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Genetic Transformation of *Bacillus brevis* 47, a Protein-Secreting Bacterium, by Plasmid DNA

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A method has been developed for introducing plasmid DNA into *Bacillus brevis* 47, a protein-secreting bacterium. Treatment of *B. brevis* 47 cells with 50 mM Tris-hydrochloride buffer of alkaline pH was effective for inducing DNA uptake competence. In the presence of polyethylene glycol, the Tris-treated cells incorporated plasmid DNA with a frequency of 10^{-4} (transformants per viable cell) when 1 μg of plasmid DNA was added to 10^9 Tris-treated cells. The pH of Tris-hydrochloride buffer as well as the concentration and molecular weight of the polyethylene glycol affected the transformation frequency. The growth phase of *B. brevis* 47 cells strongly influenced the frequency. Two plasmids, pHW1 and pUB110, have been introduced into *B. brevis* 47 by this method. The mechanism of induction of competence for DNA uptake in connection with removal of the outer two protein layers of the cell wall by treatment of *B. brevis* 47 cells with Tris-hydrochloride buffer is discussed.

Bacillus brevis 47, which was isolated as a protein-producing bacterium from soil, secretes vast amounts of protein into the medium (13). Under optimal growth conditions, the amount of protein accumulated in the medium reached 12 mg/ml (10). Since the bacterium is the only known microorganism that secretes such large amounts of protein, it seems to be a useful and interesting organism for studying the mechanism of protein secretion. Secreted protein consists mainly of two proteins with approximate molecular weights of 150,000 and 130,000. These proteins were indistinguishable from two major proteins found in the outer two protein layers of the *B. brevis* 47 cell wall (14).

Recently, a gene coding for the polypeptide that cross-reacts with antiserum against one of these two major proteins has been cloned into *Escherichia coli* (N. Tsukagoshi et al., manuscript in preparation). To study the expression of the cloned gene and to perform genetic engineering in *B. brevis* 47, it is essential to find appropriate vectors available in this bacterium and to establish methods of transformation.

Protoplast transformation has been successful in many bacteria, such as *Bacillus subtilis* (2), *Bacillus thuringiensis* (9), and *Bacillus stearothermophilus* (6), in the presence of polyethylene glycol (PEG). However, the method could not be applied directly to *B. brevis* 47 because of the difficulty in regenerating its protoplasts. Although Ca^{2+} and Mn^{2+} ions have been found to facilitate the regeneration of *B. brevis* 47 protoplasts, the regeneration involved a very long incubation period, which made the method unsuitable for practical use in transformation (Yamaguchi et al., unpublished data). Here we describe a new method for transforming *B. brevis* 47, which includes treatment of *B. brevis* cells with Tris-hydrochloride buffer of alkaline pH, followed by induction of DNA uptake with PEG. This method was much easier and gave a higher efficiency of transformation than did the protoplast method. Moreover, with the new method, transformants could be obtained within 1 day after transformation.

MATERIALS AND METHODS

Bacterial strains. *B. brevis* 47 and 481 have been described previously (13). *B. brevis* 47-5 is a uracil-requiring derivative of *B. brevis* 47. *B. brevis* ATCC 8185 and AJ3894 were obtained from the American Type Culture Collection and the Ajinomoto Co., respectively. *B. subtilis* strains carrying plasmids pHW1 (5), pTP5 (7), and pUB110 (4) were obtained from S. Horinouchi, M. Kono, and Y. Sadaie, respectively.

Media. T_2 ura medium is a T_2 medium (13) supplemented with 100 μg of uracil per ml. Solid medium contained 1.5% (wt/vol) agar. Phosphate buffer contained 1.905 g of KH_2PO_4 and 0.852 g of Na_2HPO_4 per 100 ml. The PEG solution (40%, wt/vol) contained 40 g of PEG 6000 and 50 ml of phosphate buffer in 100 ml. TP medium was a mixture (1:1) of phosphate buffer and double-strength T_2 ura medium. MT medium was T_2 ura medium supplemented with 20 mM $MgCl_2$. ET agar plate medium was T_2 ura solid medium supplemented with 10 μg of erythromycin per ml. TE buffer

contained 10 mM Tris-hydrochloride (pH 7.5) and 1 mM EDTA.

Chemicals. PEG 6000 (first grade; average molecular weight, 7,500) and PEG 4000 (first grade; average molecular weight, 3,000) were purchased from Wako Pure Chemical Industries. Erythromycin was from Boehringer Mannheim Corp. All other chemicals used were of reagent grade.

Isolation of plasmid DNA. Plasmid DNAs used for transformation were prepared from *B. subtilis* strains harboring these plasmids, as described by Tanaka et al. (12). Rapid plasmid DNA isolation for analytical purposes was performed as described by Birnboim and Doly (1).

