Transformation of Vegetative Cells of *Bacillus thuringiensis* by Plasmid DNA

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Plasmid DNA-mediated transformation of vegetative cells of *Bacillus thuringiensis* was studied with the following two plasmids: pBC16 coding for tetracycline resistance and pC194 expressing chloramphenicol resistance. A key step was the induction of competence by treatment of the bacteria with 50 mM Tris hydrochloride buffer (pH 8.9) containing 30% sucrose. Transformation frequency was strongly influenced by culture density during the uptake of DNA and required the presence of polyethylene glycol. Growth in a minimal medium supplemented with Casamino Acids gave 35 times more transformants than growth in a rich medium. The highest frequencies were obtained with covalently closed circular DNA. With all parameters optimized, the frequency was 10^{-3} transformants per viable cell or 10^4 transformants per µg of DNA. Cells previously frozen were also used as recipients in transformation experiments; such cells gave frequencies similar to those obtained with freshly grown cells. The procedure was optimized for *B. thuringiensis* subsp. *gelechiae*, but *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *galleriae*, *B. thuringiensis* subsp. *thuringiensis*, and *B. thuringiensis* subsp. *israelensis* were also transformed. Compared with protoplast transformation, our method is much faster and 3 orders of magnitude more efficient per microgram of added DNA.

Much of the recent literature on *Bacillus thuringiensis* has been focused on the crystalline δ-endotoxin and on the plasmid patterns (for reviews see references 2 and 7). The facts that the δ-endotoxin is plasmid encoded and that the gene has been cloned in *Escherichia coli* and *Bacillus subtilis* create a need for a method to reintroduce cloned material back into *B. thuringiensis*. Previously, transfer of genetic material in *B. thuringiensis* has been achieved by transduction (3, 11, 13, 14, 18, 20), by transformation of protoplasts, and by a "conjugation-like system" (2, 7). Previously described methods for transformation of *B. thuringiensis* have been based on a method developed for protoplasts of *B. subtilis* (8). Using plasmid pBC16 isolated from *Bacillus cereus* (4), Alikhanian et al. (1) transformed protoplasts of *B. thuringiensis* subsp. *galleriae*. Plasmid pC194, which originated from *Staphylococcus aureus*, has been transferred to protoplasts of *B. thuringiensis* subsp. *kurstaki* (15), as well as to some additional subspecies (9). Another *S. aureus* plasmid, pUB110, has also been used to transform protoplasts of *B. thuringiensis* subsp. *galleriae* (16). However, in all cases the numbers of transformants were very low, and the procedures were time consuming. Moreover, it has been difficult both to prepare protoplasts and to regenerate such protoplasts. For this reason we developed a plasmid transformation method which, compared with previously described procedures, is much more efficient and saves time.

In this paper we report transformation of vegetative cells of *B. thuringiensis*. We used plasmids pBC16 and pC194 with *B. thuringiensis* subsp. *gelechiae* as the recipient and with all parameters optimized obtained transformation frequencies of up to 10^{-3} (corresponding to 10^4 transformants per µg of DNA).

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MATERIALS AND METHODS

Bacterial strains, plasmids and media used. The *B. thuringiensis* strains used in this paper have been described previously (13). Strain Bt213 (*B. thuringiensis* subsp. *kurstaki*) is a spontaneous streptomycin- and nalidixic acid-resistant mutant of Bt2 (13). This strain was selected first on plates containing 100 µg of streptomycin per ml and then on plates containing 25 µg of nalidixic acid per ml. *B. subtilis* 151, containing plasmid pBC16 coding for tetracycline resistance, was provided by W. Goebel. *B. subtilis* containing plasmid pC194 expressing chloramphenicol resistance was provided by P. Martin. Bacteria were grown in minimal medium (20) which was prepared without glucose and was supplemented with 0.5% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.). When glucose was used, it was added to the minimal medium to a final concentration of 5 g/liter. Other growth media used were LB broth (13) and, for some experiments, brain heart infusion medium (Oxoid Ltd., London, England). LBP medium was a mixture (1:1) of phosphate buffer (0.2 M sodium-potassium phosphate, pH 6.4) and double-strength LB medium. The plates used for selection of transformants were LA plates (11) containing either tetracycline (25 µg/ml) or chloramphenicol (5 µg/ml). Tris hydrochloride buffer was prepared from Trizma (Sigma Chemical Co., St. Louis, Mo.). Phosphate buffer and a 40% polyethylene glycol 6000 solution (BDH Chemicals Ltd., Poole, England) were prepared as described previously (19).

