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Transformation of *Bacillus stearothermophilus* with Plasmid DNA and Characterization of Shuttle Vector Plasmids Between *Bacillus stearothermophilus* and *Bacillus subtilis*

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A thermophilic bacterium *Bacillus stearothermophilus* IFO 12550 (ATCC 12980) was transformed with each of the following plasmids, pUB110 (kanamycin resistance, Km^r), pTB19 (Km^r and tetracycline resistance [Tc^r]), and its derivative pTB90 (Km^r Tc^r), by the protoplast procedure in the presence of polyethylene glycol at 48°C. The transformation frequencies per regenerant for pUB110, pTB19, and pTB90 were 5.9×10^{-3}, 5.5×10^{-3}, and 2.0×10^{-1}, respectively. Among these plasmids, pTB90 was newly derived, and the restriction endonuclease cleavage map was constructed. When tetracycline (5 μg/ml) was added into the culture medium, the copy number of pTB90 in *B. stearothermophilus* was about fourfold higher than that when kanamycin (5 μg/ml) was added instead of tetracycline. *Bacillus subtilis* could also be transformed with the plasmids extracted from *B. stearothermophilus* and vice versa. Accordingly, pUB110, pTB19, and pTB90 served as shuttle vectors between *B. stearothermophilus* and *B. subtilis*. The requirements for replication of pTB19 in *B. subtilis* and *B. stearothermophilus* appear to be different, because some deletion plasmids (pTB51, pTB52, and pTB53) derived from pTB19 could replicate only in *B. subtilis*, whereas another deletion plasmid pTB92 could replicate solely in *B. stearothermophilus*. Plasmids pTB19 and pTB90 could be maintained and expressed in *B. stearothermophilus* up to 65°C, whereas the expression of pUB110 in the same strain was up to 55°C.

Transformation of *Bacillus subtilis* with plasmid DNA using either competent cells or protoplasts has been well documented (3, 5, 8, 12). The transformation of *Bacillus megaterium* (2, 15), *Bacillus thuringiensis* (1, 13), or *Bacillus licheniformis* (10) by the protoplast procedure has also been reported. These procedures make the cloning of a specific gene(s) possible in each strain of the above-mentioned mesophilic bacteria. For example, penicillinase genes of *B. licheniformis* have been cloned in *B. licheniformis* (10). In contrast, no reports have appeared yet on the transformation of thermophiles.

If the transformation system for a thermophile with plasmids could be established, the cloning of specific gene(s) of thermostable enzymes would be made possible and the mode of gene expression at higher temperatures could be examined.

In this paper, we described the transformation of a thermophilic bacterium *B. stearothermophilus* with plasmids by the protoplast procedure, and some characteristics on plasmids that served as shuttle vectors between *B. subtilis* (mesophile) and *B. stearothermophilus* (thermophile) were discussed.

MATERIALS AND METHODS

Media and materials. L broth contained 10 g of tryptone (Difco Laboratories, Detroit, Mich.), 5 g of yeast extract, and 5 g of NaCl in 1 liter of deionized water; it was adjusted to pH 7.3 with NaOH and solidified with 20 g of agar per liter (L agar). LG broth was L broth supplemented with glucose (2.5 g/liter). LGA agar was LG broth containing 20 g of agar per liter. LGS broth was LG broth supplemented with sucrose (0.15 M). Sucrose-magnesium-maleate buffer of Wyrick and Rogers (16) was modified as follows: SMM buffer contained 0.33 M sucrose, 0.02 M maleate, and 0.02 M $MgCl_2$ (pH 6.5). SMM-LG medium was prepared by mixing equal volumes of 2×-strength SMM buffer and 2×-strength LG broth. Regeneration agar (RGA) (pH 7.3) consisted of the following sterile solutions per liter: 700 ml of 2.86% (wt/vol throughout) agar plus 1.43% tryptone (Difco) plus 0.71% yeast extract plus 0.71% NaCl, 200 ml of 1 M sucrose, 50 ml of 3% KH_2PO_4 and 7% K_2HPO_4, 20 ml of 25% glucose, 10 ml of 1% Casamino Acids, 10 ml of 2 M $MgCl_2$, and 10 ml of filter-sterilized 2% bovine serum albumin. Agar concentration in the regeneration top agar

(RGTA) was reduced from 2.86 to 0.857%. The compositions of RGA and RGTA were the modification of DM3 regeneration medium (3), whose component of sodium succinate was replaced by sucrose (0.2 M). It was confirmed that *B. stearothermophilus* did not grow when sodium succinate was used in RGA medium.

