designated as pTB931 and pTB921, respectively. Two *EcoRI* fragments (2.9 and 1.0 MDal) of pTB931 and pTB919 were connected in the opposite

Fig. 5. A schematic diagram of the construction of pTB90 *in vitro*. This would indicate that the three *Eco*RI fragments (2.9, 2.8 and 1.0 MDal) composing pTB90 were from pTB19, pTB913, and pBSO2, respectively. Cleavage sites of *Bam*HI, *Bgl*II, *Bst*EII, *Pst*I, *Hind*III, and *Xba*I are indicated by Ba, Bg, Bs, P, H, and X, respectively. The bars inside the circles indicate the cleavage sites of *Eco*RI. The heavy line corresponds to the 1.0 MDal *Eco*RI fragment.

direction to each other. Restriction endonuclease cleavage maps of these plasmids are summarized in Fig. 6.

Transformation of *B. stearothermophilus* with various deletion plasmids from pTB90

Bacillus stearothermophilus CU21 was transformed with pTB90 and its derivatives (Table 2). The plasmids in this series of tests were all prepared from *B. subtilis*, but pTB90 used in experiment 2 was prepared from *B. stearothermophilus* to modify the *Bst*EII site.

In transformation experiment 1, about 1×10^3 transformants were obtained with 1 μ g pTB90, but no antibiotic-resistant transformants were obtained with 1 μ g pTB914 or pTB916. In experiment 2, the transformation frequency with pTB913 lacking 1.0 MDal *Eco*RI fragment was about 10^{-4} times those with pTB919 and pTB931, both of which carried 1.0 MDal *Eco*RI fragment. Of the plasmids not carrying the 1.0 MDal *Eco*RI fragment (pTB914, pTB916 and pTB913), only pTB913 gave some transformants. The difference between pTB913 and the other two plasmids (pTB914 and pTB916) might be accounted for as follows: pTB914 and pTB916 contain the restriction endonuclease cleavage site for *Bst*EII, the isoschizomer of *Bst*PI from *B. stearothermophilus* ATCC 12980, while pTB913 had no site for *Bst*EII (Fig. 6). Indeed, a few transformants were obtained when 10 μ g rather than 1 μ g DNA of pTB914 or pTB916 was used.

The results of experiments 1 and 2 suggested that the transformation frequency of *B. stearothermophilus* was significantly enhanced by the 1.0 MDal *Eco*RI fragment. To confirm this point, transformation frequencies for pTB902 and pTB921 were determined in experiment 3. No transformants were obtained from pTB902 (lacking the 1.0 MDal fragment), but 1.8×10^4 transformants were obtained with 1 μ g DNA of pTB921 carrying the 1.0 MDal fragment.

Fig. 6. Restriction endonuclease cleavage maps of plasmids. Cleavage sites of *Bgl*II, *Bst*EII, *Hind*III, and *Xba*I are indicated by Bg, Bs, H, and X, respectively. The bars inside the circles indicate the cleavage sites of *Eco*RI. The heavy line corresponds to the 1.0 MDal *Eco*RI fragment.

Table 2. Transformation of *B. stearothermophilus* CU21 with plasmid DNA

Expt no.	Plasmid	<i>Bst</i> EII	1.0 MDal <i>Eco</i> RI fragment	Antibiotic selection*	Transformants per μ g of DNA	Transformation frequency per regenerant†
1	pTB90	+	+	Km	1.9×10^3	1.6×10^{-4}
	pTB90	+	+	Tc	7.7×10^2	6.4×10^{-5}
	pTB914	+	—	Km	<1	$<8 \times 10^{-8}$
	pTB914	+	—	Tc	<1	$<8 \times 10^{-8}$
	pTB916	+	—	Km	<1	$<8 \times 10^{-8}$
	pTB916	+	—	Tc	<1	$<8 \times 10^{-8}$
2	pTB90	+‡	+	Km	1.0×10^7	1.9×10^{-1}
	pTB913	—	—	Km	4.6×10^1	8.8×10^{-7}
	pTB919	—	+	Km	2.2×10^5	4.2×10^{-3}
	pTB931	—	+	Km	5.2×10^5	1.0×10^{-2}
3	pTB90	+	+	Tc	8.6×10^3	6.1×10^{-5}
	pTB902	+	—	Tc	<1	$<7 \times 10^{-9}$
	pTB921	+	+	Tc	1.8×10^4	1.3×10^{-4}

* Selection was on kanamycin, $25 \mu\text{g ml}^{-1}$ (Km) or tetracycline, $5 \mu\text{g ml}^{-1}$ (Tc).

† Regenerated protoplasts for experiments 1, 2 and 3 were 1.2×10^7 , 5.2×10^7 , and $1.4 \times 10^8 \text{ ml}^{-1}$, respectively.

‡ Since the plasmid was prepared from *B. stearothermophilus*, the *Bst*EII site was modified.

Therefore the 1.0 MDal *Eco*RI fragment could enhance the transformation frequency for *B. stearothermophilus*. A few transformants were also obtained when $10 \mu\text{g}$ DNA of pTB902 having the site for *Bst*EII was used. The difference in transformation frequencies for pTB90 in each experiment might be due to differences in the modification of the *Bst*EII site.

