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Protoplast Transformation of *Bacillus stearothermophilus* NUB36 by Plasmid DNA

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An efficient protoplast transformation system was established for *Bacillus stearothermophilus* NUB3621 using thermophilic plasmid pTHT15 Tc^r (4·5 kb) and mesophilic plasmid pLW05 Cm^r (3 kb), a spontaneous deletion derivative of pPL401 Cm^r Km^r. The efficiency of transformation of NUB3621 with pLW05 and pTHT15 was 2×10^7 to 4×10^8 transformants per μg DNA. The transformation frequency (transformants per regenerant) was 0·5 to 1·0. Chloramphenicol-resistant and tetracycline-resistant transformants were obtained when competent cells of *Bacillus subtilis* were transformed with pLW05 [$2{\cdot}5 \times 10^5$ transformants (μg DNA)$^{-1}$] and pTHT15 [$1{\cdot}8 \times 10^5$ transformants (μg DNA)$^{-1}$], respectively. Thus, these plasmids are shuttle vectors for mesophilic and thermophilic bacilli. Plasmid pLW05 Cm^r was not stably maintained in cultures growing at temperatures between 50 and 65 °C but the thermostable chloramphenicol acetyltransferase was active *in vivo* at temperatures up to 70 °C. In contrast, thermophilic plasmid pTHT15 Tc^r was stable in cultures growing at temperatures up to 60 °C but the tetracycline resistance protein was relatively thermolabile at higher temperatures. The estimated copy number of pLW05 in cells of NUB3621 growing at 50, 60, and 65 °C was 69, 18, and 1 per chromosome equivalent, respectively. The estimated copy number of pTHT15 in cells of NUB3621 growing at 50 or 60 °C was about 41 to 45 per chromosome equivalent and 12 in cells growing at 65 °C.

INTRODUCTION

An efficient host–vector system for a thermophile will be an important tool for investigations of the biochemical, molecular and genetic basis of thermophily. A plasmid transformation system has been reported for *Clostridium thermohydrosulfuricum* (Soutschek-Bauer *et al.*, 1985), *Thermomonospora fusca* (Pidcock *et al.*, 1985) and *Thermus thermophilus* (Koyama *et al.*, 1986). The most efficient thermophile plasmid transformation systems, however, were established for strains of *Bacillus stearothermophilus* (Imanaka *et al.*, 1982; Liao *et al.*, 1986). In these transformation systems, protoplasts of *B. stearothermophilus* were transformed with plasmid DNA using a modification of the procedure described by Chang & Cohen (1979) for *Bacillus subtilis*. Imanaka *et al.* (1982) transformed *B. stearothermophilus* CU21 with plasmid pUB110 Km^r and thermophilic plasmids pTB19 Km^r Tc^r and pTB90 Km^r Tc^r, a derivative of plasmid pTB19. These plasmids also transformed *B. subtilis*. Thus, this was the first report of a shuttle vector between a mesophile and a thermophile. Transformation of a different strain of *B. stearothermophilus* with a plasmid constructed from pUB110 and a cryptic plasmid from another strain of *B. stearothermophilus* was reported by Liao *et al.* (1986). The host–vector system described by Imanaka *et al.* (1982) was used to clone structural genes of enzymes from thermophilic (Aiba *et al.*, 1983; Fujii *et al.*, 1983; Kubo & Imanaka, 1988) and mesophilic (Fujii *et al.*, 1982) bacilli and *Clostridium thermocellum* (Soutschek-Bauer & Staudenbauer, 1987), to investigate and improve the stability of plasmids at high temperature (Aiba & Koizumi, 1984; Matsumura & Aiba, 1985; Hoshino *et al.*, 1985*d*; Koizumi *et al.*, 1986; Aiba *et al.*, 1987*a*, *b*; Min

et al., 1987; Soutschek-Bauer *et al.*, 1987) to select a more thermostable enzyme (Matsumura & Aiba, 1985), to investigate the synthesis of DNA at elevated temperatures (Ano *et al.*, 1985), and to screen for novel thermophilic plasmic vectors (Hoshino *et al.*, 1985*b*). The strain of *B. stearothermophilus* used in the host–vector system described by Imanaka *et al.* (1982) was not suitable for investigations of the molecular mechanisms of thermophily because there is no genetic technology for the construction of isogenic mutant strains or for mapping of chromosomal markers.

In this report, we describe the development of a protoplast transformation system for *B. stearothermophilus* NUB36. Two genetic exchange systems have been developed for this strain (Chen *et al.*, 1986; Welker, 1988) and in combination with the molecular cloning system it will be possible to locate and analyse genes that are unique to thermophiles.