Standard procedure for transformation of *B. brevis* 47 with pHW1 DNA. An overnight culture of *B. brevis* 47-5 in T_2 ura medium was diluted 100-fold in 5 ml of the same medium and grown at 37°C with shaking. At the late logarithmic phase of growth (optical density [OD] at 660 nm = 1.9), cells were collected in a 40-ml screw-capped plastic centrifuge tube by centrifugation at 3,000 × *g* for 5 min at room temperature, washed at room temperature with 5 ml of 50 mM Tris-hydrochloride buffer (pH 7.5), suspended in 5 ml of 50 mM Tris-hydrochloride buffer (pH 8.5), and incubated for 60 min at 37°C with shaking. Cells were again collected by centrifugation at 3,000 × *g* for 5 min at room temperature and suspended in 0.5 ml of TP medium. After the addition of plasmid DNA dissolved in 100 μl of a 1:1 mixture of TE buffer and TP medium, 1.5 ml of PEG solution was added and mixed immediately (transformation mixture). After incubation at 37°C for 10 min with shaking, cells were centrifuged at 3,000 × *g* for 10 min at room temperature, suspended in 1 ml of MT medium, and incubated at 37°C with shaking. After a 30-min incubation, erythromycin was added to 0.1 μg/ml and incubation was continued for 120 min. Samples (0.1 ml each) of the culture were spread on ET agar plates, which were incubated at 37°C.

RESULTS

Transformation of *B. brevis* 47 with plasmid pHW1. Treatment of *B. brevis* 47 cells with alkaline Tris-hydrochloride buffer to remove the outer two protein layers was followed by the introduction of pHW1 DNA in the presence of PEG as described above. Erythromycin-resistant transformants were obtained with a frequency of about 10^5/ml of a transformation mixture to which 1 μg of pHW1 DNA was added. These transformants formed visible colonies on ET plates within 24 h of incubation after transformation. Figure 1 shows the numbers of transformants obtained when various amounts of pHW1 DNA were used for transformation. For up to 1 μg of pHW1 DNA, the numbers of transformants increased proportionally with the amount of DNA added. No erythromycin-resistant colonies were obtained when DNA was omitted from the transformation mixture. The presence of plasmid pHW1 in erythromycin-resistant colonies was confirmed by the rapid alkaline extraction method of Birnboim and Doly (1). Upon digestion with restriction endonucleases, plasmid DNAs isolated from the erythromycin-resistant transformants yielded the same DNA fragments as those of the original plasmid DNA, indicating that no structural alteration of the plasmid DNA had occurred in the *B. brevis* 47 cells (data not shown).

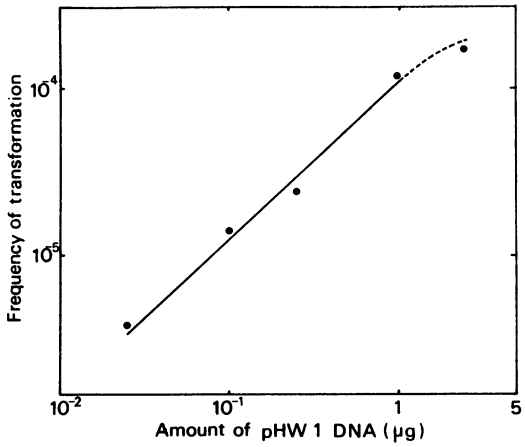

FIG. 1. Frequency of plasmid transformation as a function of the amount of DNA. The transformation procedure is described in the text. The total viable cell count in each transformation mixture was 10^9.

Effects of molecular weight and concentration of PEG on the frequency of transformation. Solutions of PEG 6000 or PEG 4000 in one-half strength phosphate buffer prepared as described above were used for the induction of pHW1 DNA uptake of *B. brevis* 47 cells that had been treated previously with Tris-hydrochloride buffer (pH 8.5) for 15 min at 37°C. Other conditions for transformation were the same as described above. The frequency of transformation was highest when 40% (wt/vol) PEG 6000 (finally 28% in the transformation mixture) was used (Table 1). No transformants were obtained when PEG was omitted from the transformation mixture.