The companies and laboratories from which the antibiotics, restriction endonucleases, and all of the reagents used here were purchased were the same, respectively, as in the previous works (9, 10), unless otherwise noted.

Bacterial strains and plasmids. Strains and plasmids used are listed in Table 1. *B. stearothermophilus* IFO 12550 (ATCC 12980) is the type strain. Since the strain carried plasmid pBSO1 (18.3 ± 0.7 megadaltons [Md]) (electron micrograph not shown) and could not grow on L agar containing any one of the following antibiotics (ampicillin, 5 µg/ml; chloramphenicol [Cm], 10 µg/ml; erythromycin, 5 µg/ml; kanamycin [Km], 5 µg/ml; and tetracycline [Tc], 5 µg/ml), a spontaneous mutant strain S1, resistant to streptomycin (Sm, 500 µg/ml in LGA) was obtained. The strain S1 was cured spontaneously of the plasmid pBSO1. The cured strain *B. stearothermophilus* CU21 was used hereafter as the recipient cells in the transformation.

S. D. Ehrlich contributed the plasmids pHV11 and pHV14. All of the plasmids shown in Table 1 that had been transferred into *B. subtilis* MI113 by transformation (see below) were used for the transformation study on *B. stearothermophilus* (also see below).

Preparation of plasmid DNA. The rapid alkaline extraction method was slightly modified as described elsewhere (9) to extract plasmid DNA. For large-scale preparations of plasmid as covalently closed circular DNA in CsCl-ethidium bromide equilibrium density gradient centrifugation, the method of Davis et al. (4) was used.

Transformation of *B. subtilis*. Competent cells of *B. subtilis* MI113 were transformed with plasmid DNA as described elsewhere (9). For pHV14 and pHV11, Cm^r transformants were selected on L agar containing Cm (5 µg/ml) at 37°C.

Transformation of *B. stearothermophilus*. *B. stearothermophilus* was transformed with plasmids, following basically the protoplast transformation procedure described by Chang and Cohen (3) for *B. subtilis*. However, a few modifications were needed in each step of the transformation of *B. stearothermophilus*.

Since the maximum and minimum temperatures for growth of *B. stearothermophilus* IFO 12550 (ATCC 12980) were 70 and 40°C, respectively (7), and also in view of the fact that the cells died quickly at room temperatures but were stably maintained at 4°C as the resting cells (data not shown), the transformation of *B. stearothermophilus* was done at temperatures higher than 40°C or lower than 4°C.

(i) Preparation of protoplasts. *B. stearothermophilus* was grown overnight in L broth at 55°C. A 0.5-ml volume of this preculture was inoculated into 50 ml of LGS broth in a conical flask (300 ml) to cultivate the bacterial cells at 55°C for about 4 h with shaking. When the optical density measured at 660 nm was around 0.4, the culture was centrifuged (8,000 × *g* at 4°C, 5 min) and the bacteria were suspended in 2 ml of SMM-LG medium (1×10^9 to 2×10^9 cells/ml). Lysozyme (50 µg/ml dissolved in SMM-LG medium) was added into the medium to the final concentration of 1 µg/ml. The mixture was gently shaken at 48°C for 20 min and centrifuged (4,000 × *g* at 4°C, 7 min). The pellet was washed in 2 ml of SMM-LG medium and centrifuged again (4,000 × *g* at 4°C, 7 min). Protoplasts prepared thus were resuspended in 2 ml of SMM-LG medium.

