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Transformation of *Bacillus thuringiensis* subsp. *galleria* Protoplasts by Plasmid pBC16

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Protoplasts of the entomopathogenic bacterium *Bacillus thuringiensis* subsp. *galleria* were transformed by plasmid pBC16. The frequency of transformation was much lower than that of *Bacillus subtilis*. All isolated *B. thuringiensis* transformants were characterized by increased sensitivity to lysozyme as compared with the original strain.

The ability of *Staphylococcus aureus* plasmids to be expressed in *Bacillus subtilis* (5, 6) as well as the examination of nonchromosomal elements of bacilli (1, 8) opened new approaches in studying these genera. Recently, cryptic plasmids have been discovered in the entomopathogen *Bacillus thuringiensis* (4, 10). The authors suggested that such plasmids might participate in the regulation of synthesis of toxic protein crystals and in spore formation. However, further experiments were difficult because of the absence of transformation. In this article we present data on the transformation of *B. thuringiensis* subsp. *galleria* 69-6 by plasmid pBC16, which determines resistance to tetracycline (1).

MATERIALS AND METHODS

Bacterial strains. *B. thuringiensis* subsp. *galleria* 69-6 (*V* serotype) was used for the production of entobacterin. *B. subtilis* 168 *trpC2* carrying plasmid pBC16 (Tc^r) and *B. subtilis* 168 HT (*his trpC2*) were kindly supplied by W. Goebel.

B. thuringiensis subsp. *galleria* 69-6 strains were examined: (i) for virulence to larvae of *Galleria melanella*; (ii) for sensitivity to specific phage g3 (2); and (iii) for the presence of crystals in sporulating cells (by microscopic examination).

Media. Nutrient broth and 1.5% nutrient agar were used. The medium for regeneration of protoplasts was prepared by the method of Chang and Cohen (3). Tetracycline was used at 100 μg/ml.

Plasmid DNA. Plasmid DNA was prepared by the procedure of Gryczan et al. (6), except that, for *B. thuringiensis*, lysozyme at a concentration of 15 mg/ml was used in the mixture containing 25% sucrose in 0.1 M Tris (pH 10.0) at 37°C for 30 min. Purification of plasmid DNA in a cesium chloride-ethidium bromide gradient and electrophoresis of DNA were performed as described previously (9).

Transformation procedure. Transformation of *B. subtilis* and *B. thuringiensis* protoplasts was carried out by the method of Chang and Cohen (3), except that, to obtain protoplasts, cells of *B. thuringiensis* were incubated for 3 h at 37°C in the presence of 15 mg of lysozyme per ml. Protoplast formation was followed under a light microscope.

Reagents. Lysozyme and polyethylene glycol 6000 were obtained from Serva.

RESULTS

The method of protoplast transformation used made it possible to transfer plasmid DNA into *B. subtilis* with high effeciency (3). The main objective was to obtain complete protoplasts, that is, removal of the whole cell wall. That would increase the probability of plasmid DNA penetration after treatment of the cells with polyethylene glycol.

It is known that the cell wall of *B. thuringiensis* is highly resistant to lysozyme (7). Therefore, a high concentration of lysozyme (15 mg/ml) was used to obtain the protoplasts. As lysozyme may irreversibly associate with DNA (11), it was necessary to wash it off the protoplasts after the treatment. Protoplasts may be obtained by the use of antimicrobial agents which block the synthesis of the cell wall. However, since *B. thuringiensis* 69-6 has a high level of resistance to such agents (e.g., penicillin derivatives), such an approach seemed of no use.

Transformants were selected on medium DM3, used for protoplast regeneration, in the presence of tetracycline. The frequency of transformation of *B. thuringiensis* by plasmid DNA was significantly lower compared with that of *B. subtilis* despite the fact that the level of survival of *B. thuringiensis* protoplasts was much higher than that of *B. subtilis* protoplasts (Table 1).

Extrachromosomal DNA was isolated from three tetracycline-resistant clones of *B. thuringiensis* and was compared with the plasmid DNA of the original *B. thuringiensis* strain and of the tetracycline-resistant transformants of *B.*

TABLE 1. *Frequency of transformation of B. subtilis and B. thuringiensis protoplasts by plasmid pBC16*

Protoplasts	Conditions of transformation	No. of regenerated protoplasts in 1 ml of transformation medium	No. of transformants in 1 ml of transformation medium	Transformation frequency
B. subtilis 168 HT	Without plasmid DNA	3.6×10^6	0	
	pBC16 DNA (1 μg/ml)	6.8×10^5	1.3×10^5	2.3×10^{-1}
B. thuringiensis subsp. *galleria* 69-6	Without plasmid DNA	1.0×10^8	0	
	pBC16 DNA (1 to 5 μg/ml)[a]	8.1×10^7 to 4.0×10^7	17-12	2.3×10^{-7} to 3.0×10^{-7}