Effects of pH and divalent cations on induction of competence for DNA uptake. *B. brevis* 47-5 cells washed with 50 mM Tris-hydrochloride buffer (pH 7.5) were suspended in 50 mM Tris-hydrochloride buffer of various pHs from 7 to 9 and were incubated further at 37°C for 15 min. The incubated cells were used for transformation with pHW1 DNA. At alkaline pHs (8.0 to 9.0), Tris-hydrochloride buffer induced the competence for DNA uptake efficiently; however, no induction of competence was observed at pH 7.0. A slight increase in the transformation frequency with an elevation in the pH between 8 and 9 was due to a loss of viability at a higher pH. The addition of 20 mM $MgCl_2$ or $CaCl_2$ to Tris-hydrochloride buffer (pH 8.5) completely

TABLE 1. Effects of the molecular weight and concentration of PEG on transformation frequency

PEG	Concn[a] (%)	No. of transformants/0.1 μg of DNA (A)	Transformation frequency (A/viable cell counts)
PEG 6000 (avg mol wt, 7,500)	0	0	$<10^{-9}$
	20	120	7.1×10^{-8}
	30	1,700	1.4×10^{-6}
	40	6,500	1.3×10^{-5}
	50	3,300	1.5×10^{-5}
PEG 4000 (avg mol wt, 3,000)	20	50	2.6×10^{-8}
	30	1,200	1.5×10^{-6}
	40	170	5.9×10^{-7}

[a] The concentrations of PEG solutions used are indicated; the final concentrations of PEG in the transformation mixtures were 0.7 times these values.

inhibited the induction (Table 2). Figure 2 shows the effect of incubation time on induction of competence. When Tris-hydrochloride buffer of pH 8.5 was used, the induction was rapid, reaching the maximum level within 15 min of incubation. Without incubation with the Tris-hydrochloride buffer, the competence was not induced (time zero, Fig. 2). On the other hand, the induction was slow and continued up to 120 min when Tris-hydrochloride buffer of pH 7.5 was used. Similar effects of pH and divalent cations on the removal of the outer two protein layers of the cell wall were observed during the Tris treatment (see below).

Effect of the growth phase of *B. brevis* 47 on the transformation frequency. *B. brevis* 47-5 cells grown in T_2 ura medium for various periods (OD at 660 nm, 0.8, 1.4, 1.9, 2.1, 2.6, and 3.0) were used for transformation with 0.1 μg of pHW1 DNA. As shown in Table 3, the number of transformants increased along with the bacterial growth, but the frequency of transformation was almost constant up to the late logarithmic phase (OD, 1.9). However, the transformation frequency decreased drastically to a level that was lower than 1/10 of the maximum level when the bacterial growth reached the early stationary phase (OD, 2.6).

General usefulness of the transformation method. The transformation method described above was used for transforming other *B. brevis* strains (481, ATCC 8185, and AJ3894) with plasmid pHW1 DNA. *B. brevis* 481, another protein-secreting *B. brevis* strain (13), was shown to be transformed with the plasmid DNA. No transformants were obtained for the non-protein-secreting *B. brevis* strains, ATCC 8185 and AJ3894.

Other plasmids, pTP5 and pUB110, were used to transform *B. brevis* 47-5 by this transformation method; T_2 ura agar plates supplemented with 60 μg of neomycin per ml or 10 μg of chlortetracycline hydrochloride per ml were used instead of ET plates. Approximately 10^4 transformants per μg of plasmid DNA were obtained when plasmid pUB110 was used. However, transformation with plasmid pTP5 has not been successful, probably because the plasmid DNA cannot replicate or because its tetracycline resistance gene cannot be expressed in *B. brevis* 47 cells.

DISCUSSION

The effect of the pH level of Tris-hydrochloride buffer on the induction of competence for DNA uptake and the time course of the induc-

TABLE 2. Effects of pH and the addition of divalent cations during Tris treatment on transformation frequency

pH of Tris-hydrochloride	Divalent cation	No. of transformants/0.1 μg of DNA (A)	Transformation frequency (A/viable cell counts)
9.0	None	1,500	7.1×10^{-6}
8.5	None	1,700	5.5×10^{-6}
8.0	None	2,000	2.8×10^{-6}
7.5	None	110	9.4×10^{-8}
7.0	None	0	$<10^{-9}$
8.5	20 mM Mg^{2+}	0	$<10^{-9}$
8.5	20 mM Ca^{2+}	0	$<10^{-9}$

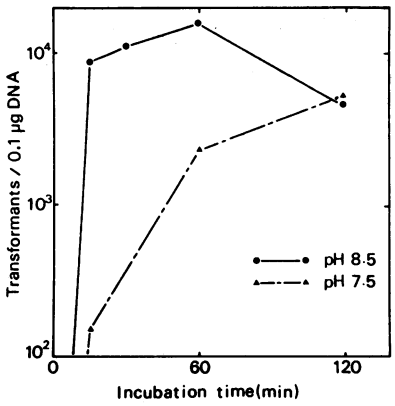

FIG. 2. Effect of duration of Tris treatment on the frequency of transformation. *B. brevis* 47-5 cells washed with 50 mM Tris-hydrochloride buffer (pH 7.5) as described in the text were suspended in 50 mM Tris-hydrochloride buffer of pH 7.5 or 8.5. Samples were taken at intervals and used for transformation by pHW1 DNA.

