APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1989, p. 1649–1652
0099-2240/89/061649-04$02.00/0

Vol. 55, No. 6

Transformation of *Bacillus cereus* Vegetative Cells by Electroporation

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Received 15 December 1988/Accepted 28 March 1989

Transformation of untreated vegetative cells of *Bacillus cereus* 569 with plasmid pC194 (1.8 megadaltons) by high-voltage electroporation resulted in a maximum of 2×10^{-5} transformants per viable cell. Transformation of a 130-megadalton plasmid occurred at a comparable frequency. The method was simple, rapid, and yielded transformant colonies in 14 to 24 h. Transformation was obtained with unpurified total plasmid DNA.

Within the genus *Bacillus*, three species, *B. anthracis*, *B. cereus*, and *B. thuringiensis*, are closely related (2, 4, 15). The primary criteria used to classify an organism as *B. cereus* or as *B. thuringiensis* or *B. anthracis* is the inability to produce the insecticidal protoxin or anthrax toxin, respectively. Taxonomic classification of species or subspecies within this group is confused at the present time because various characteristics used to make such determinations (i.e., flagellar H antigens, crystal toxin shape, size, and antigens, and/or plasmid profiles) yield conflicting results (2, 4). Many factors may be responsible for the discrepancies, but the major factor is probably the ability of the *B. anthracis-B. cereus-B. thuringiensis* group to exchange and rearrange genetic information (4). The methods for genetic exchange of chromosomal and plasmid traits in this group of organisms have been restricted to generalized transduction (25, 30), transformation of protoplasts and autoplasts (19, 20, 24), and a conjugationlike transfer process (3, 4, 6, 19).

B. cereus 569 is a useful recipient in the study of plasmid and chromosomal genes of *B. thuringiensis*. This strain expresses a variety of protoxin-encoding plasmids from *B. thuringiensis* (3, 4, 6, 13) and can replicate and express pBC16, a 2.8-megadalton (MDa) tetracycline resistance plasmid originally isolated from *B. cereus* GP7 (7), and pC194, a 1.8-MDa chloramphenicol resistance plasmid originally isolated from *Staphylococcus aureus* (10). In addition, chromosomal auxotrophic markers have also been transferred from several subspecies of *B. thuringiensis* to *B. cereus* 569 by transduction and gene transfer via cell mating, (3, 30). A previously described method for transformation of *B. cereus* 569 is based on a method developed for competent cells of *Bacillus subtilis* (30). Using chromosomal DNA isolated from a streptomycin-resistant mutant of *B. cereus* 569, Felkner and Wyss (11) transformed competent cells of *B. cereus* 569. However, this technique has not been employed to introduce plasmids from *B. thuringiensis* or other bacilli into this species.

A novel approach for transferring DNA into protoplasts or intact cells of both gram-positive and gram-negative bacteria is electroporation. This system involves the use of short, high-voltage electric discharges through a suspension of cells to induce reversible permeabilization of the cell membrane, as a result of which DNA can enter (9). Neumann et al. (22) suggested that external electric fields interact with lipid dipoles of membrane pores, thereby inducing and stabilizing penetration sites for transport of extracellular DNA across the membrane. Applications of this technique originally involved the transformation of mammalian cells and plant and yeast protoplasts (27). In an early study by Shivarova et al. (28), total plasmid DNA from *B. thuringiensis* subsp. *galleriae* harboring pUB110, a kanamycin-neomycin resistance plasmid, was used to transform protoplasts of *B. cereus* 9592 by high-voltage electroporation. The frequency of transformation obtained by electroporation was 10-fold higher (10^{-3}) than that in nonelectroporated protoplasts. Electroporation has been used to introduce plasmid DNA into bacterial species that are either poorly transformable or nontransformable by conventional methods. Among gram-positive species, transformation of intact cells by electroporation has included *Streptococcus cremoris* (23), *Streptococcus lactis* (23), *Lactobacillus casei* (9), *Lactobacillus plantarum* (5), and *Clostridium perfringens* (1). In the present report we describe a rapid, efficient method for the transformation of intact *B. cereus* 569 cells with plasmid DNA.

A derivative of *B. cereus* 569 (UM20-1; streptomycin-resistant, anthranilic acid auxotroph) was kindly provided by C. B. Thorne, University of Massachusetts, Amherst. *B. subtilis* 1E17, containing plasmid pC194, was obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus. Stock cultures were grown at 30 or 37°C on LB agar (Difco Laboratories, Detroit, Mich.) containing either streptomycin sulfate for the selection of *B. cereus* 569 UM20-1 or chloramphenicol (30 μg/ml) for the selection of *B. subtilis* 1E17 and stored at 4°C.