[a] Change in these concentrations of the plasmid DNA did not lead to an increase in transformation frequency.

subtilis HT. *B. thuringiensis* subsp. *galleria* 69-6 had three cryptic plasmids with molecular weights of more than 40×10^6 (data obtained by electrophoresis), 10.5×10^6 (data obtained by electrophoresis and electron microscopy) and 5.2×10^6 (data obtained by electrophoresis and electron microscopy), whereas the *B. thuringiensis* tetracycline-resistant clones acquired additional plasmid DNA with a molecular weight of 2.8×10^2 (pBC16) (Fig. 1). Specific phage g3 grew on the lawn of *B. thuringiensis* transformants, and the sporulating cells of these transformants produced crystal virulent for larvae of *G. melanella*. All transformants retained tetracycline resistance through at least 30 generations of growing in the absence of the antibiotic.

During the course of plasmid DNA isolation, it was found that all *B. thuringiensis* transformants were highly sensitive to lysozyme. Thus, we were able to use this agent in further experiments at a concentration of 5 mg/ml instead of 15 mg/ml for the isolation of plasmid DNA from the original strain. The plasmid DNA isolated from *B. thuringiensis* 69-6(pBC16) clones was transformed into *B. thuringiensis* 69-6 and *B. subtilis* HT protoplasts. For both strains, tetracycline-resistant transformants were again selected. However, the transformation frequency of *B. thuringiensis* subsp. *galleria* 69-6 protoplasts by plasmid pBC16 was the same, whereas that of the *B. subtilis* protoplasts was 10 times less, compared with the transformation frequencies of the strains by plasmid pBC16 isolated from *B. subtilis* 168.

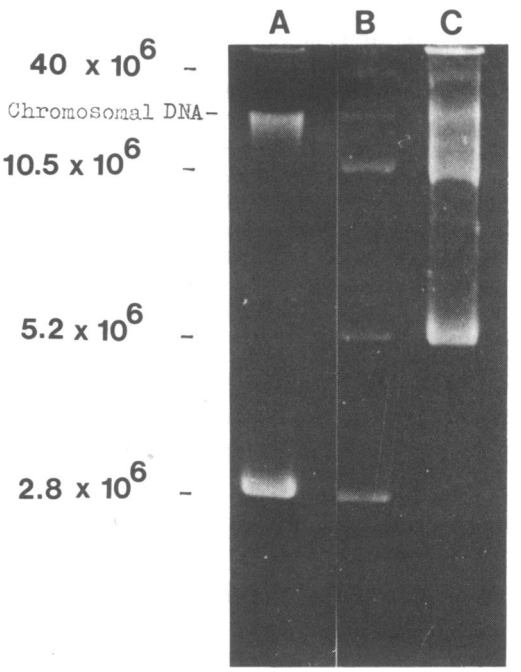

FIG. 1. *Agarose gel demonstrating the presence of pBC16 in B. thuringiensis subsp. galleria 69-6. Horizontal slab electrophoresis was carried out at 30 V for 16 h in 1% agarose gels. The molecular weights of DNA were calculated by comparison with the mobility of different plasmid DNAs with known molecular weights. The molecular weights of two cryptic plasmids of B. thuringiensis (5.2×10^6 and 10.5×10^6) were measured by using an electron microscope as well. (A) Plasmid pBC16 isolated from B. subtilis. (B and C) Patterns of plasmid DNA isolated from tetracycline-resistant transformants and the original strain of B. thuringiensis, respectively.*

DISCUSSION

Plasmid pBC16 was originally isolated from *Bacillus cereus* (1), a strain related to *B. thuringiensis*. Therefore, we expected replication and expression of pBC16 in *B. thuringiensis* cells. Our experiments showed that pBC16 DNA can be transferred into *B. thuringiensis* subsp. *galleria* by transformation and be stably maintained in the new host. Plasmid pBC16 is convenient for use in experiments on transformation because it determines resistance to those doses of antibiotic which entirely suppress the growth of *B. thuringiensis* subsp. *galleria* 69-6; *Staphylococcus aureus* plasmids do not have such a character (data not presented).

The low frequency of transformation of *B. thuringiensis* as compared with that of *B. subtilis* may be due to the following. (i) Plasmid DNA penetrates the cells with low efficiency because of incomplete protoplast formation as a

result of the high resistance of the *B. thuringiensis* cell envelope to lysozyme. (ii) Considerable degradation of plasmid DNA by a restriction enzyme(s) occurs after its introduction into *B. thuringiensis* cells. The data on the low retransformation frequency of *B. thuringiensis* protoplasts by plasmid pBC16 isolated from tetracycline-resistant *B. thuringiensis* 69-6 excluded the second suggestion, whereas the high level of regeneration of *B. thuringiensis* protoplasts on DM3 medium confirmed the first suggestion. Undoubtedly, the causes of the low frequences of transformation require further elucidation.

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