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Genetics

High-voltage electroporation of bacteria: Genetic transformation of *Campylobacter jejuni* with plasmid DNA

(gene transfer/electric field-mediated DNA transfer/shuttle vector/*Campylobacter coli*)

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ABSTRACT **Electroporation permits the uptake of DNA by mammalian cells and plant protoplasts because it induces transient permeability of the cell membrane. We investigated the utility of high-voltage electroporation as a method for genetic transformation of intact bacterial cells by using the enteric pathogen *Campylobacter jejuni* as a model system. This report demonstrates that the application of high-voltage discharges to bacterial cells permits genetic transformation. Our method involves exposure of a *Campylobacter* cell suspension to a high-voltage exponential decay discharge (5–13 kV/cm) for a brief period of time (resistance–capacitance time constant = 2.4–26 msec) in the presence of plasmid DNA. Electrical transformation of *C. jejuni* results in frequencies as high as 1.2 × 10^6 transformants per μg of DNA. We have investigated the effects of pulse amplitude and duration, cell growth conditions, divalent cations, and DNA concentration on the efficiency of transformation. Transformants of *C. jejuni* obtained by electroporation contained structurally intact plasmid molecules. In addition, evidence is presented that indicates that *C. jejuni* possesses DNA restriction and modification systems. The use of electroporation as a method for transforming other bacterial species and guidelines for its implementation are also discussed.**

Electroporation involves the application of high-intensity electric fields of short duration to reversibly permeabilize biomembranes. This technique is commonly used to transfer DNA into mammalian cells (1–5, 25), and applications for plant protoplasts and yeast have been reported (6–9). Electrical impulses have also been shown to greatly increase the frequency of eukaryotic cell fusion events (10). Since electroporation may depend on fundamental properties of biological membranes, it appeared feasible to use this technique to promote genetic transformation of intact *Campylobacter jejuni* cells.

C. jejuni, a member of the genus *Campylobacter* that contains Gram-negative, microaerophilic spiral-shaped bacilli, is a major cause of human diarrheal disease throughout the world (11, 12). Considerable effort is therefore being directed toward understanding the molecular basis of pathogenesis of this species. Genetic analysis of *C. jejuni* and its putative virulence factors has been difficult due to the absence of characterized systems for *in vivo* genetic manipulation (13). Techniques for DNA transformation or generalized transduction have not been described. Very recently our laboratory constructed plasmids that can be mobilized by conjugation between *Escherichia coli* and *Campylobacter* species (14). In this report we demonstrate the utility of high-voltage electroporation as an efficient method for genetic transformation of *C. jejuni* with plasmid DNA. In experiments designed to optimize this technique, we have systematically investigated the effect of several factors on transformation efficiency. With the conditions described here, transformation efficiencies of $>10^6$ transformants per μg of plasmid DNA have been routinely obtained.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. *C. jejuni* strain C31 was obtained from R. L. Guerrant (University of Virginia, Charlottesville) and *Campylobacter coli* strain INN183-83 was from G. M. Ruiz-Palacios (Institute Nacional de la Nutricion, Mexico). The *E. coli* strain used was HB101 (15). *Campylobacter* strains were grown on sheep blood agar (TSA II agar with 5% sheep blood, Baltimore Biological Laboratory Microbiology Systems) or Mueller Hinton agar [Mueller Hinton Broth (Baltimore Biological Laboratory) supplemented with 1.5% agar]. When required, kanamycin sulfate (Sigma) was added at 30 μg/ml. *C. jejuni* and *C. coli* were cultivated at 42°C. Microaerobic conditions were obtained by incubating plates in airtight containers with GasPak (Baltimore Biological Laboratory) H_2 + CO_2 generator envelopes.

Plasmid pILL512 (14) is a pBR322 replicon containing the cryptic *C. coli* plasmid pIP1445 (17), the *aphA-3* kanamycin-resistance determinant (16), and the RK2 *oriT* sequence (18). pILL512 was introduced into *C. jejuni* C31 by mobilization in trans from an HB101 strain containing pILL512 and the Tra^+ RK2 derivative pRK212-1 (19). Matings were performed as described (14). Plasmid DNA was isolated from HB101 as well as C31 transformants by standard procedures (20) and was purified for use in electroporation by centrifugation to equilibrium in cesium chloride/ethidium bromide density gradients. Restriction endonucleases obtained from Bethesda Research Laboratories were used to digest plasmid DNA according to supplier's recommendations. pILL512 DNA isolated from HB101 was treated with *Eco*RI methylase (New England Biolabs) and *S*-adenosylmethionine according to the manufacturer's instructions. The extent of DNA methylation was determined by assaying for resistance to digestion with excess *Eco*RI (10 units/μg).