(ii) PEG treatment. Plasmid DNA (about 1 µg) in 50 µl of TE buffer (10 mM Tris-hydrochloride [pH 7.5]-0.1 mM Na$_2$EDTA) was mixed with an equal volume of 2×-strength SMM buffer. A 0.5-ml volume of the protoplast suspension was added, followed immediately by an addition of 1.5 ml of 40% polyethylene glycol (PEG) in SMM buffer which had been prewarmed at 48°C. After the exposure of the protoplasts to the PEG for 2 min with gentle mixing at 48°C, 5 ml of SMM-LG medium was added to dilute PEG, and then the protoplasts were recovered by centrifugation (4,000 × *g* at 4°C, 10 min). The protoplasts were suspended in 1

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Mol wt (10^6)	Characteristics or phenotype	Origin or reference
B. stearothermophilus			
IFO 12550 (ATCC 12980)		Wild type, plasmid-carrier (pBSO1)	IFO[a]
S1		Sm^r, plasmid-carrier (pBSO1)	Spontaneous mutant of wild type
CU21		Sm^r, cured spontaneously of pBSO1	S1
B. subtilis MI113		*arg-15 trpC2* r_M^- m_M^-	(9)
Plasmids			
pUB110	3.0	Km^r	(8, 12)
pHV14	4.6	Cm^r	(5)
pHV11	3.3	Cm^r Tc^r	(5)
pTB19	17.2	Km^r Tc^r	(9)
pTB20	2.8	Tc^r	(9)
pTB51	8.4	Km^r	(9)
pTB52	7.0	Tc^r	(9)
pTB53	11.2	Km^r Tc^r	(9, 10)

[a] IFO, Institute for Fermentation, Osaka, Japan.

ml of SMM-LG medium containing bovine serum albumin (0.01%) and incubated for 1.5 h at 48°C in a gently shaking water bath to facilitate the expression of drug resistance gene(s) (3). Then the protoplasts were appropriately diluted with SMM-LG medium containing bovine serum albumin (0.01%) and used for regeneration.

(iii) Regeneration of protoplasts. A 100-μl volume of the protoplast sample and 3 ml of RGTA agar (50°C) were poured onto 25 ml of RGA agar plate which had been prewarmed at 48°C, and gently mixed before solidification. For direct selection of transformants, antibiotic was added into both RGA and RGTA agar. Concentrations of Km, Cm, and Tc used were 25 μg/ml, 20 μg/ml, and 5 μg/ml, respectively. Transformation frequency was scored after incubation of the protoplasts at 48°C for 5 days.

Digestion of plasmid DNA with restriction endonucleases. Restriction endonuclease *Bst*EII was purchased from Bethesda Research Laboratories, Inc., Rockville, Md. Another restriction endonuclease *Bgl*II was from Takara Shuzo Co. Ltd., Kyoto, Japan. Plasmid DNA was digested for 2 h at 60°C for *Bst*EII, whereas it was digested at 37°C for *Bgl*II in the following buffers: 6 mM Tris-hydrochloride (pH 7.9)-6 mM $MgCl_2$-50 mM NaCl-6 mM 2-mercaptoethanol for *Bst*EII; 10 mM Tris-hydrochloride (pH 7.5)-7 mM $MgCl_2$-60 mM NaCl-7 mM 2-mercaptoethanol for *Bgl*II; for digestion conditions with other restriction endonucleases used, see elsewhere (9).

Phenotypic stability of plasmid. For the stability test of plasmid, the procedure described by Imanaka et al. (11) was slightly modified. *B. stearothermophilus* carrying plasmid was first isolated as a single colony on LGA agar containing a specific antibiotic (Km, 5 μg/ml; or Tc, 5 μg/ml) at 48°C. Then a fresh colony was inoculated into 20 ml of L broth plus antibiotic (Km, 5 μg/ml; or Tc, 5 μg/ml) to precultivate the cells in a shaken flask (100 ml) at 48°C for 10 h.

The preculture was diluted with LG broth. The cells were inoculated (about 100 cells per ml) into LG broth. After about 20 generations at constant temperatures (48, 55, 60, and 65°C), the culture was diluted and samples were plated on LGA agar. One hundred colonies on LGA agar at 48°C were transferred by replica plating onto LGA agar containing a specific antibiotic to count the antibiotic-resistant colonies at 48°C. The stability of plasmid in *B. subtilis* was examined as described earlier (9). Concentrations of Km and Tc for *B. subtilis* were 5 μg/ml and 25 μg/ml, respectively.

