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Efficient DNA Transformation of *Bradyrhizobium japonicum* by Electroporation

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Intact cells of *Bradyrhizobium japonicum* USDA 110 were transformed with a 30-kilobase plasmid to efficiencies of 10^6 to 10^7 transformants per μg by high-voltage electroporation. The technique was reliable and simple, with single colonies arising from transformed cells within 5 days of antibiotic selection. Plasmid DNA from *B. japonicum* transformed the *Bradyrhizobium* (*Arachis*) sp. with high efficiency, while the same plasmid extracted from *Escherichia coli* transformed *B. japonicum* at very low efficiency. The electrical conditions that resulted in the highest efficiencies were high voltage (10.5 to 12.5 kV/cm) and short pulse length (6 to 7 ms). A linear increase in the number of transformants was observed as DNA concentration was increased over 4 orders of magnitude; saturation appeared to begin between 120 ng/ml and 1.2 μg/ml. This novel method of transformation should enhance *B. japonicum* genetic research by providing a valuable alternative to conjugal mating, which is currently the only efficient, widely used means of introducing DNA into this organism.

Bradyrhizobium japonicum, a gram-negative soil bacterium, infects soybean roots and establishes a nitrogen-fixing symbiosis. An understanding of the bacterial genes involved in this process and of how the products of these genes interact will be an important step in increasing the efficiency of this complex relationship and ultimately in improving crop performance. However, this organism is genetically recalcitrant because of the limited availability of DNA transfer alternatives and its high level of resistance to a variety of antibiotics. The $CaCl_2$ transformation procedure developed for *Escherichia coli* (11) does not work for *B. japonicum*. In addition, a procedure which transforms several members of the family *Rhizobiaceae* (i.e., *Rhizobium meliloti* and *Agrobacterium tumefaciens*) (15) has not been shown to work with *B. japonicum*. Currently, conjugation is the only efficient and broadly used technique for introducing DNA into *B. japonicum*.

Electroporation of DNA into cells was first developed by Zimmerman (18) for eucaryotic cells but has subsequently found broad applicability for several bacterial species (4, 5, 13, 17) and plants (6, 16). This relatively simple technique is especially valuable for the study of bacteria, for which existing DNA transfer methods are unreliable (14), nonexistent (13), or less efficient (4). Electroporation also has the advantage (over conjugation) of directly cloning ligated DNA into the recipient strain, as has been demonstrated for *Streptococcus lactis* (14).

Although the exact mechanism of electroporation is not known, it is believed that the electric field polarizes the membrane components and results in a voltage potential across the membrane. When the potential exceeds a threshold level, the membrane breaks down, resulting in localized reversible openings which allow the passage of macromolecules (8, 9). Various cell types differ in the conditions (i.e., pulse length and field strength) required for efficient uptake (16). For example, smaller cells generally require higher field strengths than larger cells. This makes it necessary to test a range of conditions for a given organism if the procedure is to work with maximum efficiency.

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This paper describes conditions for reproducible high-efficiency electroporation of *B. japonicum* USDA 110.

MATERIALS AND METHODS

Bacterial cells and plasmid DNA. *B. japonicum* USDA 110 (wild-type) cells were grown in modified (0.025% yeast extract) AIE medium (10) to an A_{600} of 0.4 to 0.6 at 30°C with vigorous shaking (200 rpm) and prepared for electroporation by a modification of the procedure of Dower and co-workers (4). Cells (1 liter) were chilled for 15 to 30 min on ice and then centrifuged cold (4°C) at 9,820 × *g* for 10 min. Cells and solutions were maintained at 4°C for all of the following steps. Cells were suspended in 1 liter of sterile distilled water and washed by alternate centrifugation and resuspension in 0.5 liter of water and then 20 ml of 10% glycerol. Cells were then suspended in 3 ml of 10% glycerol (approximately 10^{12} cells per ml) and stored in 40-μl volumes at −70°C. Cells stored in this manner maintained electroporation efficiency for at least 2 months.

Plasmid pZB32 (1), a 30-kilobase (kb) plasmid which encodes tetracycline resistance and contains a *lacZ* translational fusion of the *B. japonicum nodY* gene, was isolated from *B. japonicum* USDA 110 and from *E. coli* MC1061 [*hsdR mcrB araD139*Δ(*araABC-leu*)*7679*Δ *lacX74 galU galK rpsL thi*] as previously described (12). Unless otherwise indicated, all cells were transformed with plasmid pZB32 DNA, which was isolated from *B. japonicum* USDA 110 and purified on a CsCl gradient (12). DNA was suspended in 1× or 0.1× TE buffer (10 mM Trizma base plus 5 mM EDTA adjusted to pH 7.4 with concentrated HCl). Plasmid concentration was determined by linearization with restriction enzymes followed by comparison of band intensity with bands of several other linear DNA species of known mass on ethidium bromide-stained 0.8% agarose gels.

