Techniques

Application of Electroporation for Efficient Transformation of *Xanthomonas campestris* pv. *oryzae*

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ABSTRACT

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Electroporation provided an effective transformation system for strains of *Xanthomonas campestris* pv. *oryzae*. Transformation was observed for strain X37-2 with plasmid pUFR027 over a range of electric field strengths (9.2–17.1 kV/cm) and two different pulse lengths. Optimization of the compensatory relationship between the electrical parameters produced maximal transformation efficiency (3.6×10^9 transformants/μg DNA) and frequency (1.8×10^{-2} transformant/survivor) for strain X37-2. When applied to four other strains of *X. c. oryzae*, these conditions yielded lower levels of transformation (7.8×10^6 to 2.7×10^7 transformants/μg DNA). Linear relationships were observed between bacterial cell density and transformation efficiency and between plasmid DNA concentration and frequency of transformation.

Additional keywords: electrotransformation.

Xanthomonas campestris pv. *oryzae* is the causal agent of bacterial leaf blight in rice. This serious disease has been recognized in Asia since the 1800s (19), but has been confirmed only recently in the United States (13). Establishment of this pathogen in the Gulf Coast rice-growing area is indicated by disease recurrence in subsequent crop years and identification of an alternative host (10). It is hypothesized that several factors contribute to pathogenicity of *X. campestris* pathovars (5), and the involvement of extracellular enzymes has been reported (11,20,22,25). Genes that encode pathogenicity factors are of primary concern for genetic studies. A problem in conducting genetic experiments with *X. c. oryzae* is that it does not respond well to chemical treatments that induce competence necessary for transformation studies, and a highly reproducible natural transformation system has yet to be developed.

An efficient transformation system is necessary for successful genetic manipulation. Electrotransformation, a technique by which bacteria are transformed by electroporation, offers an alternative to natural transformation systems (17). Compensation between electric field strength and pulse duration provides a general relationship between these electrical parameters, and opti-

mization of this relationship allows an efficient transformation system to develop. In this report, we present conditions for the optimization of electrical parameters that allow for efficient electrotransformation of *X. c. oryzae.*

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The strains of *X. c. oryzae* are from diseased rice and include X1-5, X1-8, X7-2D, X8-3, and X37-2 (13). Strain X8-3 was previously designated as RU87-17 (1). Cultures of *X. c. oryzae, X. c. translucens, X. c. pelargonii, X. c. campestris, X. c. malvacearum,* and *X. c. vesicatoria* were routinely maintained on nutrient broth-yeast extract agar (NBY agar) (23) at 4 C. Stock cultures were maintained at −20 C in 10% glycerol. *Escherichia coli* strain DH5α, harboring plasmid pUFR027 (6), was cultured on TN agar (18) containing kanamycin (25 μg/ml).

Plasmid DNA isolation. Plasmid pUFR027 (9.3 kb) was isolated by the method of Birnboim and Doly (2) and purified twice by cesium chloride-ethidium bromide density gradient ultracentrifugation. Concentration and purity of the DNA were determined from A_{260nm} and A_{280nm} measurements (14).