DNA Transformation by Electroporation. *Campylobacter* strains were inoculated onto blood agar plates at an initial cell density of ≈5 × 10^9 colony-forming units (cfu) per plate. Plates were incubated at 42°C in a microaerobic environment. After an 8-hr growth period, cells were harvested in electroporation buffer (EPB: 272 mM sucrose/15% glycerol/2.43 mM K_2HPO_4/0.57 mM KH_2PO_4, pH 7.4) and pelleted at 6000 × *g* for 8 min at 20°C. Cells were gently resuspended in EPB to a density of ≈5 × 10^9 cfu/ml. pILL512 DNA isolated from C31 and purified by cesium chloride/ethidium bromide equilibrium gradient centrifugation was then added, and the mixture of cells and DNA was

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Abbreviation: cfu, colony-forming units.

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incubated for 10 min on ice. High-voltage pulses were delivered to ice-cold 0.5-ml samples (0.4-cm interelectrode gap) or 0.6-ml samples (0.15-cm gap) by using a Gene Pulser apparatus (Bio-Rad) set up as described below. Following electroporation, cells were incubated on ice (≈10 min), and 0.2-ml aliquots were outgrown for 4 hr on Mueller Hinton plates to allow for expression of antibiotic resistance and the establishment of plasmid maintenance. Outgrown cells were harvested, and appropriate dilutions were plated on Mueller Hinton plates containing kanamycin (30 μg/ml) for selection and quantitation of transformants. Controls included experimental trials in which either the electric pulse or pILL512 DNA was omitted. Values reported in the text are the mean (±SD) number of transformants per μg of plasmid DNA obtained from triplicate trials. The lower limit of detection in the electroporation protocol was ≈75 transformants per μg.

Electroporation Apparatus. High-voltage pulses were applied with a Gene Pulser apparatus (Bio-Rad) capable of generating pulses of up to 2500 V from a 25-μF capacitor. For experiments requiring a wider range of high-voltage capacitors, a device was constructed containing two 25-μF capacitors (film type, rated to 3500 V) switchable to series (12.5 μF) or parallel (50 μF) operation and a circuit allowing connection to the Gene Pulser apparatus.

Sample cuvettes of two types were used. Sterile, disposable cuvettes (Bio-Rad) with an interelectrode distance of 0.4 cm were used to obtain field strengths of up to 6.25 kV/cm. Higher field strengths of up to 16.7 kV/cm were obtained with cuvettes constructed to have an interelectrode distance of 0.15 cm. These cuvettes were made by attaching aluminum strips, 0.025 cm thick, to parallel walls of 0.2-cm cuvettes.

Occasionally, at very high field strengths (15 kV/cm, for example), arcing occurred in the narrow cuvettes. Arcs provide a very low resistance pathway and may allow transient currents of up to 10,000 A. This can result in explosion of the cuvette and damage to the instrument. To limit the current and protect the electronic circuit under these extreme conditions, a high-power 2-Ω resistor (consisting of four 25-W, 0.5-Ω, 1% wire-wound resistors in series and mounted in isolation) was placed in series with the sample cuvette. Since the resistance of the samples in these experiments was typically several hundred ohms, this additional 2-Ω resistance had a negligible effect on time constant or voltage applied to the sample.

A precautionary note is required for those using homemade apparatuses or electroporation devices designed for low-voltage eukaryotic electroporation. Capacitors used for low-voltage electroporation are often rated to ≈500 V. Such capacitors should not be charged to higher voltages than recommended since serious safety hazards may result.

Waveform. The pulse produced by the discharge of a capacitor has an exponential decay waveform. The electric field strength declines over time as a function of the resistance in the circuit and the size of the capacitor. The exponential decay curve is described by:

$$E_{(t)} = E_0 e^{-t/\tau}, \quad [1]$$

where $E_{(t)}$ is the electric field strength (V/cm) at any time t (sec), E_0 is the initial field strength, and τ is the resistance–capacitance (RC) time constant (sec). The time constant depends on the total resistance (R, in Ω)–capacitance (C, in F) of the system as follows:

$$\tau = RC. \quad [2]$$

Therefore, τ describes the shape of the decay waveform and is the time required for the electric field strength to decline to $1/e$ (≈37%) of the initial value. Because the resistance of the sample affects the time constant, the composition of the sample and the geometry of the cuvette have important influences on the shape of the discharge pulse. The resistance of the sample is inversely proportional to the ionic strength and temperature of the buffer and to the cross-sectional area of the solution–electrode interface. The resistance is directly proportional to the distance between the electrodes.