Isolation of plasmid DNA from *B. subtilis*. Cultures (200 ml) were grown overnight in LB medium containing either tetracycline (25 µg/ml) or chloramphenicol (5 µg/ml). Cells were harvested by centrifugation, washed once with TES (30 mM Tris hydrochloride, 5 mM disodium EDTA, 50 mM NaCl, pH 8.0), and suspended to a total volume of 8 ml in TES. To this suspension were added 1.0 ml of TES contain-

ing 5 mg of freshly dissolved lysozyme and 0.4 ml of TES containing 0.8 mg of preboiled RNase. The mixture was incubated for 30 min at 37°C. The bacteria were lysed by a 60-min incubation at 37°C with 1.4 ml of 8% (vol/vol) Triton X-100 and 0.6 ml of TES containing 3 mg of pronase K (predigested for 60 min at 37°C). Ethidium bromide (0.6 ml; 10 mg/ml) and solid CsCl were added to the lysate to give a final refractive index of 1.391. The DNA was purified by ultracentrifugation, and the concentration was determined by UV (260-nm) spectrophotometry. Generally, about 100 to 200 μg of purified plasmid DNA was obtained from a 200-ml culture.

Plasmid pBC16 DNA was cleaved with BamHI and ligated with T4 ligase according to the instructions of the manufacturer (New England BioLabs, Inc., Beverly, Mass.).

Standard procedure for transformation of B. thuringiensis by plasmid DNA. A 0.3-ml inoculum of an overnight culture was added to 10 ml of minimal medium supplemented with 0.5% Casamino Acids. The bacteria were grown with shaking at 37°C until mid or late logarithmic phase (80 to 175 Klett turbidity units, which corresponded to 1×10^7 to 5×10^7 CFU/ml). The bacteria were collected in a 12-ml glass tube by centrifugation at 3,700 rpm for 10 min at room temperature. All of the centrifugation procedures described below were carried out in the same way. The cells were washed with 5 ml of 50 mM Tris hydrochloride buffer (pH 7.5) and suspended in 50 mM Tris hydrochloride buffer containing 30% (wt/vol) sucrose (pH 8.9) to a density between 60 and 120 Klett turbidity units. A 5-ml portion of this suspension was transferred to a 50-ml Erlenmeyer flask and incubated for 35 min with slow shaking at 37°C. The bacteria were then collected by centrifugation and suspended in 0.5 ml of LBP medium. Plasmid DNA dissolved in 100 μl of a 1:1 mixture of TE buffer (50 mM Tris hydrochloride, 20 mM disodium EDTA, pH 8.0) and LBP medium was added to this suspension. After the addition of 1.5 ml of polyethylene glycol 6000 (40%, wt/vol) and gentle mixing, the bacteria were incubated for 10 min with slow shaking at 37°C. The cells were centrifuged, suspended in 1 ml of LB medium, and incubated for phenotypic expression at 37°C with slow shaking for 1.5 h. To isolate transformants, samples (0.02 to 0.2 ml) were spread in soft agar onto LA plates containing either tetracycline (25 μg/ml) or chloramphenicol (5 μg/ml). If phenotypic expression was determined on regular LA plates, an overlay of soft agar containing the appropriate antibiotic was added after incubation for 2.5 h at 37°C. Colonies were scored after incubation for 15 h at 37°C.