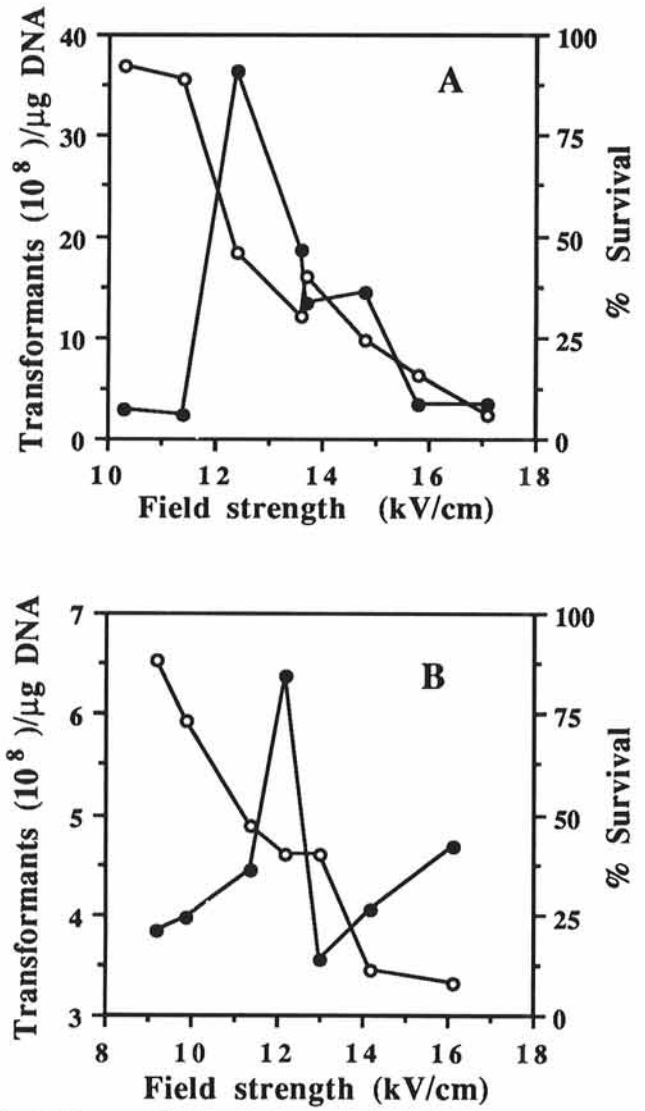

Fig. 1. Effects of field strength on transformation and survival. A concentrated cell suspension of *Xanthomonas campestris* pv. *oryzae* strain X37-2 was added to plasmid pUFR027 (1.63 μg/ml) and pulsed over a range of field strengths with pulse durations of **A,** 5 msec or, **B,** 10 msec. The transformation efficiency (●) was determined after a 1-h expression time and the percentage cell survival (○) at 0 h. Graphs are representative of duplicate trials for each data point.

Cell preparation. Bacterial strains were grown for 48 h at 28 C on NBY agar. One loopful of bacterial cells from a fresh culture was inoculated into 55 ml of NBY broth without glucose or $MgSO_4$, incubated at 28 C on a rotary shaker (225 rpm), and grown to mid-log growth phase (A_{640nm} = 0.5 to 0.65). Cells for electroporation were prepared by a modification of the procedure of Dower et al (8). A 50-ml sample was concentrated by centrifugation (3,841 *g*) at 4 C for 10 min. The cell pellet was washed in 0.5 and 0.25 volumes of cold sterile deionized distilled H_2O (ddH_2O). Cells then were resuspended in 10 ml of cold sterile 10% glycerol, concentrated by centrifugation, and resuspended in 1 ml of cold 10% glycerol. The suspension was transferred to a 1.5-ml microcentrifuge tube and centrifuged at full speed in a microcentrifuge for 1.5 min at 4 C. Cells were resuspended to a final volume of 0.5 ml in 10% glycerol and held on ice.

Electroporation protocol. Aliquots (60 μl) of concentrated cells were transferred to a cold 0.5-ml microcentrifuge tube containing 1–3 μl of plasmid DNA and mixed. Plasmid pUFR027 was used in all electrotransformation experiments. A 40-μl sample of the cell/DNA mixture was loaded between chilled electrodes of a BTX Flatpack chamber (Biotechnologies and Experimental Research Inc., San Diego, CA) (0.56 mm gap) and subjected to a single high-voltage pulse. Pulses were generated and delivered with the BTX T100 apparatus. Final plasmid DNA concentration of the sample ranged from 140 pg/ml to 20 μg/ml. During each pulse delivery, peak voltage and pulse duration were monitored by the Pulse Checker unit to determine the actual electric field strength experienced by the sample and the duration of the peak voltage. After pulse delivery, cells were immediately diluted by flushing the chamber with 1 ml of complete NBY broth into a 17 × 100 mm round bottom polypropylene tube. The pulsed sample was gently mixed and a 100-μl aliquot was transferred to a prechilled polypropylene tube containing 900 μl of ddH_2O to assess survival. The remaining culture was incubated for 1 h at 28 C with constant shaking (225 rpm) to allow for expression of antibiotic resistance.