METHODS

Media and growth conditions. B. stearothermophilus cultures were grown in supplemented LB medium (Chen *et al.*, 1986). Solid LB medium contained 1·5% (w/v) agar. Protoplasting (P) medium was as described by Chen *et al.* (1986) except it did not contain calcium chloride (modified P medium). Regeneration (R) medium was as described previously (Chen *et al.*, 1986). Chloramphenicol, tetracycline and kanamycin were used at 10, 5 and 25 μg ml^{-1}, respectively. The solid media were equilibrated and dried as described by Chen *et al.* (1986). Media, cell and protoplast suspensions, and dilutions were maintained at 50 °C.

B. subtilis cultures were grown in Difco Bacto-Penassay broth and Difco Bacto-Tryptose Blood Agar Base. Chloramphenicol, kanamycin and tetracycline were used at 5 μg ml^{-1}.

Cultures were grown on a gyratory shaker (190–200 r.p.m.) at 37 °C (*B. subtilis*) or 60 °C (*B. stearothermophilus*). For the isolation of plasmid DNA, cultures of *B. stearothermophilus* were grown at 50 °C. Growth was monitored by using a Klett–Summerson colorimeter with a no. 42 blue filter.

Bacterial strains and plasmids. Bacterial strains used were *B. subtilis* BR151 *trpC2 metB10 lys-3* and *B. stearothermophilus* NUB3621 $Rif^{r}Hsr^{-}Hsm^{-}$ (Chen *et al.*, 1986). Plasmids used are listed in Table 1.

Isolation of DNA. Plasmid DNA was isolated by the alkaline lysis procedure described by Maniatis *et al.* (1982) followed by centrifugation in a caesium chloride/ethidium bromide density gradient. Rapid isolation of plasmid DNA from small scale cultures of *B. stearothermophilus* and *B. subtilis* was as described by Maniatis *et al.* (1982) and Rodriguez & Tait (1983), respectively. DNA was dissolved in 10 mM-Tris/HCl buffer, pH 8·0, containing 1 mM-EDTA.

Transformation of B. subtilis. Competent cells were prepared as described by Bott & Wilson (1967) and transformation was as described by Sullivan *et al.* (1984).

Transformation of B. stearothermophilus. Cells from a late exponential phase culture of NUB3621 (Chen *et al.*, 1986) were collected by centrifugation at 1800–1900 *g* for 5 min at room temperature (about 20 °C) and suspended in modified P medium to a cell density of 2×10^{9}–4×10^{9} c.f.u. ml^{-1}.

(*i*) *Protoplast formation.* Cells were converted to protoplasts as described by Chen *et al.* (1986) except that the cell suspension was incubated with lysozyme on a gyratory shaker (130 r.p.m.) for 10 min at 50 °C. Protoplasts were diluted with 5 ml modified P medium and collected by centrifugation at 700–800 *g* for 7 min at room temperature. Protoplasts were gently suspended in the same volume of modified P medium used for the preparation of the concentrated cell suspension.

(*ii*) *Polyethylene glycol (PEG)–induced transformation.* Plasmid DNA (5–20 μl) was gently mixed with 0·1 ml protoplast suspension and transformation was initiated by the addition of 0·9 ml freshly prepared 40% (w/v) PEG 6000 (Serva Fine Biochemicals) in modified P medium. The mixture was gently shaken on a gyratory shaker at 130 r.p.m. for 2 min at 50 °C. The transformation mixture was diluted with 2·5 ml modified P medium and the protoplasts were collected by centrifugation at 700–800 *g* for 7 min at room temperature. Protoplasts were gently suspended in 0·1 ml modified P medium. The volume of the transformation mixture can be doubled without a significant change in the efficiency of transformation.

(*iii*) *Phenotypic expression.* The transformation mixture was gently shaken on a gyratory shaker at 130 r.p.m. for 1 h at 50 °C.

(*iv*) *Regeneration of protoplasts and selection of transformants.* Protoplasts were diluted in modified P medium, plated on plates of R medium containing the selective antibiotic, and incubated for 12 h at 50 °C and then at 60 °C until protoplast regeneration was complete (about 24–48 h). The number of viable cells in transformed regenerant colonies of R medium plates decreased when the plates were stored at 5 °C or room temperature for 12 h or more. Thus, transformant colonies were immediately transferred to LB plates containing the appropriate antibiotic and incubated for 16–18 h at 60 °C. The number of cells not converted to protoplasts, the number of L colonies and the regeneration frequency were determined as described by Chen *et al.* (1986).