tion described here closely resembled those observed for the removal of the outer two protein layers of the *B. brevis* 47 cell wall (14). Divalent cations added to Tris-hydrochloride buffer inhibited the induction of competence; their effect resembled inhibition of the removal of the outer protein layers. These results strongly suggested that the outer two layers of the *B. brevis* 47 cell wall were a barrier for DNA uptake and that treatment of *B. brevis* 47 cells with Tris-hydrochloride buffer facilitated the uptake by removing the barrier. Recently, the induction of competence for DNA uptake by washing cells with Tris buffer has been reported for *Rhodopseudomonas sphaeroides* (3). Tris buffer of neutral pH and of high concentration (around 0.5 M) was used, and the presence of $CaCl_2$ as well as PEG in the transformation mixture was essential in the system of Fornari and Kaplan. In contrast, Tris-hydrochloride buffer of alkaline pH and relatively low concentration (lower than 50 mM) was effective in our system. $CaCl_2$ was inhibitory for the induction of competence and was not required for DNA uptake in our system. Thus, the mechanism of induction of competence for DNA uptake in *B. brevis* 47 with Tris treatment should be distinct from that in *R. sphaeroides*. The mechanism seems to work commonly in protein-producing *B. brevis*, since this method of transformation was applicable to *B. brevis* 481, another protein-secreting *B. brevis* strain, but not to other non-protein-secreting *B. brevis* strains such as ATCC 8185 and AJ3894. The method described here might also be useful for the transformation of other microorganisms such as *Bacillus* sp. strain C.I.P. 76-111 (8) and *Bacillus polymyxa* (11) that have multilayered cell walls similar to that of *B. brevis* 47. Another plasmid, pUB110, was also introduced into *B. brevis* 47 by this method. The intensity of the band of plasmid pUB110 DNA in cleared lysates prepared from the transformants and analyzed by agarose gel electrophoresis suggested that plasmid pUB110 existed at a multicopy number in *B. brevis* 47 cells (data not shown).

TABLE 3. Effects of the growth phase of *B. brevis* 47 on transformation frequency

OD at 660 nm	No. of transformants/0.1 μg of pHW1 DNA (A)	Transformation frequency (A/viable cells)
0.8	4.3×10^2	1.03×10^{-5}
1.4	7.5×10^2	1.05×10^{-5}
1.9	4.0×10^3	1.1×10^{-5}
2.1	5.5×10^3	5×10^{-6}
2.6	4.0×10^2	3×10^{-7}
3.0	1.0×10^2	7×10^{-8}

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LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Chang, S., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Mol. Gen. Genet. **168**:111–115.
3. **Fornari, C. S., and S. Kaplan.** 1982. Genetic transformation of *Rhodopseudomonas sphaeroides* by plasmid DNA. J. Bacteriol. **152**:89–97.
4. **Gryczan T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134**:318–329.
5. **Horinouchi, S., and B. Weisblum.** 1982. Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide, and streptogramin type B antibiotics. J. Bacteriol. **150**:804–814.
6. **Imanaka, T., M. Fujii, I. Aramori, and S. Aiba.** 1982. Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **149**:824–830.
7. **Kono, M., M. Sasatsu, and H. Hamashima.** 1978. Transformation of *Bacillus subtilis* with staphylococcal plasmid DNA. Microbios Lett. **5**:55–59.
8. **Leduc, M., M. Rousseau, and J. Heijenoort.** 1977. Structure of the cell wall of *Bacillus* species C.I.P. 76-111. Eur. J. Biochem. **80**:153–163.
9. **Martin, P. A. W., J. R. Lohr, and D. H. Dean.** 1981. Transformation of *Bacillus thuringiensis* protoplasts by plasmid deoxyribonucleic acid. J. Bacteriol. **145**:980–983.
10. **Miyashiro, S., H. Enei, K. Takinami, Y. Hirose, T. Tsuchida, and S. Udaka.** 1980. Stimulatory effect of inhibitors of cell wall synthesis on protein production by *Bacillus*

brevis. Agric. Biol. Chem. **44**:2297–2303.
11. **Nermut, M. V., and R. G. E. Murray.** 1967. Ultrastructure of the cell wall of *Bacillus polymyxa*. J. Bacteriol. **93**:1949–1965.
12. **Tanaka, T., M. Kuroda, and K. Sakaguchi.** 1977. Isolation and characterization of four plasmids from *Bacillus subtilis*. J. Bacteriol. **129**:1487–1494.
13. **Udaka, S.** 1976. Extracellular production of proteins by microorganism. Part I. Screening for protein producing bacteria. Agric. Biol. Chem. **40**:523–528.
14. **Yamada, H., N. Tsukagoshi, and S. Udaka.** 1981. Morphological alterations of cell wall concomitant with protein release in a protein-producing bacterium, *Bacillus brevis* 47. J. Bacteriol. **148**:322–332.