Survivors were enumerated at 0-h incubation time by serial dilution plating of a portion of the pulsed sample on NBY agar not amended with antibiotic. Plasmid pUFR027 transformants were selected on NBY agar containing kanamycin (25 μg/ml) at the end of expression time. Transformation efficiency was calculated as transformants per microgram of plasmid DNA. An additional nonselective plating was performed after a 1-h incubation time to assess the frequency of transformation (transformant/survivor) after expression. Controls consisted of a cell/DNA mixture to which no electric pulse was delivered. The colony-forming units/ml (cfu/ml) of the cell suspension were determined by nonselective plating of control sample at 0-h incubation time. The physical presence of plasmid pUFR027 in transformants was determined by agarose gel electrophoresis of plasmid preparations obtained by the method of Birnboim and Doly (2).

RESULTS

Initial studies. We evaluated the application of electroporation as an alternative method for the introduction of plasmid DNA into *X. c. oryzae.* An electric field of 6 kV/cm applied across the cell/DNA suspension for 5 msec resulted in 6.7×10^4 transformants/μg DNA with a frequency of 10^{-5} (data not shown). Expression time trials determined that the doubling time of bacterial strain X37-2 was 4.3 h after pulse delivery. Selective and nonselective platings of electroporated cells at 0, 1, 2, and 3 h after pulsing indicated that transformant recovery increased with expression time. After a 3-h expression time, survivors of the pulse increased 77% over the 0-h plating (data not shown).

Optimization of electrical parameters. Strain X37-2 was used as a representative strain to conduct optimization experiments. During these trials, culture age (mid-log growth phase), buffer conditions, plasmid DNA concentration (1.63 μg/ml), bacterial cell concentration (1.5×10^{11} cfu/ml), and expression time (1 h) were held constant. Cell/DNA suspensions were subjected

to a range of electric field strengths, 9–17 kV/cm, for 5 or 10 msec. Figure 1 illustrates the efficiency of transformation for the actual field strength experienced by the sample and the resulting survivability of the cells. At a set duration of 5 msec, electroporation of X37-2 at 12.4 kV/cm yielded a maximum efficiency of 3.6×10^9 with 46% survival of cells. When a 10-msec pulse was used, maximum efficiency was attained at 12.2 kV/cm, yielding 6.4×10^8 transformants per microgram of plasmid DNA and 40% survival of pulsed cells. Frequency of transformation at the optimal field strength for 5 or 10 msec was determined to be 1.8×10^{-2} and 1.5×10^{-2} transformant/survivor, respectively. All subsequent electroporation experiments were conducted at 12 kV/cm for 5 msec.

DNA and cell concentration effects. Electrotransformation of prepared cells was conducted with pUFR027 plasmid DNA concentrations ranging from 140 pg/ml to 20 μg/ml. A linear relationship between the proportion of cells transformed and DNA concentration was observed (Fig. 2).

Prepared cells of strain X37-2 were diluted (1:1, 1:10, 1:100, 1:1,000) to determine the effect of cell concentration on transformation efficiency. Cell numbers ranged from 1.1×10^8 to 1.1×10^{11} cfu/ml and were electroporated in the presence of a fixed DNA concentration (1.63 μg/ml). Figure 3 depicts a steady and approximately linear increase in transformation efficiency in response to cell concentration.

Strain variability. Table 1 summarizes percent survival, transformation efficiency, and frequency of transformation for four additional strains of *X. c. oryzae* when optimized electrical parameters for strain X37-2 were applied. Cells were harvested and concentrated as previously described and exposed to a single 5-msec pulse of 12 kV/cm. The percentage of survival for the additional strains was similar to that previously observed for X37-2, except for strain X1-8, which had greater survivability. Transformation efficiencies and frequencies for the additional strains of *X. c. oryzae* were lower than those obtained for the optimized strain (Table 1). These same parameters were applied to five other *X. campestris* pathovars that used plasmid pUFR027 (1.63 μg/ml) and resulted in greater variation of transformation rates. Efficiencies of 2.1×10^7, 1.2×10^6, 1.3×10^6, 7.6×10^4, and 0 were obtained for *X. c. translucens, X. c. pelargonii, X. c. campestris, X. c. malvacearum,* and *X. c. vesicatoria*, respectively. Data represent averaged duplicate trials.