RESULTS

Transforming Plasmid pILL512. To determine conditions required for electroporation of *C. jejuni*, plasmid pILL512 (14) was used as a source of transforming DNA. After mobilization into *Campylobacter* C31, pILL512 DNA was purified from a C31 transconjugant for use in subsequent electroporation experiments. *In vivo* modification of pILL512 would therefore ensure resistance to host restriction systems that may be expressed by C31 (see below).

Electroporation: Pulse Amplitude and Duration. The effect of the initial electric field strength on electroporation efficiency is shown in Fig. 1*A*. *Campylobacter* C31 cells subjected to electroporation in the absence of pILL512 DNA did not produce kanamycin-resistant colonies, and the addition of plasmid DNA without an electric pulse did not result in detectable transformation. The voltage densities shown in Fig. 1*A* were achieved by using specially constructed cuvettes with a 0.15-cm interelectrode distance. Time constants remained nearly constant throughout the voltage range tested (1.8–2.1 msec). For field strengths between 5 kV/cm and 13 kV/cm, the efficiency of transformation increased in an exponential manner with the frequency approximately doubling every 1000 V/cm. The amplitude of the electric pulse therefore had a dramatic effect on the efficiency of electroporation. At 13 kV/cm (2.0 msec), an average transformation efficiency of $1.2 \pm 0.9 \times 10^6$ transformants per μg was obtained.

The effect of time constant on electroporation efficiency was also examined. Capacitors ranging from 3 μF to 50 μF were used to generate waveforms with increasing RC time constants as shown in Fig. 1*B*. The electric field strength was held constant at 5.25 kV/cm, and the ionic strength of all samples tested was identical. Cuvettes with an interelectrode distance of 0.4 cm were used. The results in Fig. 1*B* demonstrated an increase in the efficiency of transformation as the time constant increases from 3.5 msec to 26 msec. No transformants were detected at 5.25 kV/cm with time constants at or below 1.2 msec. Similar results were obtained when the time constant was varied by adjusting the ionic strength of the electroporation buffer, and capacitance and electric field strength were held constant (data not shown).

Cell Survival During Electroporation. Increased electric field strengths as well as longer exposure to high-voltage fields resulted in greater transformation efficiencies. It was expected that this elevation in transformation frequency would eventually be limited by lethality. Survival values for strain C31 are given in Table 1. Although slightly lower cell numbers were observed following a very high voltage pulse, the difference in survival following a 12 kV/cm pulse and survival of the no-pulse control was not statistically significant (see Table 1 legend). Lethality therefore did not seem to be a major limiting factor within the range of electric field strengths and time constants tested.

Cell Growth and Electroporation Conditions. We have also investigated the effect of various physiological variables on electroporation efficiency. Transformation was best with cells grown on sheep blood agar or Mueller Hinton agar under microaerobic conditions. We have not been able to transform cells grown in Brucella broth or Mueller Hinton broth. We have also monitored the effect of growth stage on

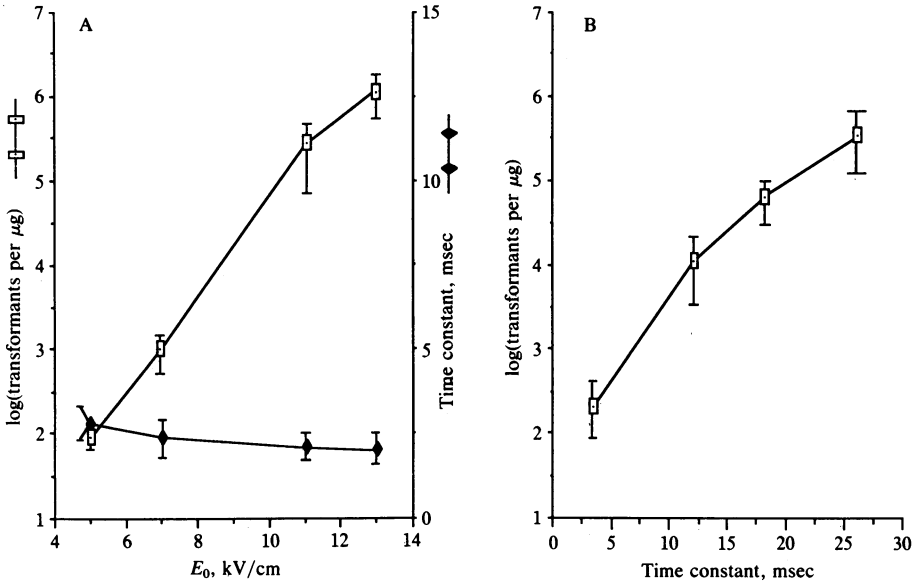

FIG. 1. Dependence of transformation efficiency on pulse amplitude and duration. (*A*) Initial electric field strength (E_0). High voltage densities were achieved by using electroporation cuvettes with a 0.15-cm interelectrode distance and the 25-μF internal capacitor. (*B*) Exponential decay time constant. The initial electric field strength was 5.25 kV/cm, and cuvettes with a 0.4-cm interelectrode distance were used. A time constant of 3.5 msec was obtained with the 3-μF internal capacitor, and time constants of 12.1 msec, 18.3 msec, and 26 msec were produced by using an external capacitor assembly set at 12.5 μF, 25 μF, and 50 μF, respectively. Values are means ± SD from triplicate trials.