Table 1. *Plasmids used in this study*

Plasmid	Phenotype	Source*	Reference
pUB110	Km^r	G. Wilson[1]	Gryczan *et al.* (1978)
pPL401	$Cm^r Km^r$	U. Streips	Ambulos *et al.* (1984)
pPL708	$Cm^r Km^r$	P. Lovett[2]	
pTB19	$Km^r Tc^r$	T. Imanaka[3]	Imanaka *et al.* (1981)
pTB90	$Km^r Tc^r$	T. Imanaka	Imanaka *et al.* (1982)
pTHT15	Tc^r	T. Hoshino[4]	Hoshino *et al.* (1985*b*)
pIH41	$Cm^r Km^r Tc^r$	T. Hoshino[4]	
pLW05	Cm^r	This study	

* The sources of the strains were as follows: (1) Dr Gary Wilson, Miles Laboratories, Inc., USA; (2) Dr P. Lovett, University of Maryland, USA; (3) Dr T. Imanaka, Osaka University, Japan; (4) Dr T. Hoshino, Fermentation Research Institute, Japan.

DNA procedures. Plasmid DNA was digested with restriction endonucleases under the conditions recommended by the supplier. Digests were analysed by agarose gel (1%) electrophoresis with Tris/borate buffer (98 mM-Tris/borate, pH 8·3, 28 mM-EDTA). The size of the DNA fragments was estimated from the mobility of the fragments relative to those fragments in *Hin*dIII or *Bst*EII digests of λ DNA. Restriction fragments, separated by agarose gel electrophoresis, were transferred to nitrocellulose paper and hybridized with nick-translated radioactive DNA probes according to Maniatis *et al.* (1982).

Plasmid stability and expression of antibiotic resistance. A 300 ml triple-baffled shake flask containing 20 ml LB medium with antibiotic was inoculated with cells of a plasmid-containing strain from an LB plate with antibiotic (11–14 h, 50 °C). The culture was grown on a gyratory shaker (190–200 r.p.m.) for 2 h at 50 °C to 1×10^8–5×10^8 c.f.u. ml^{-1}.

To test for plasmid stability, a 2 h culture was diluted to a cell density of 100–400 c.f.u. ml^{-1} in 20 ml LB and grown to the late exponential phase at 50, 60, or 65 °C. The growth cycle was repeated as required. Samples were removed at intervals, diluted in LB medium, plated in triplicate on LB plates with and without antibiotic, and incubated overnight at 50 °C to determine the percentage of cells that carried plasmid.

To determine the expression of the antibiotic resistance phenotype, a 300 ml triple-baffled nephelometer flask containing 20 ml LB medium with and without antibiotic was inoculated with cells of a 2 h culture to a Klett reading of 10–15. The culture was grown on a gyratory shaker (190–200 r.p.m.) at 50, 60, 65, or 70 °C.

Inactivation of antibiotics in solid media at high temperature. LB medium and R medium plates containing kanamycin, tetracycline or chloramphenicol were incubated for 17–26 h at 50, 60, or 65 °C. Control plates were held at 5 °C. The plates were seeded with 1×10^7–5×10^7 c.f.u. of exponential phase cells of NUB3621 and incubated for 18–20 h at 50, 60, or 65 °C. Growth of NUB3621 on the preincubated plates and not on the control plates indicates that the antibiotic was inactivated by the incubation conditions.