Cell transformation. For electroporation, the Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) apparatus, which generates field strengths up to 12.5 kV/cm, was used with the 0.2-cm cuvette supplied by the manufacturer. Cells were removed from −70°C storage, thawed at room temperature, and placed on ice. Plasmid DNA (1 to 3 μl) was then mixed thoroughly with a 40-μl cell sample, placed on ice for 1 min,

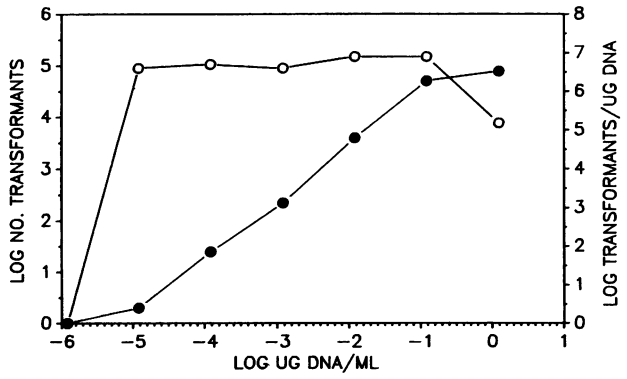

FIG. 1. Effect of DNA concentration on number of transformants (●) and transformation efficiency (○) of *B. japonicum* USDA 110. A quantity of 0.05 pg to 50 ng of pZB32 DNA (1.2 pg/ml to 1.2 μg/ml) was mixed with a 40-μl cell suspension and pulsed at 12.5 kV/cm (0.2-cm gap) with a 4.7- to 6.6-ms pulse length. The pulse was generated by a 25-μF capacitor and directed through a pulse controller (20 Ω in series, 200 Ω in parallel with the sample) prior to passing it through the sample. Transformants were selected on RDY plates with 150 μg of tetracycline per ml. Values shown are the means from four experiments.

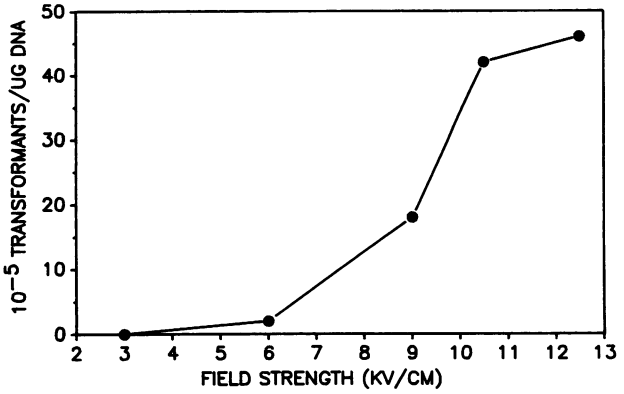

FIG. 2. Effect of field strength on transformation efficiency of *B. japonicum* USDA 110. Field strengths of 3, 6, 9, 10.5, and 12.5 kV/cm were generated by directing 0.6, 1.2, 1.8, 2.1, and 2.5 kV of electric discharge, respectively, from a 25-μF capacitor through a pulse controller and then through the cuvette with a 0.2-cm electrode gap. Pulse lengths of 4.7 to 6.6 ms were routinely obtained with cells suspended in 10% glycerol and the pulse controller (20 Ω in series with the sample) set at 200 Ω in parallel with the sample. Values shown are the means from three experiments.

and then transferred to a chilled cuvette. The pulse was applied, and cells were immediately suspended in 1 ml of cold (4°C) RDY (consisting of the minimal salts-vitamin base of Bishop et al. [2] containing 5 g of gluconic acid, 1 g of glutamic acid, and 1 g of yeast extract per liter) and placed on ice until all samples were pulsed. Cells were then incubated (200 rpm at 30°C for 20 h) and then dilution plated on nonselective (RDY) and selective (RDY + 150 μg of tetracycline per ml) media. CFU were scored after 5 to 7 days at 30°C. Controls consisted of cells from which pZB32, the pulse, or both (to measure pulse lethality) had been omitted prior to incubation in RDY and dilution plating. Values shown in the figures are averages from at least three determinations. The mean standard error for dual replicate plates of a single electroporation was 13%, while for electroporations done on different days with various cell and pZB32 plasmid preparations the mean standard error was 37%. Confirmation of plasmid transformation was obtained by assaying the activity of the *nodY-lacZ* fusion on pZB32 after induction with genistein (1).