DISCUSSION

An efficient, reproducible transformation system is necessary for genetic research of *X. c. oryzae*. Introduction of plasmid DNA by electropermeabilization of cell membranes provides an effective means of bacterial transformation. This technique has been successfully applied to both eukaryotes and prokaryotes (3,4,7–9, 16,21), including some phytopathogenic bacteria (12,15,24), and promises to be one of the most versatile tools available to introduce DNA into cells.

Our trials indicated that electroporation can be employed to introduce plasmid DNA into *X. c. oryzae*. This allows for greater flexibility in genetic studies since *X. c. oryzae* is especially recalcitrant to most genetic techniques. We examined a range of electrical parameters. Optimization of field strength and pulse duration was based on maximal transformation efficiencies after 1 h of expression in relation to percent survival. Longer expression times increase the recovery of transformants and may be useful in the recovery of slower-growing xanthomonads, such as strain X8-3. After pulse delivery, an expression period of 1 h allowed for cell recovery and expression of antibiotic resistance genes and represented, as accurately as possible, the transformants from the initial cell population exposed to the electric pulse.

Cultures of *X. c. oryzae* were grown in the presence of incomplete NBY broth (no glucose or $MgSO_4$) to reduce production of extracellular polysaccharides. These culture conditions had previously been established by Xu and Gonzalez (*unpublished*) for use in conjugational transfer experiments. Also, cultures were harvested during mid-log growth phase, the stage

TABLE 1. Effects of X37-2 optimized electrical parameters on electroporation of other strains of *X. c. oryzae*[a]

Strain	Survival (%)	Efficiency (transformants/μg DNA)	Frequency (transformant/survivor)
X37-2	42	3.6×10^9	1.8×10^{-2}
X1-5	32	1.8×10^7	2.9×10^{-4}
X7-2D	44	1.3×10^7	2.2×10^{-4}
X1-8	76	2.7×10^7	4.1×10^{-4}
X8-3	41	7.8×10^6	1.8×10^{-4}

[a]Electrical parameters: 12 kV/cm, 5 msec. Efficiency and frequency were determined after expression for 1 h. Data are representative of duplicate trials.

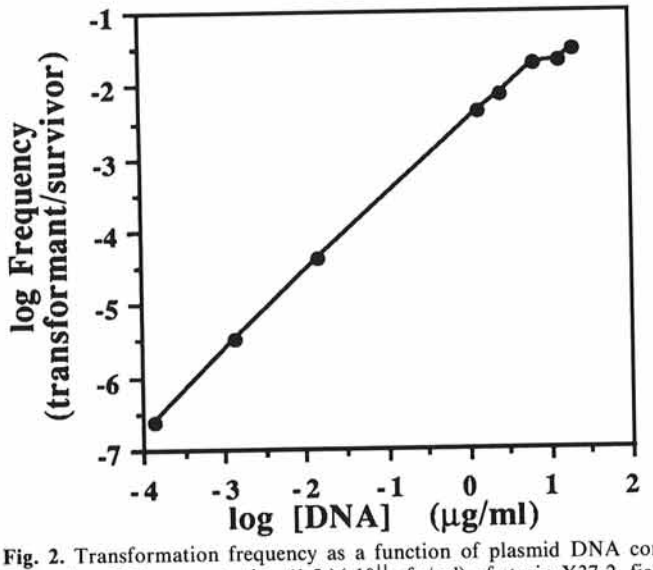

Fig. 2. Transformation frequency as a function of plasmid DNA concentration. Cell concentration (1.5×10^{11} cfu/ml) of strain X37-2, field strength (12 kV/cm), and pulse duration (5 msec) were held constant in the presence of various concentrations of plasmid pUFR027 (140 pg/ml to 20 μg/ml). Graph is representative of duplicate trials for each data point.