Other procedures. Agarose gel electrophoresis of plasmid DNA, ligation of DNA with T4 ligase, electron microscopy of plasmid, and assessment of the plasmid copy number have been described, respectively, in the preceding work (9).

RESULTS

Optimal conditions for growth, protoplast formation, and regeneration of *B. stearothermophilus*. Although the previous study (6) on mesophilic *B. subtilis* pointed out that sucrose concentration in the medium for the bacterial growth and that for the protoplast formation could be equally taken as 0.5 M, different concentrations of 0.15 M for the growth and 0.33 M for the protoplast formation were used in this work on *B. stearothermophilus*. If 0.5 M sucrose were used in LG broth, *B. stearothermophilus* could not grow at 48°C. The use of an extremely low concentration of lysozyme, 1 μg/ml, in contrast to 2 mg/ml for *B. subtilis* (3) for the protoplast formation was to enhance the regeneration frequency of protoplasts. Even under the extremely low concentration of the enzyme, the conversion ratio of intact cells to protoplasts was more than 99.99%, if colonies of intact cells on LGA agar before and after the lysozyme treatment were counted. The microscopic observation showed that protoplasts of *B. stearothermophilus* were quite stable morphologically in SMM-LG medium at 48°C even after 1 week. Regeneration frequencies of protoplasts were around 10%.

Transformation of *B. stearothermophilus* with plasmids. Plasmid DNA prepared from *B. subtilis* MI113 by CsCl-ethidium bromide equilibrium density gradient centrifugation was used to transform *B. stearothermophilus*. Selection markers in the transformation using *B. stearothermophilus* CU21 were: Cm for pHV11 and pHV14; Km for pUB110, pTB19, pTB51, and pTB53; Tc for pHV11, pTB19, pTB20, pTB52, and pTB53. Antibiotic-resistant transformants were obtained with pUB110 or pTB19. None of the other plasmids could transform *B. stearothermophilus*. pUB110 conferred Km resistance (Km^r) on *B. stearothermophilus*, whereas the transformants with pTB19 were resistant to both Km and Tc (Km^r Tc^r). Transformed clones of *B. stearothermophilus* were examined by agarose gel electrophoresis for the presence of plasmid DNA. DNAs of pUB110 and pTB19 from *B. stearothermophilus* corresponded, respectively, to those extracted from *B. subtilis* (Fig. 1, lanes A, B, E, and F).

Transformation frequencies of *B. stearothermophilus* CU21 were determined for plasmids pUB110 and pTB19 (Table 2). The plasmids in this series of tests were prepared from both *B. subtilis* and *B. stearothermophilus*. The transformation frequency for pUB110 from *B. subtilis* was nearly the same as that (5.9×10^{-3} per regenerant) from *B. stearothermophilus*. The frequency for pTB19 from *B. subtilis* was much lower than that (5.5×10^{-3} per regenerant) from *B. stearothermophilus* (Table 2). These results suggest that DNA of pTB19 from *B. subtilis* was restricted in *B. stearothermophilus* CU21. As a matter of fact, when restriction endonuclease *Bst*EII, the isoschizomer of *Bst*PI from *B. stearothermophilus* ATCC 12980 (14), was used to digest pTB19 from *B. subtilis* MI113, two fragments were observed (Fig. 1, lane G), whereas the plasmid from *B. stearothermophilus* CU21