RESULTS AND DISCUSSION

B. japonicum USDA 110 was successfully electroporated to a maximum efficiency of 10^7 transformants per μg with a 30-kb plasmid (Fig. 1). No colonies were ever observed on selective plates (150 μg of tetracycline per ml) on which control treatments (omission of the plasmid, pulse, or both) were plated. Shake incubation of cells in 1 ml of RDY for 4 versus 20 h resulted in the same number of transformants on selective plates. Because it was convenient, a 20-h incubation was used for all subsequent experiments. Highest efficiencies were obtained with field strengths of 12.5 kV/cm (Fig. 2), pulse lengths of 5 to 8 ms (Fig. 3), and DNA concentrations of 125 ng/ml (5 ng/40 μl of reaction mixture) (Fig. 1). However, DNA concentration (except at the highest and lowest concentrations tested) did not have a significant effect on efficiency, since only a twofold reduction in the number of transformants per microgram was observed at concentrations from 125 ng/ml to 12.5 pg/ml. The efficiency decreased nearly 100-fold between 125 ng/ml and 1.25 μg/ml, and no transformants were detected at 1.25 pg/ml (Fig. 1) (the lowest DNA concentration tested). The efficiency of transformation increased sharply with field strengths increasing from 6 to 10.5 kV/cm (5-ms pulse length) and then increased less rapidly with field strengths from 10.5 to 12.5 kV/cm (Fig. 2). No transformants were observed at a field strength of 3 kV/cm. Increasing the pulse length from 6.6 to 30.5 ms (12.5-kV/cm field strength) resulted in a 20-fold decrease in transformation efficiency (Fig. 3), while a decrease from 6.6 to 3.4 ms resulted in a 2-fold drop in efficiency. A linear relationship was observed between log DNA concentration and log number of transformants from 12.5 pg/ml (lowest concentration at which transformants were detected) to 125 ng/ml (Fig. 1). Between 125 ng/ml and

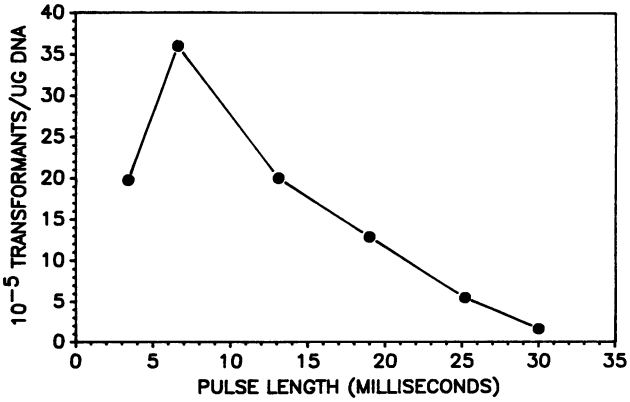

FIG. 3. Effect of pulse length on transformation efficiency of *B. japonicum* USDA 110. Pulse lengths of 3.4, 6.6, 13, 19, 25, and 30 ms were obtained by directing a 2.5-kV (12.5-kV/cm) electric discharge from a 25-μF capacitor through a pulse controller with 20 Ω in series with the sample and 100, 200, 400, 600, 800, and 1,000 Ω, respectively, in parallel with the sample (which was suspended in 10% glycerol in a cuvette with a 0.2-cm gap). Since pulse lengths varied slightly from pulse to pulse, mean pulse lengths are shown. Transformants were selected on RDY plates with 150 μg of tetracycline per ml. Values shown are the means from three experiments.

1.25 μg/ml, there was only a 50% increase in the number of transformants, with the maximum number of transformants being 7.74×10^4/40-μl sample at the highest DNA concentration tested. This is contrary to results obtained with other bacteria such as *E. coli* (4) and *Campylobacter jejuni* (13), in which a linear relationship with no sign of saturation was observed with DNA concentrations of up to 7.5 and 10 μg/ml, respectively. However, *S. lactis* behaves more like *B. japonicum* in that the yield of transformants increases linearly up to a DNA concentration of 1 to 1.25 μg/ml and then levels off as the concentration approaches 5 μg/ml (14). There could be several explanations for this, as mentioned by Shigekawa and Dower (16), including the presence of fewer permeabilized cells in the *Streptococcus* and *B. japonicum* preparations or the presence in the DNA preparation of deleterious chemicals (phenol, sodium dodecyl sulfate, EDTA, ethidium bromide, etc.) which could enter the cells during electroporation. Regardless, it appears that various bacterial species require different DNA concentrations to maximize electroporation efficiency.