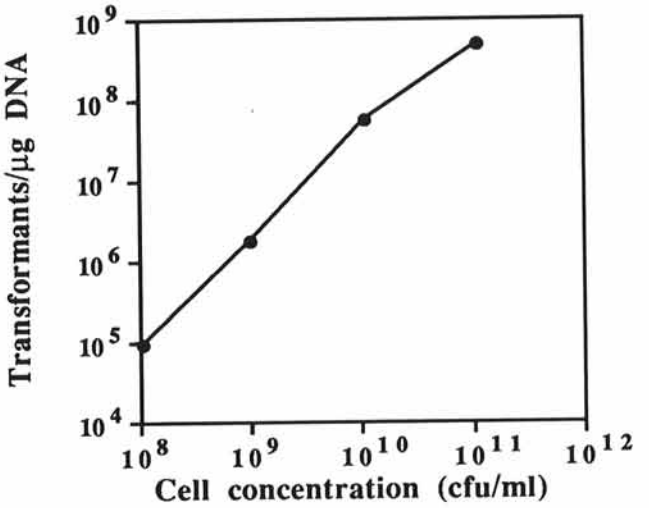

Fig. 3. Effect of cell concentration on transformation efficiency. Cells of *Xanthomonas campestris* pv. *oryzae* strain X37-2 were grown and harvested as described in Materials and Methods. The concentrated cell suspension (1.1×10^{11} cfu/ml) was diluted 1:1, 10^{-1}, 10^{-2}, and 10^{-3}. Cells (40 μl) were electroporated in the presence of 1.63 μg of plasmid DNA/ml at 12 kV/cm for 5 msec. Transformants were selected on medium containing kanamycin (25 μg/ml). Graph is representative of duplicate trials for each data point.

found most suitable for conjugational transfer studies. The growth conditions and culture age used during this study were found to provide electro-competent cells.

A large window of transformability was found for *X. c. oryzae* strain X37-2. Transformation efficiencies of 2.5×10^8 to 3.6×10^9 were detected spanning the range of 10.3–17.1 kV/cm at 5 msec. When a 10-msec pulse was used, field strengths of 9.2 to 16.1 kV/cm yielded efficiencies of 3.5×10^8 to 6.4×10^9. For each pulse length, a narrow region of field strength within this window provided the optimal electrical conditions for maximal transformation efficiencies. A pulse of 6 kV/cm was delivered across the sample for 50 msec to determine the effect of a longer pulse duration on transformation rates. Lower efficiencies and frequencies indicate the necessity of a large potential difference for maximal membrane permeabilization of strain X37-2.

The application of X37-2-optimized electrical parameters to four other strains of *X. c. oryzae* and to five different pathovars of *X. campestris* also was investigated. The efficiencies and frequencies for these strains were lower than those observed for X37-2. No transformants were recovered for *X. c. vesicatoria*. However, because the percent survival for *X. c. vesicatoria* was comparable to the other pulsed pathovars, it should be expected that some level of electrotransformation would occur. Other parameters involved in the electrotransformation of this particular strain may have influenced its ability to be electrotransformed. Specifically, the electrical parameters applied here may not have resulted in maximal membrane permeabilization and not allowed for efficient uptake of plasmid DNA. Therefore, it may be essential that electrical parameters be individually determined for each bacterial strain to yield optimal efficiencies and frequencies of transformation.

The parameters presented in this study provide an effective starting point for optimization trials on these strains and other xanthomonads. Conditions established during our study will facilitate the efficient introduction of plasmid DNA into *X. c. oryzae* and permit increased genetic manipulation. Future applications currently being investigated include the introduction of poorly mobilizing plasmid DNA, the introduction of suicide vectors for transposon mutagenesis studies, and the introduction of transposon-trapping plasmids.

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