Plasmid pC194 was extracted from *B. subtilis* 1E17 by a combined adaptation of the procedures described by Battisti et al. (6), Kado and Liu (18), and Gonzalez and Carlton (14). Cells for plasmid extraction were grown in 10 ml of brain heart infusion broth (Difco) supplemented with 20 μg of chloramphenicol per ml. Cultures were incubated for 16 h at 37°C at 100 rpm. Cells from 1.5 ml of culture were collected by centrifugation at 15,600 × *g* for 5 min at room temperature in a microcentrifuge and suspended in 0.1 ml of E buffer (40 mM Tris hydrochloride, 2 mM EDTA [disodium salt], 6.7% [wt/vol] sucrose [pH 7.9]) containing lysozyme (2 mg/ml) by gentle vortexing. Cell suspensions were incubated for 15 min at 37°C to generate spheroplasts. Cells were lysed by adding 0.2 ml of lysis buffer, prepared by the addition of 3 g of sodium dodecyl sulfate and 5 ml of 3 N NaOH to 100 ml of 6.7% (wt/vol) sucrose in 50 mM Tris hydroxide, to the spheroplast suspension. The tube contents were inverted 20 times and placed in a 60°C water bath for 20 min. Cell lysates

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were neutralized with 0.1 ml of 2 M Tris hydrochloride (pH 7.0), and the tube contents were mixed as described above and incubated at 37°C for an additional 20 min. Sodium chloride (5 M in distilled H_2O) was added in 50-μl quantities to the lysates, which were mixed gently and stored at 4°C for 1 h. The sodium dodecyl sulfate-protein complexes and most chromosomal DNA were pelleted by centrifugation at 15,000 × *g* for 5 min at 4°C. The supernatant fluids, containing plasmid DNA, were pipetted into sterile tubes. RNA was removed by treating the cleared lysates with 5 μl of RNase A (10 mg/ml in TES buffer [30 mM Tris hydroxide, 5 mM disodium EDTA, 50 mM NaCl, pH 8.0; Sigma]) (final concentration, 100 μg/ml). The lysates were mixed gently and incubated for 15 min at 37°C. Twenty microliters of proteinase K (1 mg/ml in sterile distilled H_2O) was added (final concentration, 50 μg/ml), and the lysates were incubated for an additional 15 min at 37°C. The lysates were extracted once with 0.6 ml of 3% (wt/vol) NaCl-saturated phenol by inverting the tubes 40 times. The emulsions were centrifuged at 15,000 × *g* for 5 min at 4°C, and the aqueous phases were removed and extracted twice with an equal volume of chloroform-isoamyl alcohol (24:1 [vol/vol]). The aqueous phases from six extracts were pooled into one 40-ml polypropylene tube, and the DNA was precipitated with 2 volumes of absolute ethanol at −20°C for at least 12 h. The DNA was centrifuged at 17,300 × *g* for 20 min at 4°C, and the pellet was rinsed with 75% ethanol to remove any remaining NaCl. The pellets were air dried and dissolved in 0.1 ml of TE buffer (10 mM Tris hydrochloride, 1 mM disodium EDTA [pH 7.0]). Chromosomal DNA remaining in the plasmid preparations was denatured by the method of Jarrett (17). Dissolved DNA samples were placed in a boiling water bath for 3 min and cooled on ice for 5 min to randomly renature the denatured DNA. Covalently closed plasmids are not denatured under these conditions and remain biologically active (17).

Intact cells of *B. cereus* 569 UM20-1 were transformed with plasmid pC194 by using the Gene Pulser electroporation apparatus (Bio-Rad Laboratories, Richmond, Calif.). Various field strengths (2,500 to 6,250 V/cm) were tested at capacitance settings of 3 and 25 μF to determine optimum transformation conditions. Cells for electroporation were grown in 10 ml of LB broth (Acumedia Manufacturers Inc., Baltimore, Md.) and incubated for 16 h at 37°C with shaking at 100 rpm. Two milliliters of the 16-h culture was transferred to 10 ml of fresh LB broth and further incubated at 37°C with shaking at 100 rpm. Culture turbidity was monitored at 600 nm until it reached an optical density of 1.0 (2.5 h; 2.2×10^8 CFU/ml). Cells were collected by centrifugation at 4,000 × *g* for 10 min at 4°C, washed three times in 1 ml (0.1 volume) of ice-cold electroporation buffer (10 mM *N*-2-hydroxymethylpiperazine-N-2-ethanesulfonic acid [HEPES] [pH 7.0]) and resuspended in 4 ml (0.4 volume) of the same buffer to yield 5.5×10^8 CFU/ml. The cells were kept on ice for no longer than 30 min before electroporation. A 0.8-ml volume of cell suspension was transferred to a small sterile glass test tube, and 30 μl of DNA mixture containing 0.5 μg of the desired plasmid was added, mixed gently with the cells, and kept on ice for 5 min.