Freezing of bacteria to be used in transformation. By using a rotatory shaker at 37°C bacteria were grown to late logarithmic phase (140 to 190 Klett turbidity units) in minimal medium supplemented with 0.5% Casamino Acids. The culture was centrifuged for 10 min at 3,700 rpm, and the bacteria were concentrated about five times in LB medium containing 10% (vol/vol) glycerol. Samples (1.5 ml) were frozen in liquid nitrogen and stored at −70°C. Before the bacteria were used in transformation experiments, they were washed once to remove the glycerol.

Detection of plasmid DNA in transformants. Plasmid DNA was isolated by a modification of the protocol of Hansen and Olsen (10). Bacteria were grown in 10 ml of LB medium at 37°C to stationary phase, washed once in TE buffer at room temperature, and suspended in 0.3 ml of TE buffer containing 25% (wt/vol) sucrose (pH 8.0). To these cells we added 0.2 ml of TE buffer containing 1 mg of freshly dissolved lysozyme and 50 U of mutanolysin (Sigma). The suspension was incubated for 40 min at room temperature. After the

TABLE 1. Effects of pH and divalent cations on transformation frequency

pH of Tris hydrochloride–20% sucrose	Divalent cation addition	Transformation frequency[a]
7.50	None	$<1.0 \times 10^{-8}$
8.00	None	5.0×10^{-7}
8.45	None	1.6×10^{-6}
8.75	None	2.8×10^{-6}
8.90	None	5.1×10^{-6}
9.50	None	2.3×10^{-6}
8.90	20 mM Mg^{2+}	2.1×10^{-8}
8.90	20 mM Ca^{2+}	$<4.0 \times 10^{-8}$

[a] The recipient was strain Bt13, and the DNA used was plasmid pBC16 coding for tetracycline resistance. The transformation frequency was calculated as the number of transformants per viable cell. In each transformation mixture there was 2×10^7 CFU, and 5 μg of pBC16 DNA was used. In experiments in which the pH was 7.50 or the buffer was supplemented with Mg^{2+}, 10^8 viable cells were used.

addition of 0.2 ml of 250 mM disodium EDTA (pH 8.0), the mixture was put on ice for 10 min. The bacteria were lysed by adding 0.5 ml of 15% sodium dodecyl sulfate and heating for 5 min in a 55°C water bath with gentle inversions of the tube. After treatment with RNase (final concentration, 50 μg/ml) for 5 min at room temperature, the membrane-bound chromosomal DNA was precipitated with 0.5 ml of 5 M NaCl, and the mixture was kept on ice overnight. The precipitated chromosomal DNA was spun down, and the supernatant, containing mainly plasmid DNA, was concentrated by adding polyethylene glycol 6000 (42%, wt/vol, in 10 mM phosphate buffer, pH 7.0) to a final concentration of 10%. The mixture was left on ice for at least 6 h, after which the DNA was collected by centrifugation and suspended in 25 μl of water. Plasmid DNA was analyzed on a 0.6% (wt/vol) agarose gel.

RESULTS

Need for a Tris-sucrose treatment. Except when otherwise stated, in all transformation experiments we used plasmid pBC16, which codes for tetracycline resistance. The recipient, B. thuringiensis strain Bt13, was first treated with Tris hydrochloride buffer as described by Takahashi et al. (19), but with a reduced incubation time. This procedure gave only a few tetracycline-resistant transformants. When the pH was varied, the highest transformation frequency, 5.1×10^{-6} transformant per viable cell (transformation frequency was always calculated as the number of transformants per viable cell), was obtained at pH 8.9 (Table 1), a somewhat higher pH than that used for transformation of Bacillus brevis (19). The increase in frequency was not due to a decrease in the number of viable cells, which was the case for B. brevis. When the bacteria were treated with Tris hydrochloride buffer containing 20 mM MgCl₂ or 20 mM CaCl₂, the transformation frequency decreased by 2 orders of magnitude.