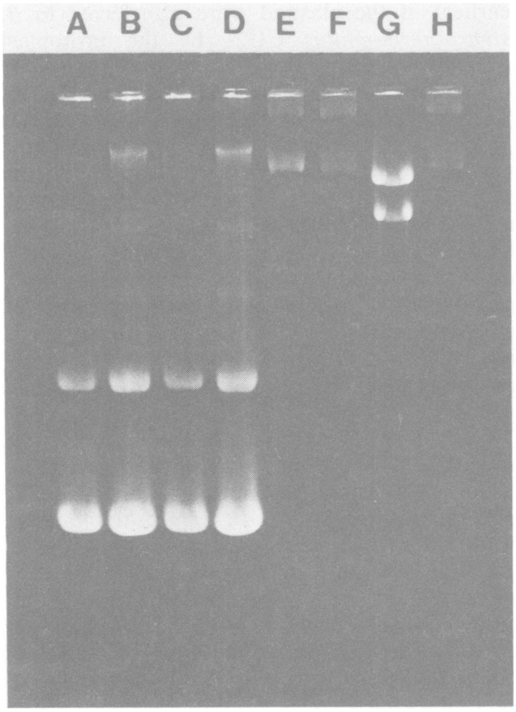

FIG. 1. Agarose gel electrophoresis of plasmid. Lanes: A, pUB110 from *B. subtilis* MI113; B, pUB110 from *B. stearothermophilus* CU21; C, pUB110 from *B. subtilis* treated with *Bst*EII; D, pUB110 from *B. stearothermophilus* treated with *Bst*EII; E, pTB19 from *B. subtilis*; F, pTB19 from *B. stearothermophilus*; G, pTB19 from *B. subtilis* treated with *Bst*EII; H, pTB19 from *B. stearothermophilus* treated with *Bst*EII. Electrophoresis was performed in 0.7% [wt/vol] agarose gel.

(transformant) could not be cleaved (Fig. 1, lane H). In contrast, neither pUB110 from *B. subtilis* MI113 nor that from *B. stearothermophilus* CU21 (transformant) were cleaved by *Bst*EII (Fig. 1, lanes C and D). These results are evidence that pUB110 was not subjected to restriction regardless of the plasmid source, whereas pTB19 from *B. subtilis* MI113 suffered restriction in transformation of *B. stearothermophilus*.

Preliminary studies on the transformation of *B. stearothermophilus* with plasmid DNA revealed an indispensable role of PEG in the transformation procedures; that is, no drug-resistant colonies were obtained in the absence of either plasmid DNA or PEG.

Construction of deletion plasmid in *B. stearothermophilus*. Although deletion plasmids (pTB51, pTB52, and pTB53) were constructed from *Eco*RI digests of pTB19 in *B. subtilis* (9), all of the plasmids could not transform *B. stearothermophilus*. Hence construction of another deletion plasmid was attempted in *B. stearothermophilus*.

After digestion of pTB19 (from *B. stearothermophilus*) with *Eco*RI, followed by ligation with T4 ligase, *B. stearothermophilus* CU21 protoplasts were transformed with the ligation mixture (see above). Transformants were selected on RGA agar containing either Km or Tc. All of the Tc^r transformants exhibited Km^r. However, only about 10% of the Km^r transformants were Tc^r. Plasmid DNAs were extracted from the transformants (Km^r Tc^r) by the rapid alkaline extraction procedure and examined by agarose gel electrophoresis. The smallest and the second smallest plasmids were selected and designated as pTB90 and pTB92, respectively.

DNAs of pTB90 and pTB92 were prepared, respectively, from the transformants (Km^r Tc^r) by CsCl-ethidium bromide equilibrium density gradient centrifugation, and the DNAs of each plasmid were used to transform *B. subtilis* MI113 and *B. stearothermophilus* CU21. Both DNAs transformed *B. stearothermophilus* CU21, implying that all of the transformants were Km^r Tc^r. However, only pTB90 rather than pTB92 could be transferred to *B. subtilis*

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

Plasmid	Source	Antibiotic (μg/ml)	Transformants per μg of DNA	Transformation[a] frequency per regenerant
pUB110	*B. subtilis* MI113	Km (25)	1.6×10^5	1.6×10^{-3}
pUB110	*B. stearothermophilus* CU21	Km (25)	5.9×10^5	5.9×10^{-3}
pTB19	*B. subtilis* MI113	Km (25)	4.9×10^3	4.9×10^{-5}
pTB19	*B. subtilis* MI113	Tc (5)	9.0×10^3	9.0×10^{-5}
pTB19	*B. stearothermophilus* CU21	Km (25)	1.0×10^5	1.0×10^{-3}
pTB19	*B. stearothermophilus* CU21	Tc (5)	5.5×10^5	5.5×10^{-3}
pTB90	*B. subtilis* MI113	Km (25)	2.2×10^4	2.2×10^{-4}
pTB90	*B. subtilis* MI113	Tc (5)	2.6×10^4	2.6×10^{-4}
pTB90	*B. stearothermophilus* CU21	Km (25)	1.3×10^7	1.3×10^{-1}
pTB90	*B. stearothermophilus* CU21	Tc (5)	2.0×10^7	2.0×10^{-1}
pTB92	*B. stearothermophilus* CU21	Km (25)	6.9×10^6	6.9×10^{-2}
pTB92	*B. stearothermophilus* CU21	Tc (5)	3.9×10^6	3.9×10^{-2}

[a] Number of regenerated protoplasts was 1.0×10^8 per ml.