The entire sample was pipetted into a prechilled Gene Pulser cuvette (0.4-cm interelectrode gap) and subjected to single-pulse electroporation. Immediately after administration of the pulse, the transformation mixture was dispensed into 7.2 ml (10^{-1} dilution) of LB broth and incubated at 30°C for 1 h with shaking at 60 rpm to allow expression of the plasmid-encoded resistance determinant and recovery of the electroporated cells. Prior to the expression period, a sample (0.1 ml) was serially diluted in peptone diluent (1 g/liter; Difco) and plated on LB agar plates to determine the number of CFU per milliliter that survived electroporation. After recovery, cells were diluted in peptone diluent and plated on Difco LB selective agar plates containing 10 μg of chloramphenicol per ml for the selection of transformants. To determine the frequency of mutation to chloramphenicol resistance, cells were pulsed without transforming DNA. All agar plates were incubated at 30°C, and CFU were enumerated after 24 h.

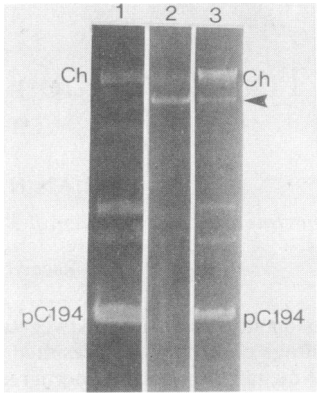

FIG. 1. Detection of plasmid DNA by agarose gel electrophoresis. Lanes: 1, *B. subtilis* 1E17 harboring plasmid pC194; 2, *B. cereus* 569 UM20-1 recipient; 3, *B. cereus* 569 UM20-1 transformant. Twenty transformants were examined, and all yielded a plasmid profile identical to that in lane 3. The arrowhead indicates a plasmid native to *B. cereus* 569 UM20-1, with an estimated molecular mass of 12 MDa. Ch, Chromosomal DNA.

Plasmid pC194 transformed *B. cereus* 569 UM20-1 to chloramphenicol resistance at high frequencies. Transformation frequency was calculated as the number of transformants per recovered cell (26). Chloramphenicol-resistant colonies were observed only with cells electroporated with plasmid DNA. Plasmid DNA was isolated from transformants by the method described above but with the following modifications: (i) the RNase and proteinase K treatments were eliminated, and (ii) the DNA from 1.5 ml of cells was directly precipitated in Microfuge tubes with 2 volumes of absolute ethanol, and the resulting DNA pellet was dissolved in 10 μl of TE buffer and mixed with 5 μl of tracking dye (0.25% bromophenol blue, 40% [wt/vol] sucrose). Total samples were applied to horizontal 0.8% (wt/vol) agarose gels prepared and run in Tris borate electrophoresis buffer (89 mM Tris borate, 2.5 mM disodium EDTA, 89 mM boric acid [pH 8.2]). Electrophoresis was carried out at 6 V/cm for 3 h at room temperature. The gels were stained with ethidium bromide (0.5 μg/ml) and destained in distilled water prior to photography. The electrophoretic mobilities of the multiple forms of pC194 (i.e., monomers and multimers) isolated from *B. cereus* 569 UM20-1 transformants were identical to those observed with preparations isolated from *B. subtilis* 1E17 (Fig. 1).

Variation in field strength and capacitance had an effect on both cell survival and transformation frequency. At 2,500 V/cm and 25 μF, 68.9% of the starting CFU were recovered and the transformation frequency was low (Table 1). Increasing the field strength to 3,750 and 5,000 V/cm at 25 μF lowered the survival to 5.7 and 0.9%, respectively, but no transformants were observed. Decreasing the capacitance to

TABLE 1. *B. cereus* 569 UM20-1 survival under various electroporation conditions and transformation with pC194 DNA[a]

Field strength (V/cm)	Capacitance (μF)	Time constant (ms)	CFU rec[b]	CFU obs[c]	% Survival ([CFU rec/CFU tot[d]] × 100)	Transformation frequency (CFU obs/CFU rec)
2,500	25	19.3	3.8×10^8	2.0×10^2	68.9	5.3×10^{-7}
3,750	25	17.1	3.1×10^7	0	5.7	0
5,000	25	15.2	5.2×10^6	0	0.9	0
2,500	3	2.4	5.5×10^8	4.5×10^2	100.0	8.2×10^{-7}
3,750	3	2.7	2.6×10^8	5.1×10^3	46.4	2.0×10^{-5}
5,000	3	2.4	2.4×10^8	1.0×10^2	43.6	4.2×10^{-7}
6,250	3	2.4	2.7×10^7	2.0×10^2	5.0	7.4×10^{-6}

[a] Source of pC194 DNA was *B. subtilis* 1E17. All values are averages for two experiments.

