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Genetic Transformation of the Extreme Thermophile *Thermus thermophilus* and of Other *Thermus* spp.

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Genetic transformation of auxotrophs of the extreme thermophile *Thermus thermophilus* HB27 to prototrophy was obtained at high frequencies of 10^{-2} to 10^{-1} when proliferating cell populations were exposed to chromosomal DNA from a nutritionally independent wild-type strain. The transformation frequency was proportional to the DNA concentration from 10 pg/ml to 100 ng/ml. *T. thermophilus* HB27 cells did not require chemical treatment to induce competence, although optimal transformation was obtained by the addition of a divalent cation (Ca^{2+} or Mg^{2+}). Competence was maintained throughout the growth phase, with the highest transformation frequencies at pH 6 to 9 and at 70°C. *T. thermophilus* HB27 and four other typical *Thermus* strains, *T. thermophilus* HB8, *T. flavus* AT62, *T. caldophilus* GK24, and *T. aquaticus* YT1, were also transformed to streptomycin resistance by DNA from their own spontaneous streptomycin-resistant mutants. A cryptic plasmid, pTT8, from *T. thermophilus* HB8 was introduced into *T. thermophilus* HB27 Pro$^-$ at a frequency of 10^{-2}.

Thermus spp. are extremely thermophilic bacteria which can grow at temperatures over 75°C. They are aerobic, rod-shaped, nonsporulating, gram-negative bacteria. These organisms synthesize macromolecules which are not only heat stable but also resistant to many other drastic conditions, including organic solvents and high concentrations of urea and detergents. For these reasons, a number of enzymes (3, 4, 9) and tRNAs (20) from *Thermus* spp. have been purified and extensively characterized. Because of the lack of mutant strains and DNA transfer systems, genetic studies on *Thermus* spp. are limited, an exception being that the leucine gene from *Thermus thermophilus* was cloned in *Escherichia coli* and sequenced (7, 8, 18). Several plasmids of *Thermus* spp. have been reported (6, 19), but their functions are unknown. We have found natural DNA transformation events in *Thermus* spp., and moreover we have demonstrated introduction of a cryptic plasmid, pTT8, into *T. thermophilus* by using the same DNA transformation system. We report here some characteristics of natural transformation by using chromosomal and plasmid DNA in these extremely thermophilic organisms.

Mutants auxotrophic for proline (K102 Pro$^-$), leucine (K103 Leu$^-$), methionine (K104 Met$^-$), lysine (K105 Lys$^-$), and tryptophan (K106 Trp$^-$) were obtained after exposure of wild-type *T. thermophilus* HB27 cells (16) to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine by the method of Adelberg et al. (1). Auxotrophic mutants of *T. thermophilus* HB27 were transformed to prototrophy as follows. The TM broth medium was used for transformation experiments and routine cultivations. It consisted of 0.4% Polypeptone (Daigo-Eiyo Chemical Co. Ltd., Osaka, Japan), 0.2% yeast extract (Difco Laboratories, Detroit, Mich.), 0.1% NaCl, and basal salt (13), and the pH was adjusted to 7.5 with NaOH at room temperature. An overnight TM broth culture of the auxotrophic mutant was diluted 1:100 into fresh TM broth and incubated with shaking at 70°C for 2 h. Then, a 0.45-ml portion of culture (about 5×10^7 cells per ml) was removed and mixed with 50 μl of chromosomal DNA solution from the prototrophic parental strain. DNA was isolated by the method of Saito and Miura (15). The final DNA concentration in the mixture was 10 μg/ml. The mixture was then incubated with shaking at 70°C for 1 h, followed by cooling on ice for 5 min. DNA uptake was terminated by incubating the mixture at 37°C for 15 min after the addition of DNase I (50 μg/ml). Samples were appropriately diluted with 0.9% NaCl and plated on minimal medium plates (18) for the detection of transformants. Transformants were counted after 36 to 48 h of incubation at 70°C.

All the auxotrophs were transformed to prototrophy. The transformation frequency of the Pro$^-$ strain was very high (11.9%), and transformation to Trp$^+$ was 2.7%, whereas for Leu$^-$, Met$^-$, and Lys$^-$, transformation frequencies were lower (0.75, 0.92, and 0.95%, respectively). The results indicate that transformation events are not limited to a few particular genetic markers.

That transformation to prototrophy was due to the exogenous DNA was demonstrated by the following results. The transforming activity was completely eliminated by prior treatment of the DNA solution with DNase and greatly reduced by sonication, whereas treatment with RNase and pronase had little effect (data not shown). The absence of any transformants when donor DNA from the same auxotrophic strain as the recipient (K102, Pro$^-$) was used in place of DNA from the prototrophic strain clearly suggests that exogenous DNA is responsible for the transformation.