MI113 at a frequency of about 10^{-5} to 10^{-4} per viable cell, indicating that all of the transformants were Km^r Tc^r. Transformation frequencies of *B. stearothermophilus* determined so far for these plasmids are summarized in Table 2. It is noted that the transformation frequency with pTB90 from *B. subtilis* was much lower than that (around 2 × 10^{-1} per regenerant) with pTB90 from *B. stearothermophilus*, whereas the transformation frequency with pTB92 was slightly lower than that with pTB90.

Consequently, pTB90, the plasmid that was newly constructed in this work, could serve as a shuttle vector between *B. subtilis* MI113 and *B. stearothermophilus* CU21 in addition to pUB110 and pTB19. Characterizations of the latter two plasmids are described elsewhere (8, 9, 12). Here it is worthwhile to characterize pTB90.

Cleavage map of pTB90. DNA of pTB90 extracted from *B. stearothermophilus* was digested with several restriction enzymes. The digestion pattern was analyzed by agarose gel electrophoresis (Fig. 2). The restriction enzyme *Hin*dIII was found to cut pTB90 at a single site when compared with covalently closed circular DNA of the plasmid (Fig. 2, lanes A and B). *Eco*RI and *Bgl*II cleaved pTB90 DNA at three and two sites, respectively (Fig. 2, lanes C and D), although the two *Eco*RI fragments (2.9 and 2.8 Md) in lane C were not clearly separated. These results and further restriction analysis of double digestion led to the cleavage map of pTB90 (Fig. 3).

It is clear from the cleavage maps of pTB90 and pTB19 that the determinant of Tc^r in pTB90 was associated with the 2.8-Md *Eco*RI fragment, i.e., R3 fragment of pTB19 (9). Neither *Bam*HI site in *Eco*RI fragment R1b (determinant of Km^r) of pTB19 (9) nor *Pst*I site in fragment R1a (determinant of DNA replication) (9, 10) was detected in pTB90. Additionally, the *Eco*RI fragments (2.9 and 1.0 Md in pTB90) were not detected in the original plasmid pTB19. These results indicate that the *Eco*RI fragments (2.9 and 1.0 Md) of pTB90 might have emerged incidentally in the construction of the deletion plasmid from pTB19 in *B. stearothermophilus* (see above).

pTB90 from *B. subtilis* MI113 (transformant) gave the same digestion pattern of restriction enzymes as that for DNA of pTB90 from *B. stearothermophilus* (transformant) (photographs not shown here) except for the following. Although pTB90 DNA from *B. stearothermophilus* underwent no cleavage by the restriction enzyme *Bst*EII, DNA of the same plasmid from *B. subtilis* MI113 (transformant) was cleaved at one site by *Bst*EII (Fig. 2, lanes E and F). This fact indicates that the restriction would be imposed on pTB90 from *B. subtilis* MI113 (as referred to earlier), if the plasmid were transferred to *B. stearothermophilus* CU21 by the protoplast transformation.

Copy number of plasmids pTB19 and pTB90. The copy number of pTB19 in *B. stearothermophilus* CU21 was about 1 per chromosome in the presence of Tc, and the value was nearly the same as in *B. subtilis* MI113 (9). The copy numbers of pTB90 in *B. stearothermophilus* CU21 in LG broth containing either Tc (5 μg/ml) or Km (5 μg/ml) were about 18 and 5 per chromosome, respectively. The copy number of pTB90 in *B. subtilis* MI113 in LG broth containing Tc (25 μg/ml) or Km (5 μg/ml) was about 9 per chromosome irrespective of the antibiotic species.