Estimation of plasmid copy number. Plasmid copy number was estimated by a modification of the procedure described by Kieser *et al.* (1982). Protoplasts were prepared from cells of an exponential phase culture (5 ml) grown in LB medium with antibiotic at 50, 60, or 65 °C as described above. Protoplasts were collected by centrifugation at 700–800 ***g*** for 5 min at room temperature and suspended in 0·25 ml 50 mM-Tris/HCl buffer pH 8·0, containing 15% (w/v) sucrose and 50 mM-Na_2EDTA. Protoplasts were lysed at room temperature by the addition of an equal volume of lysis buffer (1%, w/v, SDS, 50 mM-Tris/HCl, pH 8·0, 50 mM-Na_2EDTA), followed by incubation for 10 min at 70 °C. The mixture was cooled to room temperature and DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1, by vol.). The solution was vigorously mixed for 1 min and the phases were separated by centrifugation. The extraction procedure was repeated twice and sodium acetate was added to the aqueous phase to a final concentration of 0·25 M. Two volumes of absolute ethanol was added and, after 30 min at −20 °C, the precipitate was collected by centrifugation. The pellet was washed with 70% ethanol/water (v/v), and suspended in 20–30 µl and diluted with a 14% (w/v) sucrose, 10 mM-Na_2EDTA solution containing 0·1% bromophenol blue and heat-treated ribonuclease A (100 µg ml^{-1}). After incubation for 1 h at 37 °C, the samples were analysed by agarose gel electrophoresis as described above. The gels were stained for 1 h in ethidium bromide (1 µg ml^{-1}), irradiated with short wavelength UV to nick closed-circular DNA, and stained for an additional 1 h in ethidium bromide. The gels were destained in water and photographed on Polaroid type 665 P/N film. The negatives were scanned with an LKB Bromma Ultrascan XL laser densitometer. The ratio of plasmid to chromosomal DNA was determined from density tracings of those dilutions in which the intensity of the densitometer tracings of each DNA species was proportional to the dilution. The molecular mass of the chromosome of *B. stearothermophilus* was taken as 2×10^9 (Imanaka *et al.*, 1981). The molecular masses of pLW05 and pTHT15 are $1{\cdot}98 \times 10^6$ and $2{\cdot}97 \times 10^6$, respectively.

RESULTS

Optimal conditions for protoplast transformation

The protoplast transformation procedures established for *B. subtilis* (Chang & Cohen, 1979) and for other strains of *B. stearothermophilus* (Imanaka *et al.*, 1982; Liao *et al.*, 1986) would not work with NUB36. In addition, attempts to transform protoplasts of NUB36 using the protoplasting and regeneration techniques established for protoplast fusion in this strain (Chen *et al.*, 1986) were also unsuccessful. In the protoplast transformation systems already established for *B. stearothermophilus*, the temperature of incubation for transformation and regeneration of protoplasts was 47–48 °C. In order to duplicate this condition, it was necessary to use a strain that would regenerate or form L colonies at or near these temperatures. Although the majority of NUB36 strains exhibit optimal regeneration at 65 °C and optimal L colony growth at 60 °C (Chen *et al.*, 1986), some exhibit optimal L colony growth and regeneration at lower temperatures. NUB3621 was selected because it exhibits optimal L colony growth at 45–50 °C and optimal regeneration at 60 °C and has a restriction-deficient phenotype.

Plasmids that specify resistance to kanamycin could not be used in initial studies because kanamycin in R medium was inactivated at 50 °C. In contrast, kanamycin in LB medium was not inactivated at temperatures up to 70 °C. The inactivation of kanamycin at high temperatures was caused by the relatively high concentration of lactose and calcium in the R medium (data not presented). Tetracycline and chloramphenicol in LB medium or R medium were not inactivated at high temperatures.

We attempted to transform protoplasts of NUB3621 by the protoplast fusion procedure described by Chen *et al.* (1986) modified by adding a phenotypic expression step and incubation of all steps at 50 °C. No transformants were obtained with the hybrid plasmids containing both the thermophilic cryptic plasmid pNU01 from NUB36 and a mesophilic or thermophilic plasmid. However, a few chloramphenicol-resistant L colonies were obtained with mesophilic plasmid pPL401 Cm^r Km^r and several regenerant transformant colonies were obtained when incubation of the R medium plates was continued for 20–24 h at 60 °C. One transformant colony that subsequently also grew well on LB medium with chloramphenicol at 60 °C was selected and the plasmid in this isolate, designated pLW05, was used as a model plasmid to establish the transformation procedure.

Optimal conditions for the transformation of protoplasts of NUB3621 with pLW05 Cm^r were established by a systematic investigation of each step of the modified protoplast fusion procedure described above. In initial experiments, R medium plates with antibiotic were incubated at 50 °C. At this temperature, protoplasts in L colonies will not regenerate. Thus, the conditions that affect transformation were determined independently from those conditions that affect regeneration.

The protoplasting medium and PEG used by Chen *et al.* (1986) for protoplast fusion were not suitable for protoplast transformation. Elimination of calcium from the P medium (modified P medium) and the substitution of PEG 6000 for PEG 1550 enhanced the efficiency of transformation 10-fold and 100-fold, respectively.