The transforming activity of several different concentrations of DNA is shown in Fig. 1. A linear relationship of transformation frequency to DNA concentration was observed over a 10,000-fold range. The number of transformants reached a maximum at about 5 μg of DNA per ml. The transformation frequency was proportional to the length of incubation time up to 30 min. Maximum frequency was obtained with a 60-min incubation. Transformation in TM broth over a pH range from 4 to 10 revealed a broad, flat, optimal pH of 6 to 9. Transformation frequencies were similarly high throughout this range, and a sharp optimum peak was not obtained. The optimal temperature for transformation was 70°C, and transformation frequencies were sharply reduced at 45 and 80°C.

The transforming medium consisted of base medium

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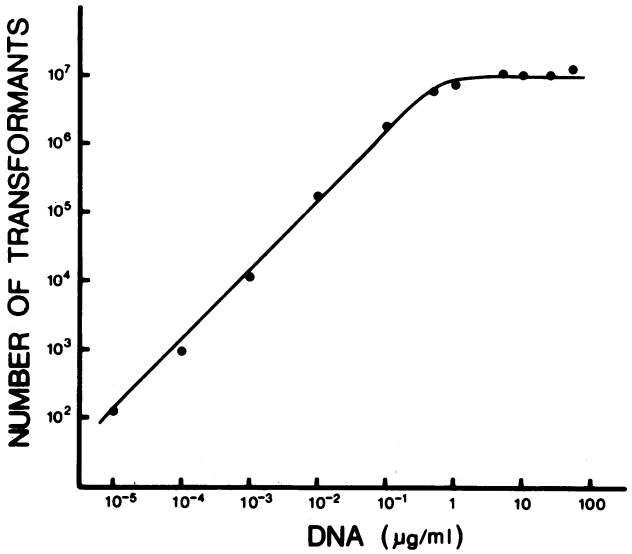

FIG. 1. Dependence of transformation upon DNA concentration. The number of K102 (Pro$^-$) cells (●) transformed to Pro$^+$ with various concentrations of the wild-type DNA in a cell-DNA mixture is shown. The total number of recipient cells was 8.6×10^7.

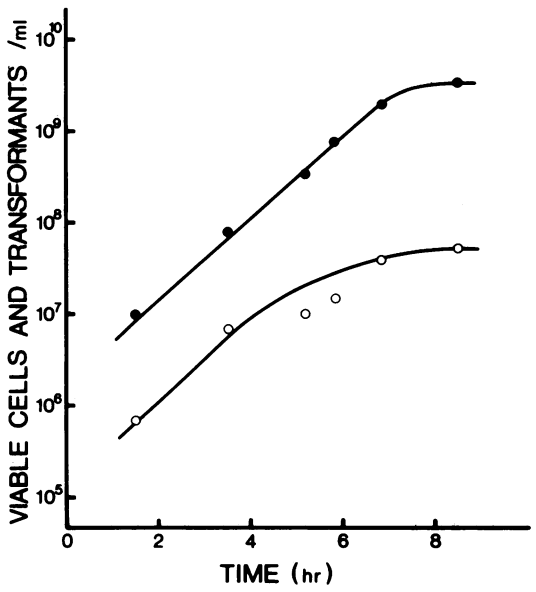

FIG. 2. Transformation at various stages of growth. At the indicated intervals, 0.45 ml of the growing bacterial culture of K102 (Pro$^-$) in TM broth was withdrawn and transformed by mixing with 50 μl of the wild-type DNA. The final DNA concentration was 10 μg/ml. The incubation time of the mixture was shortened to 30 min to reveal the effect of the growth phase clearly. Symbols: ●, CFUs; ○, Pro$^+$ transformants.

(Polypeptone, yeast extract, and NaCl) plus basal salts, including 0.35 mM Ca^{2+} and 0.4 mM Mg^{2+}. Omission of basal salts from the transforming medium decreased transformation by 77%. We determined which component(s) of the basal salts was required (Table 1). High-frequency transformation required addition of Ca^{2+} or Mg^{2+}. Transformation was greatly reduced by EDTA, but only in the absence of added Ca^{2+} and Mg^{2+}.

To investigate the relationship of the growth phase to competence, strain K102 (Pro$^-$) was grown in a TM broth medium, and portions were removed at chosen intervals. Then, without concentration or dilution, cells were transformed with DNA (10 μg/ml) (Fig. 2). Competence was essentially uniform during all phases of growth and showed no evidence for waves of increased or decreased transformability. The transformation frequency of cells in stationary phase, however, was not so high (about 1.5%), but they could be transformed at a high frequency (about 10%) if they were diluted appropriately with fresh transforming medium and immediately transformed by being mixed with DNA (data not shown).