[b] CFU rec, CFU per milliliter recovered after electroporation, dilution, and plating.

[c] CFU obs, CFU per milliliter on LB agar plates containing 10 μg of chloramphenicol per ml; equals number of recovered cells transformed by electroporation.

[d] CFU tot, CFU per milliliter used in the electroporation experiment, determined by dilution and plating on LB agar. Each electroporation mixture initially contained 5.5×10^8 CFU/ml.

3 μF and maintaining the field strength at 2,500 V/cm increased cell survival to 100%, with a transformation frequency of 8.2×10^{-7}. Increasing the field strength to 3,750 V/cm (3 μF) raised the transformation frequency to maximal levels while lowering the survival to 46.4%. Further increases in field strength resulted in both poorer survival and lower transformation frequencies.

Given the effects of field strength and capacitance on the survival and transformation frequency of *B. cereus* 569 UM20-1, the theoretical argument that the magnitude of an external electric field required to generate membrane pore formation is inversely related to cell size may apply to bacteria as well as eucaryotic cells. High-frequency transformation of eucaryotic cells by electroporation generally requires lower field strengths than those used for bacterial transformation (21). The field strengths necessary for maximal transformation of *Escherichia coli*, *Pseudomonas putida*, *L. casei*, *L. plantarum*, and *Campylobacter jejuni* range from 5,000 to 13,000 V/cm, and capacitances range from 25 to 50 μF (5, 9, 12, 21). The results of the present study indicate that for larger-size *B. cereus* 569 UM20-1 intact cells, lower field strengths and capacitances (3,750 V/cm and 3 μF) may be required to obtain maximal transformation.

Total plasmid DNA isolated from a mercury-resistant *B. cereus* 5 strain (16) was also used to transform *B. cereus* 569 UM20-1. Electroporation-mediated transformation was performed as previously described except that only one field strength and one capacitance were used (3,750 V/cm and 3 μF) and transformants were selected on Difco LB agar containing 25 μM $HgCl_2$. A plasmid of approximately 130 MDa was transformed into *B. cereus* 569 UM20-1 at a frequency of 8×10^{-6} transformants per recovered cell. Mercury volatilizing ability was detected in all transformants assayed for mercuric reductase activity (16), suggesting that the inorganic mercury resistance determinant in *B. cereus* 5 is probably encoded on the 130-MDa plasmid. A report on the biology of this large plasmid is in preparation (B. H. Belliveau and J. T. Trevors). The present report is the first on the transformation of a *Bacillus* species with a relatively large plasmid (greater than 100 MDa), and it is also the first account of a plasmid larger than 40 kilobases (ca. 27 MDa; 9) being introduced into a bacterial host by high-voltage electroporation. Furthermore, very little is known about the disruptive effect of high-voltage electroporation on large plasmids and the effect of competing plasmids on electroporation efficiency.

The present study demonstrated that electroporation can be used to achieve genetic transformation of intact, untreated cells of *B. cereus* 569 UM20-1 by both small (pC194) and large (130-MDa) plasmids. The procedure is rapid, reproducible, easy to perform, and requires minimal sample preparation, and transformant colonies appear after 14 to 24 h of incubation. Highly purified plasmid DNA was not necessary to obtain high frequencies of transformation, which is in contrast to the purity of DNA necessary for protoplast (8) and competence (29) transformation systems. *B. cereus* 569 may become a useful host for studying plasmid-encoded traits and for broadening the range of useful hosts of plasmids. For example, the introduction of several insecticidal-protoxin genes (on one or more plasmids) into the same *B. cereus* 569 recipient, or possibly other *B. thuringiensis* strains once electroporation parameters are defined, could lead to the development of a multipurpose agent with, perhaps, increased toxicity and activity against lepidopteran, dipteran, and/or coleopteran insect pests. The major barrier to obtaining high-frequency transformation of *B. cereus* and *B. thuringiensis* seems to be an inability to efficiently employ conventional gene exchange techniques, including competent cell or protoplast transformation systems (4). Electroporation-induced transformation may be an alternative and efficient means to allow molecular genetic experiments with *B. thuringiensis* (and related species), whose present genetic potential as a host for plasmid vectors is only in the developmental stage, as are the techniques for its genetic manipulation.

ACKNOWLEDGMENTS

We thank W. J. Dower of Bio-Rad Laboratories for helpful suggestions. Sincere appreciation is expressed to S. Sprowl for typing the manuscript.

This work was supported by an operating grant to J.T.T. from the Natural Sciences and Engineering Research Council of Canada (NSERC). B.H.B. was a recipient of a postgraduate scholarship from NSERC.

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