Sucrose added to the Tris hydrochloride buffer increased the transformation frequency from 2.5×10^{-8} to about 2×10^{-5} transformant per viable cell (Fig. 1). A plateau was reached when the final sucrose concentration was over 25%. Microscopic observations showed some protoplast formation after centrifugation of the Tris-sucrose-treated cells. In 20% sucrose there were about 15% protoplasts, compared with 5% protoplasts in 30% sucrose.
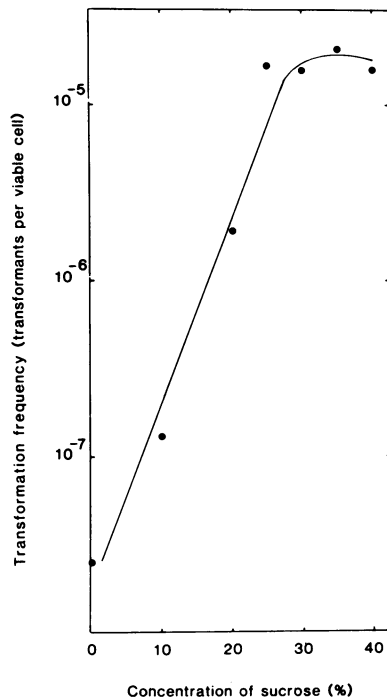
FIG. 1. Effect of sucrose on induction of competence. Bacteria were treated for 35 min with Tris hydrochloride buffer (pH 8.9) containing different concentrations of sucrose. The recipient, strain Bt13, had been grown in minimal medium containing 0.5% Casamino Acids. In each transformation mixture 5 μg of pBC16 DNA, which conferred tetracycline resistance, was used. Each value represents the average from two experiments.

The optimum time of incubation in Tris hydrochloride buffer containing 30% sucrose was 35 min (Fig. 2). Experiments performed with bacteria previously frozen gave a similar optimum.
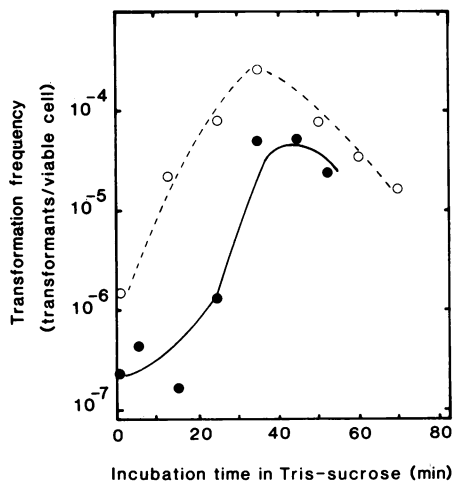

FIG. 2. Time dependence for induction of competence by treatment with Tris hydrochloride buffer (pH 8.9) containing 30% sucrose. The recipient was strain Bt13, which was either freshly grown in minimal medium containing 0.5% Casamino Acids (●) or frozen before being treated with Tris-sucrose buffer (O). In each transformation mixture 5 μg of pBC16 DNA, which conferred tetracycline resistance, was used.

TABLE 2. Effect of DNA configuration on the transformation frequency[a]

pBC16 DNA	No. of transformants per 5 μg of DNA	Transformation frequency
CCC	854	2.0×10^{-5}
Multimers	114	2.7×10^{-6}
Linear	8	1.9×10^{-7}
No DNA	2	4.8×10^{-8}

[a] The recipient was strain Bt13, and the total number of viable cells in each transformation mixture was 4.2×10^7. The bacteria were grown in LB medium and were treated with Tris hydrochloride buffer (pH 8.9) containing 20% sucrose. The transformation frequency was calculated as the number of tetracycline-resistant transformants per viable cell. Plasmid pBC16 (CCC form) was linearized by cleavage with endonuclease BamHI.