Incubation temperature had the greatest effect on protoplast transformation of the conditions examined (Table 2). The fraction of the protoplasts expressing the plasmid-encoded antibiotic resistance increased 10-fold to a plateau after incubation for 1 h in antibiotic-free medium. Since the number of L colonies did not increase after incubation in P medium for 1 h at 50 °C, the transformant L colonies detected on R medium plates must represent individual clones. The efficiency of transformation was reduced when transformation (74-fold) or phenotypic expression (7-fold) or both (2600-fold) were carried out at 60 °C rather than 50 °C. However, the total number of L colonies detected on R medium without antibiotic was not significantly affected by the temperature of protoplast transformation or phenotypic expression. Therefore, transformation of protoplasts of NUB3621 with plasmid pLW05 is most efficient when transformation, phenotypic expression and the early stages of L colony growth are carried out at 50 °C. This temperature is near to the minimum temperature of growth for this strain (45–48 °C). Transformation of protoplasts of *B. stearothermophilus* CU21 (Imanaka *et al.*, 1982) and

Table 2. *Effect of temperature on PEG-induced transformation of protoplasts and phenotypic expression*

Protoplasts of NUB3621 were transformed with plasmid pLW05. The R medium plates with antibiotic were incubated at 50 °C. At this temperature, protoplasts in L colonies did not regenerate. Each value is the mean of three experiments; SD values were less than 5% of the mean.

Transformation temperature (°C)	Phenotypic expression		Number of L colonies ml^{-1}*	Efficiency of transformation†
	Temperature (°C)	Time (h)		
50	50	0	$4{\cdot}3 \times 10^8$	$9{\cdot}6 \times 10^6$
50	50	1	$7{\cdot}5 \times 10^8$	$8{\cdot}1 \times 10^7$
50	50	2	$4{\cdot}1 \times 10^8$	$7{\cdot}2 \times 10^7$
50	60	1	$2{\cdot}8 \times 10^8$	$1{\cdot}1 \times 10^7$
60	50	1	$4{\cdot}8 \times 10^8$	$1{\cdot}1 \times 10^6$
60	60	1	$2{\cdot}9 \times 10^8$	$3{\cdot}1 \times 10^4$

* The number of L colonies detected on R medium plates without antibiotic.
† The number of transformant L colonies $(\mu g\ DNA)^{-1}$.

NRRL 1174 (Liao *et al.*, 1986) was also the most efficient at temperatures near the minimum temperature of growth for these strains. Thus, there is a direct relationship between the temperature for efficient transformation and the minimum temperature of growth for the three strains of *B. stearothermophilus*.

The final step was to initiate the regeneration of the protoplasts in transformant L colonies. Protoplasts of *B. stearothermophilus* NUB3621 regenerated at 60 °C but not at 50 °C. Protoplasts in L colonies of NUB3621 incubated for 12 h at 50 °C followed by incubation at 60 °C regenerated with a high frequency (72–95%). Between 60 and 90% of the transformants were regenerants.

The regeneration frequency of protoplasts of NUB3621 at 60 °C was generally between 50 and 100%. In contrast, protoplasts of *B. stearothermophilus* CU21 and NRRL 1174 regenerated at 48 and 47 °C, respectively, but the regeneration frequency was only about 10% (Imanaka *et al.*, 1982; Liao *et al.*, 1986).

Transformation of protoplasts of NUB3621 with plasmid DNA

Mesophilic plasmids pUB110, pPL401 and pPL708 and thermophilic plasmids pTB19, pTB90 and pIH41 transformed NUB3621 at a frequency of $1{\cdot}8 \times 10^4$–$2{\cdot}9 \times 10^5$ and $1{\cdot}2 \times 10^2$–$2{\cdot}4 \times 10^6$ transformants $(\mu g\ DNA)^{-1}$, respectively (data not presented). A majority of the transformants obtained with plasmids that carry two or three antibiotic resistance determinants did not retain the antibiotic resistance phenotype of the parent plasmid.

The highest efficiency of transformation (number of transformant L colonies and regenerant colonies $(\mu g\ of\ DNA)^{-1}$ was obtained with mesophilic plasmid pLW05 Cm^r (2×10^8), a spontaneous derivative of pPL401 $Km^r\ Cm^r$, and thermophilic plasmid pTHT15 Tc^r (4×10^8) isolated from NUB3621. The transformation frequency (transformants per regenerant) was 0·5–1·0. Chloramphenicol-resistant and tetracycline-resistant transformants were obtained when competent cells of *B. subtilis* were transformed with pLW05 [$2{\cdot}5 \times 10^5$ transformants $(\mu g\ DNA)^{-1}$] and pTHT15 [$1{\cdot}8 \times 10^5$ transformants $(\mu g\ DNA)^{-1}$], respectively. In the same experiment, pUB110 isolated from *B. subtilis* transformed *B. subtilis* with an efficiency of $2{\cdot}5 \times 10^5$ transformants $(\mu g\ DNA)^{-1}$. These results indicate that *B. subtilis* BR151 does not restrict plasmid DNA from *B. stearothermophilus* NUB3621.