Table 3. *Properties of plasmids*

Plasmid	Characteristics	Rep	1.0 MDal <i>Eco</i> RI fragment	Copy no. in <i>B. subtilis</i>	Transformation of <i>B. stearothermophilus</i>	Copy no. in <i>B. stearothermophilus</i>
pTB19	Km ^r Tc ^r	A, B	—	1*	+	1*
pTB51	Km ^r	A	—	8	—	—
pTB52	Tc ^r	A	—	9	—	—
pTB53	Km ^r Tc ^r	A	—	8	—	—
pTB90	Km ^r Tc ^r	B	+	11	+	5
pTB914	Km ^r Tc ^r	B	—	13	+†	43
pTB916	Km ^r Tc ^r	B	—	15	+†	—‡
pTB913	Km ^r	B	—	25	+†	39
pTB919	Km ^r	B	+	13	+	5
pTB931	Km ^r	B	+	14	+	4
pTB902	Tc ^r	B	—	24	+†	60
pTB921	Tc ^r	B	+	13	+	7

* Data from Imanaka *et al.* (1982).

† Low transformation frequency.

‡ Deletion plasmid appears frequently.

Copy number of plasmids

The copy numbers of plasmids carrying the 1.0 MDal *Eco*RI fragment were about one-tenth of those of plasmids lacking this fragment in *B. stearothermophilus*. However, copy numbers of plasmids with and without the 1.0 MDal *Eco*RI fragment did not differ appreciably in *B. subtilis* (Table 3).

DISCUSSION

It is shown in this paper that pTB19 isolated from a thermophilic bacillus had two distinct DNA segments capable of autonomous replication. This finding is the first report of a specific plasmid from the genus *Bacillus*, having two replication determinants on the same replicon. A deletion plasmid pTB913 (Km^r) was isolated from pTB19 (Km^r Tc^r) in *B. stearothermophilus* (Fig. 5). The same deletion plasmid (Km^r, 2.9 MDal) was also obtained from pTB19 in *B. subtilis*. In addition, another deletion plasmid (Tc^r, about 14 MDal) was observed in *B. subtilis* (data not shown). These results suggest that the two deletion plasmids emerged from pTB19 (Km^r Tc^r, 17.2 MDal) by *in vivo* recombination and, conversely, pTB19 might have been constructed by *in vivo* fusion of two distinct plasmids specifying resistance to Km and Tc, respectively, in a host bacterium. It has been reported that many drug-resistance plasmids (R plasmids) of *Enterobacteriaceae* are composite replicons (Danbara *et al.*, 1980; Perlman & Rownd, 1976; Yoshikawa, 1974).

Deletion plasmids containing RepA (pTB51, pTB52 and pTB53) derived from pTB19 could replicate only in *B. subtilis*, while other plasmids containing RepB (pTB19, pTB90 and its derivatives) could replicate in both *B. subtilis* and *B. stearothermophilus*. Further, copy numbers of RepA⁺ plasmids in *B. subtilis* were somewhat lower than those of RepB⁺ plasmids (Table 3). These facts would justify that the two determinants, RepA and RepB, responsible for DNA replication, are different. The precise nature of these determinants, such as whether they are replication origins, is now under investigation.

The 1.0 MDal *Eco*RI fragment which combined RepB from pTB90 significantly enhanced the transformation frequency for *B. stearothermophilus* and reduced copy number (Tables 2 and 3). The increase of transformation frequency might be partly explained by the possibility of recombinational rescue during transformation due to homology between the 1.0 MDal *Eco*RI fragment of the incoming plasmid and like-sequences on the *B. stearothermophilus* chromosome. To examine this possibility, another recombinant plasmid, pUB111, was constructed by inserting the 1.0 MDal *Eco*RI fragment into *Eco*RI cleavage site of pUB110. The transformation frequency of *B. stearothermophilus* with pUB111 was nearly the same as that

with pUB110 (data not shown). Thus, it seems most unlikely that the 1.0 MDal *Eco*RI fragment assists transformation by homology of the incoming plasmid with the chromosome. Copy numbers of pUB110 and pUB111 in *B. stearothermophilus* were nearly the same. Once the plasmids were satisfactorily transferred into the host cells, each plasmid was stably maintained in *B. stearothermophilus* irrespective of the presence or absence of the 1.0 MDal *Eco*RI fragment (data not shown). These results suggest that the 1.0 MDal *Eco*RI fragment of pTB90 could be responsible for the regulation of RepB function, including plasmid DNA replication and copy number control. Possible genetic information encoded in this 1.0 MDal *Eco*RI fragment might include the following: (i) a protein which acts negatively for the initiation of the plasmid replication (because it reduces the copy number in *B. stearothermophilus*); (ii) a small RNA which inhibits the initiation of plasmid replication as reported for plasmids ColE1 and R1 (Stougaard *et al.*, 1981; Tomizawa & Itoh, 1981); and (iii) a replication origin available only in *B. stearothermophilus* but not in *B. subtilis*. The growth rate of *B. subtilis* (pUB111) in L broth was 40% lower than that of *B. subtilis* (pUB110). The colonies of these two strains on L agar were clearly different in size and appearance, suggesting that the gene product of the 1.0 MDal *Eco*RI fragment gave rise to a stress to host cells. However, this kind of difference was not observed between *B. stearothermophilus* (pUB111) and *B. stearothermophilus* (pUB110). These phenomena indicate that the phenotypic expression of this 1.0 MDal *Eco*RI fragment is considerably influenced by both the vector plasmid and the host bacterium. DNA sequencing and analysis of the gene products are now in progress to provide an insight into the mechanism that controls plasmid DNA replication in these mesophilic and thermophilic bacteria.

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