Transformation frequency depends on DNA concentration and configuration. There was a linear dependence over a 20-fold concentration range of added plasmid DNA. Saturation was slowly approached with 10 μg of pBC16 DNA (data not shown). Based on these results, we used in all subsequent transformation experiments 5 μg of DNA in a total volume of 0.5 ml. Table 2 shows that the highest transformation frequency, 2.0×10^{-5} transformant per viable cell, was obtained with covalently closed circular (CCC) DNA. Linear plasmid pBC16 cleaved with BamHI endonuclease had almost no transformation activity. BamHI cleaves this plasmid at only one site, which is outside the gene coding for tetracycline resistance (17). The religated plasmid DNA, which contained a majority of multimeric forms (as judged by agarose gel electrophoresis), showed a 10-fold decrease in transformation frequency compared with the CCC DNA. Transformation experiments performed with chromosomal DNA in which we selected for resistance to streptomycin and rifampin gave no transformants (data not shown).

Effect of growth medium on transformation frequency. When recipient strain Bt13 was cultivated in LB medium or brain heart infusion medium, the transformation frequency was 10^{-5} transformant per viable cell (Table 3). Minimal medium supplemented with Casamino Acids gave a frequency which was at least 1 order of magnitude higher. Not only the frequencies but also the numbers of transformants were clearly higher (about 35 times) when the bacteria were grown in minimal medium. Addition of glucose to minimal medium decreased the frequency by about 2 orders of magnitude compared with medium without glucose.

Influence of culture density and growth phase on the transformation frequency. The culture density was varied between 15 and 300 Klett turbidity units during the Tris-sucrose treatment (Fig. 3). Early and late logarithmically growing bacteria were used. When these cultures were

TABLE 3. Effect of the growth medium on the transformation frequency

Medium	Addition(s)	No. of transformants per 5 μg of DNA	Transformation frequency[a]
Minimal	0.5% Casamino Acids	10,500	1.5×10^{-4}
Minimal	0.5% Casamino Acids and 0.5% glucose	830	4.4×10^{-6}
Brain heart infusion	None	318	1.0×10^{-5}
LB	None	288	9.0×10^{-6}

[a] The recipient was strain Bt13, and the plasmid used was pBC16. The transformation frequency was calculated as the number of tetracycline-resistant transformants per viable cell.
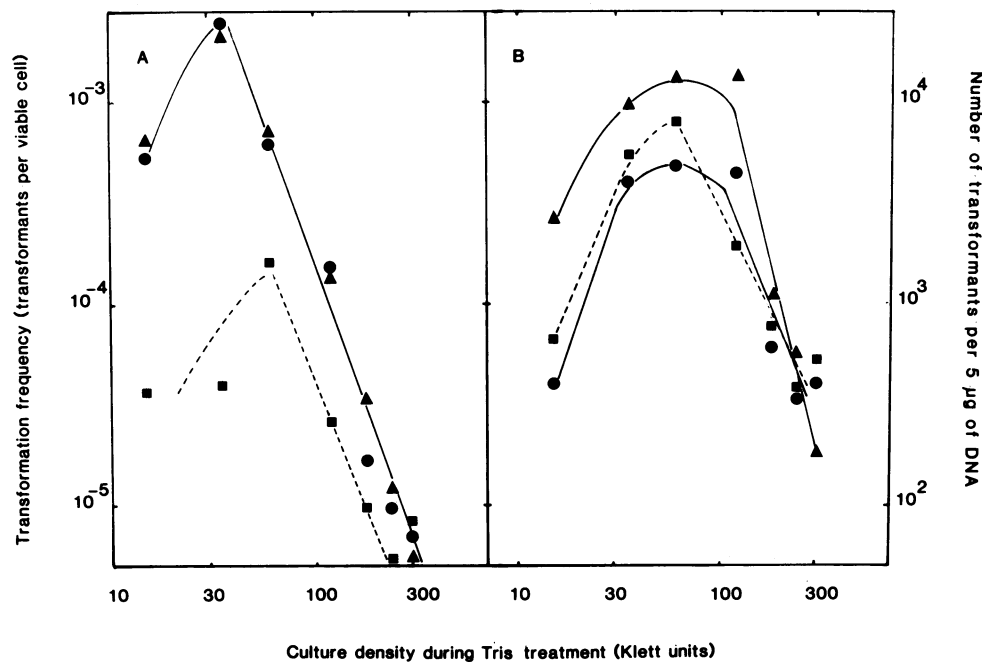
Culture density during Tris treatment (Klett units)