With plasmid pLW05 the number of transformants was proportional to the amount of DNA up to 2 $\mu g\ ml^{-1}$. Thus, 0·5–1·5 μg DNA ml^{-1} was used in all experiments. The efficiency of transformation with relatively crude preparations of pTHT15 obtained from small-scale cultures of NUB3621(pTHT15) was 5×10^6–1×10^7 transformants $(\mu g\ DNA)^{-1}$. A similar efficiency was obtained when plasmid pTHT15 was completely digested with *Eco*R1, the two

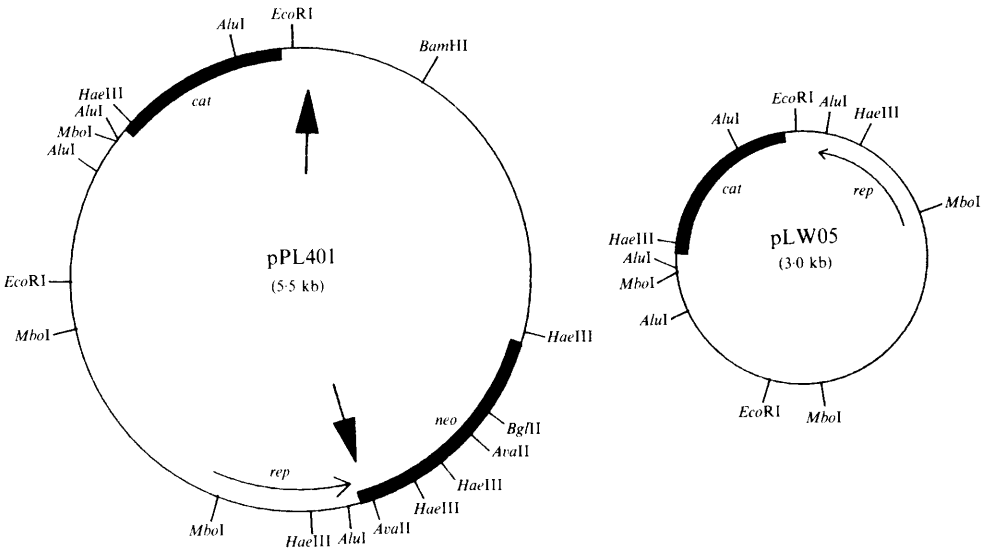

Fig. 1. Restriction map of pPL401 and pLW05. Plasmids were analysed by different restriction enzymes. Only the *Alu*I and *Mbo*I sites that are present in pLW05 are shown in pPL401. The position and orientation of the replication (*rep*), chloramphenicol (*cat*) region, and neomycin (*neo*) region was taken from Scheer-Abramowitz *et al.* (1981), Ambulos *et al.* (1984), and Matsumara *et al.* (1984), respectively. The segment of pPL401 to the right of the arrows was missing in pLW05. The drawings are to scale.

fragments ligated using the procedure described by Maniatis *et al.* (1982), and protoplasts of NUB3621 transformed with the ligation mixture. These results indicate that NUB3621 can be used for plasmid screening and for shotgun cloning experiments.

The efficiency of transformation of protoplasts of NUB3621 with pLW05 and pTHT15 isolated from *B. subtilis* was always lower than that observed when the plasmids were isolated from *B. stearothermophilus* NUB3621 (data not presented). However, since NUB3621 is a restriction-deficient mutant, mechanisms other than restriction must contribute to this lower efficiency.

Restriction analysis of pLW05 and pTHT15

A restriction map of plasmids pPL401 and pLW05 is shown in Fig. 1. Ambulos *et al.* (1984) reported that pPL401 was constructed by inserting a 1·5 kb *Eco*RI fragment of pPL401A that carries the pC194 *cat* gene into the *Eco*RI site of pUB110 (4·5 kb). Thus, the estimated size of pPL401 would be 6·0 kb. We found by restriction analyses that the size of the *Eco*RI fragment and of pPL401 was 1·35 kb and 5·5 kb, respectively. Plasmid pLW05 (3·0 kb) was missing a 2·5 kb segment of pPL401 that contains the neomycin region (segment to the right of the arrows).

The restriction map of pTHT15 isolated from NUB3621(pTHT15) was identical to that reported by Hoshino *et al.* (1985*b*) (data not presented). These results indicate that pTHT15 did not undergo deletions or rearrangements in NUB3621.