Gene expression and stability of plasmids in *B. stearothermophilus* at elevated temperatures. Gene expression and stability of pUB110, pTB19, and pTB90 in *B. stearothermophilus* were tested, respectively (Table 3), to check the validity as vector plasmid at elevated temperatures. pUB110 was stably maintained at 48 and 55°C, whereas the plasmid became unstable at 60 and 65°C. Since about 10% of the total population still carried pUB110 after about 20 generations at 60 or 65°C, the replication of the plasmid might not have been totally damaged at these higher temperatures. The ability of *B.*

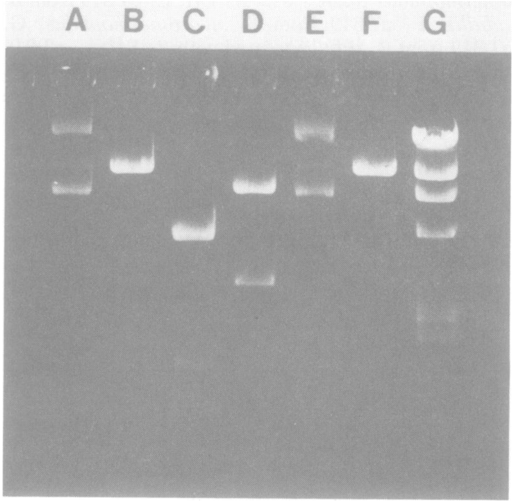

FIG. 2. Agarose gel electrophoresis of restriction endonuclease-digested DNA of pTB90. Plasmid DNA was extracted from *B. stearothermophilus* CU21 (A to E): A, no enzyme; B, *Hin*dIII; C, *Eco*RI; D, *Bgl*II; E, *Bst*EII; F, pTB90 (from *B. subtilis* MI113) digested with *Bst*EII; G, λ *c*I857 *S*7 digested with *Hin*dIII. Molecular weights of λ-*Hin*dIII fragments (in Md) are 14.63, 6.13, 4.05, 2.85, 1.45, 1.26, and 0.36 (9). Electrophoresis was performed in 1.0% [wt/vol] agarose gel.

TABLE 3. Gene expression and stability of plasmids in *B. stearothermophilus*

Plasmid	Growth temp (°C)	No. of generations	Plasmid carrier (%)	Growth in LG broth plus drug (Km or Tc)
pUB110	Preculture	—	100	+
	48	18	100	+
	55	21	100	+
	60	19	8	−
	65	20	13	−
pTB19	Preculture		90	+
	48	19	68	+
	55	20	57	+
	60	19	54	+
	65	20	26	+
pTB90	Preculture		100	+
	48	23	98	+
	55	22	98	+
	60	20	58	+
	65	19	1	+

stearothermophilus (pUB110) to grow in LG broth containing Km (5 μg/ml) was examined at elevated temperatures. The strain carrying pUB110 could grow in LG broth plus Km at 48 and 55°C. However, no growth was observed in the medium at 60 and 65°C, even when the inoculum size was increased to about 10^6 cells/ml.

Plasmid pTB19 became unstable in *B. stearothermophilus* with an increase in temperature of cultivation, as noted clearly in Table 3. pTB90 was stably maintained at 48 and 55°C, whereas the plasmid became unstable at 60 and 65°C. *B. stearothermophilus* strains carrying either pTB19 or pTB90 could grow in LG broth containing Km (5 μg/ml) or Tc (5 μg/ml) even at 65°C, irrespective of the drug species added into LG broth.

DISCUSSION

A protoplast transformation procedure was developed for a thermophile, *B. stearothermophilus*. The frequency and efficiency of transformation in *B. stearothermophilus* protoplasts with plasmid pTB90 (20% transformants per regenerant and 2×10^7 transformants per μg of plasmid DNA, respectively) were as high as those (80% transformants per regenerant and 4×10^7 transformants per μg of plasmid DNA, respectively) in *B. subtilis* protoplasts with plasmid (pC194 or pUB110) (3). However, the frequency and efficiency in *B. megaterium* protoplasts transformed with pUB110 are reported to be considerably lower (about 2% transformants per regenerant and 2×10^5 transformants per μg of plasmid DNA, respectively) (2). Further, the transformation frequency of *B. thuringiensis* protoplasts with plasmid pBC16 is also reported to be very low (3×10^{-7} per regenerant) (1).