FIG. 3. (A) Effect of culture density on induction of competence by the Tris-sucrose treatment. The recipient was strain Btl3. Early-log-phase bacteria (●) were harvested at 60 Klett turbidity units. Late-log-phase bacteria (▲) were harvested between 120 and 175 Klett turbidity units. The density of stationary-phase bacteria (■) was 240 Klett turbidity units. (B) Number of tetracycline-resistant transformants per 5 μg of pBC16 DNA. Each value is the average from three experiments.

adjusted to the different densities, a sharp peak was obtained with a maximum transformation frequency of 2×10^{-3} transformant per viable cell at 35 Klett turbidity units. If stationary-phase bacteria were treated in the same way, the maximum was around 60 Klett turbidity units. The highest number of transformants (1.3×10^4 transformants per 5 μg of DNA) was obtained if late-log-phase bacteria were adjusted to densities between 35 and 120 Klett turbidity units. Transformation of an overnight culture of strain Btl3 gave less than 10 transformants per μg of pBC16 DNA.

Importance of low culture density during DNA uptake. We determined whether the culture density was important during the Tris-sucrose treatment or during some later step in the transformation protocol (Table 4). Regardless of the densities used during the Tris-sucrose treatment, the highest transformation frequencies were obtained if the culture density during DNA uptake was low. Higher densities decreased the frequency by 2 orders of magnitude. These experiments were performed with cells that were previously frozen. Compared with freshly grown cells, where a maximum transformation frequency was obtained at a density

corresponding to 35 Klett turbidity units (as shown in Fig. 3), no such maximum was found for frozen cells. Instead, a plateau was reached when the culture density was decreased further (data not shown). Varying the culture density during phenotypic expression had no effect on the transformation frequency (data not shown).

If the polyethylene glycol was omitted, no transformants were obtained (data not shown). The length of treatment with polyethylene glycol was not critical. Similar transformation frequencies were obtained with bacteria incubated in polyethylene glycol from 5 up to 30 min.

Phenotypic expression on LA plates. The selected marker on pBC16 was tetracycline resistance, which requires time for phenotypic expression. For this purpose we normally used liquid medium in which the shortest time for obtaining optimal transformation frequency was 1 h (Fig. 4). Transformants could also be obtained after phenotypic expression on regular LA plates. In this case the time needed for expression of antibiotic resistance was 2 h (Fig. 4).

Transformation with pC194 DNA and transfer of pBC16 DNA to different *B. thuringiensis* subspecies. We also per-

TABLE 4. Transformation frequency as a function of culture density during Tris-sucrose treatment and during uptake of DNA[a]

Culture density during Tris-sucrose treatment (Klett units)	Transformation frequency as a function of culture density during DNA uptake[b]		
	12 Klett units	60 Klett units	300 Klett units
12	9.3×10^{-4} (495)[c]	NT[d]	NT
60	1.3×10^{-3} (918)	8.9×10^{-5} (340)	3.0×10^{-6} (48)
300	1.6×10^{-3} (835)	3.4×10^{-5} (583)	5.1×10^{-6} (108)

[a] The recipient, strain Btl3, was frozen before Tris-sucrose treatment. The transformation frequency was calculated as the number of tetracycline-resistant transformants per viable cell.

[b] Different cell densities were obtained by either diluting or concentrating the culture to the Klett unit values indicated.

[c] The numbers in parentheses are numbers of transformants per 5 μg of pBC16 DNA.