Plasmid stability and copy number and the expression of antibiotic resistance

Plasmid stability was determined in cultures growing in antibiotic-free medium at 50, 60, and 65 °C (Table 3). Mesophilic plasmid pLW05 was not stably maintained in cultures of NUB3621 growing in antibiotic-free medium for 12–14 generations at 50, 60, or 65 °C. NUB3621(pLW05), however, exhibited growth in LB medium with chloramphenicol at temperatures up to 70 °C. The growth rate of cultures growing in LB medium with antibiotic at each temperature was not significantly different from the growth rate of cultures growing in LB medium without

Table 3. *Plasmid stability and copy number and expression of antibiotic resistance in B. stearothermophilus NUB3621*

Plasmid	Growth temperature (°C)	Plasmid stability*		Expression of antibiotic resistance: growth rate (divisions h^{-1}) of culture growing in LB medium†		Plasmid copy number‡
		No. of divisions	Plasmid carrier (%)	With antibiotic	Without antibiotic	
pLW05	50	12	83	1·54	1·62	69
		19	<1			
	60	13	3	2·19	2·28	18
		20	<1			
	65	14	<1	2·97	3·38	1
	70	ND	ND	1·39	1·51	ND
pTHT15	50	7	100	1·43	1·91	41
		18	100			
	60	11	100	1·89	3·50	45
		22	100			
	65	11	13	1·66	3·77	12
		22	<1			
	70	ND	ND	0·47	1·42	ND

ND, Not done.

* Cultures of a plasmid-containing strain growing in LB medium were plated on LB medium plates with or without 100 μg chloramphenicol ml^{-1} (pLW05) or 5 μg tetracycline ml^{-1} (pTHT15). The percentage of cells that carry plasmid (plasmid carrier) is the number of colonies detected on LB antibiotic plates divided by the number of colonies detected on LB plates, multiplied by 100. Each value is the mean of triplicate samples of two separate experiments; SD values were less than 2% of the mean.

† Cultures of a plasmid-containing strain were grown in LB medium and LB medium containing 10 μg chloramphenicol ml^{-1} (pLW05) or 5 μg tetracycline ml^{-1} (pTHT15) to the late exponential phase of growth. Cultures in the exponential phase of growth undergo three to five divisions before entering the stationary phase. Each value is the mean of triplicate samples of two separate experiments; SD values were less than 5% of the mean.

‡ The data are the means of triplicate samples; SD values were less than 2% of the mean.

antibiotic. The estimated copy number of pLW05 in cells growing under selective conditions at 50, 60, and 65 °C was 69, 18, and 1 per chromosome equivalent, respectively. All the cells of late exponential phase cultures growing under selective conditions at each temperature were resistant to chloramphenicol. We conclude that cells carrying a reduced copy number of pLW05 are not significantly inhibited by chloramphenicol at high temperature because the chloramphenicol acetyltransferase was active at high temperature. Shaw & Brodsky (1968) reported that purified chloramphenicol acetyltransferase encoded by the pC194 *cat* gene was remarkably resistant to thermal inactivation at 75 °C.

Thermophilic plasmid pTHT15 was stable in cells of NUB3621 growing for 18–22 generations in antibiotic-free medium at 50 and 60 °C and unstable in cultures growing at 65 °C. NUB3621 (pTHT15) grows poorly in LB medium with tetracycline at 50, 60, 65, and 70 °C. The growth rate of cultures growing in LB medium with antibiotics at each temperature was significantly lower than the growth rate of cultures growing in LB medium without antibiotic. The inhibitory effect of the antibiotic was more pronounced at high temperatures. All the cells of late exponential phase cultures growing under selective conditions at each temperature were resistant to tetracycline. These results indicate that the tetracycline resistance determinant was not stable at high temperatures.

The estimated copy number of pTHT15 in cells growing under selective conditions at 50 and 60 °C was about 41–45 copies per chromosome equivalent and 12 in cells growing at 65 °C. Hoshino *et al.* (1985*a*) reported that the estimated copy number of pTHT15 in *B. stearothermophilus* CU21 growing under selective conditions was about 110 per cell. Although the temperature at which the culture was grown was not specified, we assume that the culture was grown at a temperature at which pTHT15 was stable (48–60 °C; Hoshino *et al.*, 1985*a*). The

discrepancy may be explained by the different procedures used to estimate plasmid copy number or to the mechanisms that regulate plasmid copy number in the two strains of *B. stearothermophilus*.