The transformation frequency of *B. stearothermophilus* relative to the amount of donor DNA was examined. Using pUB110 as an example of donor, a linear relationship was observed between the transformation frequency and the amount of the DNA from 0.01 to 1 μg, leveling off then (data not shown). Consequently, about 1 μg of the plasmid DNA was used throughout in the transformation.

pUB110, pTB19, and the deletion plasmid pTB90 could transform both *B. subtilis* and *B. stearothermophilus* reciprocally; that is, these plasmids harbored by either strain could transform, respectively, the other. The stability of pTB90 in *B. subtilis* MI113 was 33% after about 20 generations without drug; the stability was nearly the same as pTB19 in *B. subtilis* (9). It

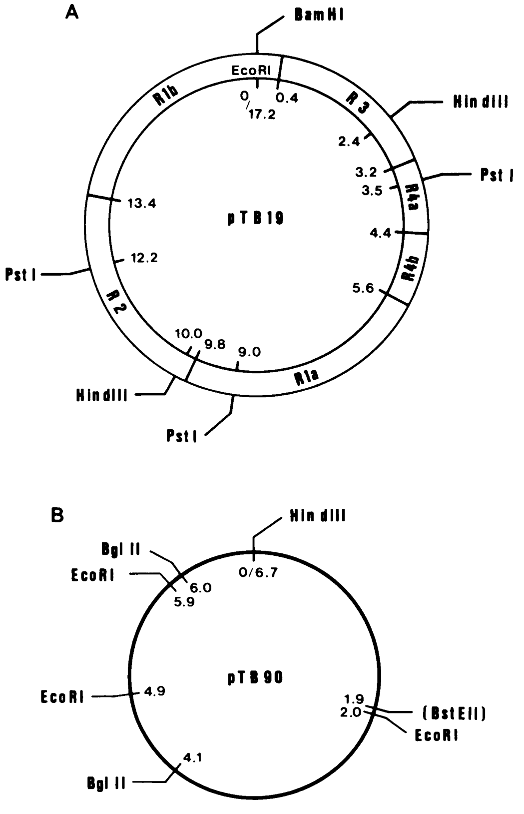

FIG. 3. Restriction endonuclease cleavage maps of pTB19 (A) and pTB90 (B). Cleavage sites and their map positions (in Md) are indicated. *Bst*EII does not cleave pTB90 extracted from *B. stearothermophilus* CU21. Cleavage sites in pTB19 for *Bgl*II and *Bst*EII were not determined.

was also confirmed that pUB110 was stably maintained in *B. subtilis* (data not shown). Consequently, pUB110, pTB19, and pTB90 are potentially useful as shuttle vectors between mesophilic and thermophilic bacilli. In connection with the transformation in a thermophile, it is interesting that the copy number of pTB90 in *B. stearothermophilus* CU21 in LG broth containing Tc was about fourfold higher than the same system of host and plasmid in the presence of Km.

B. stearothermophilus strains carrying either pTB19 or pTB90 could grow in LG broth containing Km or Tc even at 65°C. On the contrary, *B. stearothermophilus* (pUB110) could grow in LG broth plus Km at 48 and 55°C, but not at 60 and 65°C. Supposing that the Km resistance gene(s) of pUB110 was expressed normally even at temperatures higher than 60°C, the abovementioned result on the growth of *B. stearothermophilus* (pUB110) suggests that the protein product of the gene(s) might have been thermolabile at the elevated temperatures. The different characteristics among these plasmids would be attributable to the difference of the source, that is, pUB110 from a mesophile (8), and pTB19 from a thermophilic *Bacillus* spp. (9).

Several deletion plasmids were constructed from pTB19. A deletion plasmid pTB90 could transform both *B. subtilis* and *B. stearothermophilus*, whereas pTB51, pTB52, and pTB53 could transform only *B. subtilis*. pTB92 transformed only *B. stearothermophilus*. The establishment of shuttle vector plasmids between *B. stearothermophilus* and *B. subtilis* is deemed to be significant per se, and the success or failure of transformation in both strains depended clearly on the species of plasmid DNAs. Although intriguing, this fact would present a stimulus for further study on the difference or identity of regulation mechanisms of plasmid DNA replication between these meso- and thermophiles.

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