[d] NT, Not tested.

formed transformation experiments with plasmid pC194 by using 5 μg of pC194 DNA and strain Bt13 as the recipient. In one experiment 1.5×10^4 transformants were obtained, which corresponded to a frequency of 1.8×10^{-4} transformant per viable cell. Preparation of total plasmid DNA from six of these chloramphenicol-resistant transformants and electrophoretic analysis on agarose gels revealed the 2.7-kilobase band corresponding to pC194. The plasmid was stably maintained and could be detected in transformants grown in the absence of chloramphenicol (data not shown).

Transformation frequencies were also determined for four other subspecies of *B. thuringiensis* (*B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *galleriae*, *B. thuringiensis* subsp. *thuringiensis*, and *B. thuringiensis* subsp. *israelensis*) (Table 5). The frequency obtained for strain Bt13 (*B. thuringiensis* subsp. *gelechiae*) was 2 to 3 orders of magnitude higher than the frequencies obtained for the other subspecies tested. Strain Bt213 (*B. thuringiensis* subsp. *kurstaki*) gave the highest transformation frequency with Tris hydrochloride buffer containing 35% sucrose. Strain Bt3 (*B. thuringiensis* subsp. *galleriae*) was grown in minimal medium supplemented with both Casamino Acids and 2% (vol/vol) LB medium.

To demonstrate the presence of pBC16 in transformants from the different subspecies, plasmid-enriched DNAs were isolated from four different strains and compared with a sample of purified pBC16 DNA by using agarose gel electrophoresis (data not shown). The recipient strains of *B. thuringiensis* subsp. *gelechiae*, *B. thuringiensis* subsp. *galleriae*, *B. thuringiensis* subsp. *israelensis*, and *B. thuringiensis* subsp. *kurstaki* lacked the 4.2-kilobase band of pBC16, whereas in the four transformants from these subspecies this band was clearly present. Plasmid pBC16 was stably maintained and was also detected in transformants which were grown without tetracycline (data not shown).

DISCUSSION

The method which we describe for transformation of vegetative cells of *B. thuringiensis* requires optimization of a number of different parameters. One of these is the induction

TABLE 5. Transformation of different subspecies of *B. thuringiensis*[a]

Strain	Subspecies	Transformation frequency
Bt13	*B. thuringiensis* subsp. *gelechiae*	1.5×10^{-3}
Bt213	*B. thuringiensis* subsp. *kurstaki*	7.3×10^{-5}
Bt3	*B. thuringiensis* subsp. *galleriae*	3.8×10^{-5}
Bt4	*B. thuringiensis* subsp. *thuringiensis*	4.1×10^{-5}
Bta1	*B. thuringiensis* subsp. *israelensis*	6.7×10^{-7}

[a] The transformation frequency was calculated as the number of tetracycline-resistant transformants per viable cell. In each transformation mixture 5 μg of pBC16 DNA was used.

of competence by treatment of the bacteria with Tris-sucrose. In *B. brevis* treatment with Tris buffer without sucrose induces competence due to the removal of two crystalline protein layers (S layers) from the outside of the cell wall (21). However, in *B. thuringiensis* no S layers are found (12). Thus, the mechanisms by which Tris treatment induces competence are different in the two species.

High concentrations of sucrose during the Tris treatment are necessary for a high transformation frequency (Fig. 1). Since incubation of bacteria in an isotonic medium has been reported to result in formation of protoplasts (2), we suspected that we might have protoplast transformation. However, this possibility was ruled out for the reasons described below. (i) Optimal transformation frequency was obtained with 30% sucrose, despite the fact that 20% sucrose gave somewhat more protoplasts. (ii) There was no increase in transformation frequency if experiments were performed with an osmotic support during the uptake of DNA and during phenotypic expression (data not shown). (iii) We obtained efficient transformation after phenotypic expression on regular LA plates (Fig. 4), conditions which do not allow protoplast regeneration. (iv) Transformants on LA plates could be scored after 7 to 8 h of incubation, whereas protoplast regeneration normally requires 2 to 3 days (15). For the reasons described above we are confident that we transformed vegetative cells.