To determine whether *Thermus* strains other than *T. thermophilus* HB27 could be transformed, early log phase cultures (0.9 ml) of *T. thermophilus* HB27 and four typical *Thermus* strains, *T. thermophilus* HB8 (10), *T. flavus* AT62 (14), *T. caldophilus* GK24 (17), and *T. aquaticus* YT1 (2), were mixed with DNA (10 μg/ml) from their own spontaneous streptomycin-resistant mutants, which were obtained by plating the wild-type strain on a TM plate containing 500 μg of streptomycin per ml. Each mixture was incubated at 70°C for 1 h before the reaction was terminated by addition of DNase. An additional 3-h incubation at 70°C with shaking was needed to get the maximum transformation frequency for the streptomycin resistance marker (data not shown). TM plates containing streptomycin (50 μg/ml) were used for the detection of Str^r transformants. All the strains tested could be transformed (Table 2), though the transformation frequencies were somewhat lower than those found with the transformation of *T. thermophilus* HB27 auxotrophs to prototrophy. The transformation frequency of *T. aquaticus* YT1, which was isolated from a hot spring in Yellowstone National Park, was lower than those of four other strains, all of which were isolated from hot springs in Japan.

A cryptic plasmid, pTT8 (6), from *T. thermophilus* HB8 was introduced into plasmid-free *T. thermophilus* K102 (Pro$^-$). After *T. thermophilus* K102 cells were grown as for

TABLE 1. Effect of divalent cations and EDTA on transformation[a]

Medium supplement	Transformation frequency (%)	Relative transformation
None	2.1	23
Basal salts	9.3	100
0.35 mM Ca^{2+}	9.4	101
0.4 mM Mg^{2+}	5.4	60
0.35 mM Ca^{2+} + 0.4 mM Mg^{2+}	10.8	116
1 mM EDTA	0.0083	0.089
1 mM EDTA + 1 mM Ca^{2+} + 1 mM Mg^{2+}	9.3	100

[a] Pro$^-$ cells were grown in TM medium and washed once in 0.9% NaCl. Cells were then suspended in the indicated medium with or without supplement(s) and transformed.

TABLE 2. Transformation to streptomycin resistance

Recipient	Viable CFU	No. of Str^r transformants (%)[a]
T. thermophilus HB27	2.5×10^9	2.6×10^7 (1.0)
T. thermophilus HB8	4.1×10^8	1.0×10^6 (0.24)
T. flavus AT62	2.4×10^9	2.1×10^7 (0.88)
T. caldophilus GK24	5.1×10^8	1.4×10^6 (0.27)
T. aquaticus YT1	2.2×10^8	1.4×10^5 (0.064)

[a] Frequencies of spontaneous mutation to Str^r of five strains were all less than 10^{-7}.

transformation with chromosomal DNA, a 50-μl portion of culture was added to dried pTT8 plasmid DNA (5 μg) and incubated at 70°C for 1 h. The solution was then appropriately diluted and spread onto nonselective TM plates. After overnight incubation at 70°C, colonies on TM plates were then tested for plasmid possession by colony hybridization (5) by using nick-translated ^{32}P-labeled pTT8 plasmid DNA as a probe. Nine clones were positive among 1,000 tested. All positive clones were auxotrophic for proline, as was the recipient strain used, and bore plasmids with the same size and restriction-digest pattern as authentic pTT8 plasmid (data not shown). We conclude that plasmid pTT8 was introduced into a recipient without deletion of DNA. The plasmid was stably maintained in the recipient cells for more than 20 generations.

Genetic transformation has been reported in several genera of gram-negative bacteria, including *Haemophilus*, *Neisseria*, *Moraxella*, *Acinetobacter*, and *Pseudomonas*. In the present study, we demonstrated natural transformation in the genus *Thermus*.

T. thermophilus HB27 cells in TM broth culture were highly competent, and competence was maintained throughout the growth phase. The transformation frequencies of *Thermus* strains were very high and comparable to other bacterial systems. Plasmid DNAs are typically inefficient at transforming naturally competent cells. In *Bacillus subtilis* and *Haemophilus influenzae*, transformation with plasmid DNA is 10^{-3}- to 10^{-4}-fold less efficient than transformation with chromosomal markers. These inefficiencies are based on the conversion of transforming DNA into single-stranded form in *B. subtilis* (11) and the processing of DNA during translocation from a membrane vesicle to cytoplasm in *H. influenzae* (12). However, in *T. thermophilus* HB27, plasmid introduction was relatively efficient. This suggests that the transformation mechanism of *Thermus* spp. may be different from those of *B. subtilis* and *H. influenzae*. Although the transformation system of *Thermus* spp. was not fully characterized, the system will be a useful tool for genetic studies of the extremely thermophilic organisms, since it appears to be applicable to many *Thermus* strains.

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