The type of apparatus used in this experiment was previously used by Dower and co-workers (4) to electroporate *E. coli* to an efficiency of 10^{10} transformants per μg. Interestingly, the electrical conditions (field strength and pulse length) for efficient results with *E. coli* also resulted in the highest efficiency observed for *B. japonicum* (12.5-kV/cm field strength and 6.6-ms pulse length) (Fig. 2 and 3). However, under optimal conditions, much higher cell mortality (50 to 75%) was observed for *E. coli* than for *B. japonicum* (25%) (data not shown). This indicates that *B. japonicum* cells, like those of *C. jejuni* (13), are more resistant to high-voltage electric impulses than are mammalian cells (3), carrot protoplasts (6), yeast cells (7), and bacterial cells such as *E. coli* (4). However, cell survivability of *B. japonicum* was lowered to 25% as the pulse length was increased from 6 to 30 ms (at 12.5 kV/cm) but recovery of transformants dropped nearly 100-fold (data not shown). This indicates that different cell types vary in their responses to electric impulses and that the death of a large proportion of cells is not required for efficient transformation.

The combination of higher survival rates and lower numbers of transformants for *B. japonicum* resulted in much lower transformation frequencies (transformants per survivor) for *B. japonicum* (9×10^{-7}) than for *E. coli* (as high as 7.8×10^{-1}) (3). The electroporation values are perhaps better understood when compared with those for conjugation; plasmid pp375 (20 kb [1]), similar in size to pZB32 (28 kb), transformed strain USDA 110 by electroporation to an efficiency of 10^6 transformants per μg of DNA and at a frequency of 1.7×10^{-7} transconjugants per survivor (Mark Barbour, unpublished data). The mating frequency with the same plasmid was similar at approximately 10^{-7} transconjugants per USDA 110 recipient cell. We chose to present results in terms of efficiencies (transformants per microgram) rather than frequencies (transformants per survivor) since in most experiments the total number of transformants that can be obtained is the critical parameter and DNA is usually limiting. The resistance to damage and the lower transformation frequencies described above tend to support the hypothesis, introduced in the previous paragraph, that fewer *B. japonicum* cells than *E. coli* become permeabilized by the pulse. Such observations, combined with the fact that the field strength curve (Fig. 2) did not completely level off up to the highest level tested (12.5 kV/cm), suggest that transformation efficiencies and frequencies could possibly be enhanced in *B. japonicum* by increasing the field strength or by permeabilizing the cells by some treatment or both. However, the field strengths used here are the maximum available from our instrument, and extensive prior treatment of cells could reduce the viability and negate the convenience of the method described.

The bacterial source of the DNA to be introduced appears to be more important for successful electroporation of *B. japonicum* than the method of DNA preparation. pZB32 DNA extracted from *B. japonicum* USDA 110 by small-scale alkaline lysis transformed this strain as efficiently as did the same plasmid extracted in large-scale preparations purified on CsCl-ethidium bromide gradients (data not shown). However, pZB32 DNA extracted from *E. coli* MC1061 (small-scale alkaline lysis preparation) could only occasionally be introduced into *B. japonicum* (one to three transformants per electroporation event) even when adding microgram quantities of DNA. In these few cases, the presence of the plasmid was confirmed by a positive β-galactosidase assay (1). The lack of incorporation of *E. coli*-derived plasmids is possibly due to the presence of a restriction-modification system in *B. japonicum* which degrades heterologous DNA (DNA derived from an alternative bacterial species or strain). Restriction modification has been shown to be a limiting factor in electroporation of other bacterial species (13). On the other hand, plasmid pZB32 extracted from *B. japonicum* USDA 110 transformed *Bradyrhizobium* (*Arachis*) sp. to an efficiency (10^6/μg) similar to that of *B. japonicum* USDA 110. Therefore, it may be possible to efficiently transfer DNA among several *Bradyrhizobium* species.

One important limiting factor in genetic studies with *B. japonicum* has been the lack of efficient, rapid, and reliable techniques for introducing exogenous DNA into cells. This study shows that electroporation is an efficient, simple, and reliable method for introducing relatively large plasmids (30 kb) into *B. japonicum*. With routine efficiencies as high as 10^7/μg, this procedure should become very useful for reliably obtaining large numbers of transformants. Despite the apparent restriction-modification problem, this technique should facilitate genetic studies of this organism by providing an alternate means of DNA introduction which is simpler, faster, and more reliable than conjugation. Since conjugal mating is presently the only means of introducing DNA into this organism, electroporation should assume an increasingly important role in *Bradyrhizobium* genetic research as the usefulness of the technique becomes recognized. Electroporation will become even more valuable when a means of introducing heterologous DNA from other bacterial species is found.

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