The addition of glucose to the minimal medium decreased the transformation frequency by 2 orders of magnitude compared with medium without glucose. This suggests that in *B. thuringiensis* the synthesis of some factor(s) of importance for competence is repressed by glucose. The occurrence of glucose in the LB medium could also explain why this medium was less efficient than minimal medium containing Casamino Acids.

Table 4 shows that the same degree of competence was induced regardless of cell density during the Tris-sucrose treatment. These data also show that it was crucial to use a low culture density during the uptake of DNA. The decrease in number of transformants with higher cell densities could have been due to an observed aggregation of bacteria, which could have influenced the uptake of DNA.

In *B. subtilis* competence can only be induced during late log phase (5), whereas in *B. thuringiensis* there was a broad optimum between early and late logatithmic phases (Fig. 3A). However, the highest number of transformants was obtained if a late-log-phase culture was used (Fig. 3B).

Transformation of *B. subtilis* by plasmid DNA required multimeric forms, and CCC DNA was inefficient (6). We found that the CCC form of plasmid DNA was more efficient than linear forms of DNA (Table 2). Chromosomal DNA,
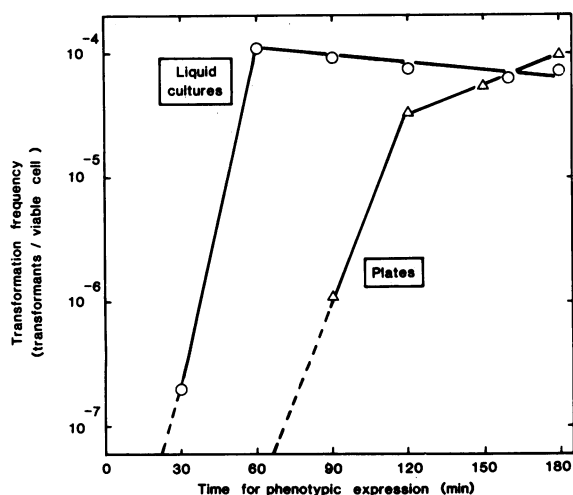

FIG. 4. Transformation frequency as a function of time for phenotypic expression in liquid LB medium (○) or on LA plates (△). The recipient strain was Bt13, and in each transformation mixture 5 μg of pBC16 DNA was used.

which is always linear, gave no transformants. The 10-fold decrease in frequency for the multimeric DNA probably reflects the lower number of molecules compared with CCC DNA. The transformation protocol worked well for two different plasmids, and the method is potentially suitable for cloning experiments.

The transformation of vegetative cells of *B. thuringiensis* was optimized for *B. thuringiensis* subsp. *gelechiae*. For *B. thuringiensis* subsp. *kurstaki*, *B. thuringiensis* subsp. *galleriae*, and *B. thuringiensis* subsp. *thuringiensis* we obtained frequencies between 4×10^{-5} and 7×10^{-5} transformant per viable cell, (Table 5), results which probably can be improved by further study of the transformation protocol. These results were all obtained with cells which had been frozen. We observed that *B. thuringiensis* subsp. *kurstaki* frozen cells survived the Tris-sucrose treatment better than freshly grown cells.

Protoplast transformation of *B. thuringiensis* is very inefficient, with about 10 transformants per μg of plasmid DNA (1, 9, 15, 16). With our method for transformation of vegetative cells, we obtained about 1,000 times more transformants per microgram of DNA, which means that our frequencies are similar to those obtained for *B. subtilis* and *B. brevis* (6, 19). In addition to the high frequencies, our method has the advantage of being time saving because transformants can be scored after only 7 to 8 h and because frozen cells can be used.

ACKNOWLEDGMENT

ADDENDUM IN PROOF

An efficient transformation of protoplasts of *B. thuringiensis* subsp. *israelensis* was recently reported by Loprasert et al. (J. Invert. Pathol. 48:325–334, 1986).

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