DISCUSSION

A highly efficient and reliable protoplast transformation system was developed for *B. stearothermophilus* NUB3621 using mesophilic plasmid pLW05 Cm^r and thermophilic plasmid pTHT15 Tc^r. The efficiency of transformation obtained with *B. stearothermophilus* NUB3621 [2×10^7–4×10^8 transformants (μg DNA)$^{-1}$] was higher than that reported for *B. stearothermophilus* CU21 [2×10^7 transformants (μg DNA)$^{-1}$; Imanaka *et al.*, 1982] or *B. stearothermophilus* NRRL 1174 [4×10^4 transformants (μg DNA)$^{-1}$; Liao *et al.*, 1986]. Chen *et al.* (1986) reported that a majority of the protoplasts of NUB36 formed L colonies when plated on R medium. The unique viability of the protoplasts may account for the high efficiency of transformation observed with NUB3621. Plasmids pLW05 and pTHT15 also transform *B. subtilis*. Hoshino and colleagues reported that pTHT15 transformed (Hoshino *et al.*, 1985*b*) and was stably maintained (Hoshino *et al.*, 1985*d*) in *B. subtilis* and *B. stearothermophilus* CU21. Thus, pLW05 and pTHT15 are shuttle vectors for mesophilic and thermophilic bacilli.

Plasmid pLW05 Cm^r was not stably maintained in cells growing under non-selective conditions but the relatively thermostable chloramphenicol acetyltransferase was active at temperatures up to 70 °C. Soutschek-Bauer *et al.* (1987) constructed a plasmid (pGS13 Cm^r Km^r, 3·4 kb) that is similar to pLW05. Plasmid pGS13 contains the replication region and the kanamycin-resistance gene of pUB110 and the *cat* gene of pC194 and was unstable in *B. stearothermophilus* CU21 under non-selective conditions. Thermophilic plasmid pTHT15 Tc^r was stable in cells growing under non-selective conditions at temperatures up to 60 °C but the tetracycline resistance (Tet) protein did not function well at higher temperatures. Similar results were found for pTHT15 in *B. stearothermophilus* CU21 (Hoshino *et al.*, 1985*d*). Sakaguchi *et al.* (1986) reported that the Tet protein encoded on mesophilic plasmid pNS1981 from *B. subtilis* was identical to the Tet protein encoded on thermophilic plasmid pTHT15 except for two consecutive base pairs. Hoshino *et al.* (1985*c*) proposed that the Tet protein of thermophilic plasmid pTHT15 and the Tet proteins of mesophilic plasmids may have a common origin. No information is available as to the thermostability of the Tet protein of pNS1981 and pTHT15.

Plasmid pLW05 carries the replication region and two (BA1 and BA2) of the *in vitro* membrane-binding regions of mesophilic plasmid pUB110 (Tanaka & Sueoka, 1983). Although pTHT15 was isolated from a thermophilic bacillus (Hoshino *et al.*, 1985*c*), the replication region of this plasmid was identical to the replication region of pUB110 and shows extensive homology to *in vitro* membrane-binding region BA3 of this plasmid (Hoshino *et al.*, 1985*a*; McKenzie *et al.*, 1986). The relationship between plasmid copy number and stability and the binding of plasmid to specific membrane sites requires clarification.

The transformation system described in this paper will be useful for molecular cloning in strains of the genus *Bacillus*. While plasmids pLW05 and pTHT15 may be suitable for preliminary molecular cloning experiments, ideally we need a vector that is stably maintained and expresses antibiotic resistance at temperatures above 65 °C. The search for the ideal thermophilic vector has been carried out by three different types of investigation: (i) isolation and characterization of plasmids from thermophilic bacilli (Bingham *et al.*, 1979; Imanaka *et al.*, 1981; Hoshino *et al.*, 1985*b*); (ii) construction of hybrid plasmids from cryptic plasmids of thermophilic bacilli and mesophilic plasmids (Liao *et al.*, 1986); and (iii) isolation of mutant plasmids or construction of hybrid plasmids that either have enhanced stability (Koizumi *et al.*, 1986; Aiba *et al.*, 1987*a*, *b*; Min *et al.*, 1987; Soutschek-Bauer *et al.*, 1987) or the thermostability of the antibiotic resistance determinant is enhanced (Matsumura & Aiba, 1985; Liao *et al.*, 1986). To date the plasmids used in *B. stearothermophilus* protoplast transformation systems either are not stable or do not express antibiotic resistance at temperatures much above 65 °C, or both.

Experiments are under way to construct hybrid plasmids that contain unique restriction sites and are stably maintained and express antibiotic resistance at temperatures above 65 °C.

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