transformation by electroporation (data not shown). *C. jejuni* strain C31 cultures grown on blood agar were harvested at various times after inoculation with ≈5 × 10^9 cfu per plate, adjusted to equivalent cell densities, and electroporated in the presence of pILL512 DNA. The efficiency of transformation was optimal with cells in the early to mid-exponential phase of growth (6–8 hr at 42°C). The difference in electroporation efficiencies over a 24-hr growth period varied by a maximum of about 10-fold. Growth phase, in comparison with electric field strength and time constant, appears to have a relatively minor effect on transformation efficiency.

We found that the temperature of the sample during electroporation was important. Preincubation of samples at 22°C (10 min) followed by electroporation at 5.25 kV/cm with a 50-μF capacitor resulted in a transformation efficiency of 5.0 ± 0.9 × 10^3 transformants per μg, as compared to an efficiency of 3.3 ± 1.9 × 10^5 transformants per μg obtained with cells preincubated at 4°C (10 min) and electroporated under otherwise identical conditions.

Divalent Cations. Since divalent cations have been shown to affect the efficiency of electroporation of eukaryotic cells (1, 6), we tested the effect of several divalent cations on bacterial transformation by electroporation (Fig. 2). The total resistance of each of the samples was adjusted to an approximately equivalent value, resulting in a relatively narrow set of time constants averaging 20.6 ± 0.87 msec at 5.25 kV/cm. There was a marked decrease in transformation efficiency observed in the presence of $CaCl_2$ that could not be attributed to the small fluctuations in the time constant. Similar results were seen with $MgCl_2$ and $MnCl_2$ (data not

Table 1. Survival of *C. jejuni* strain C31 after electroporation

Initial electric field strength, kV/cm	Capacitance, μF	Time constant, msec	Viable cells, cfu/ml
No pulse	—	—	2.8 ± 0.4 × 10^9
5.25	1	1.2	3.6 ± 0.7 × 10^9
5.25	25	16.9	3.2 ± 0.3 × 10^9
6.0	25	2.7	2.9 ± 0.8 × 10^9
12.0	25	2.1	1.7 ± 0.7 × 10^9

Cells for electroporation were prepared as described in the text, except that no DNA was added. Following electroporation, dilutions of the cell suspensions were plated for enumeration of viable cells. Cuvettes with 0.4-cm interelectrode distances were used to generate electric field strengths of 5.25 kV/cm. Higher field strengths of 6 kV/cm and 12 kV/cm were obtained with 0.15-cm cuvettes. Values are means ± SD of triplicate samples. For each treatment, mean values did not differ significantly from the no treatment control by Student's *t* test ($P > 0.05$).

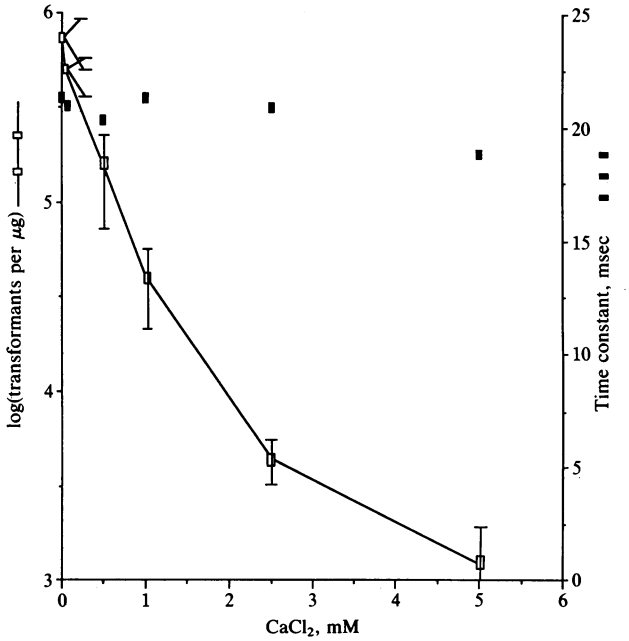

FIG. 2. Effect of $CaCl_2$ on transformation efficiency. $CaCl_2$ was added to the electroporation buffer at the concentrations indicated and the total ionic strength of the buffer was adjusted to give an approximately equivalent resistance for each sample. The electric field strength was held constant at 5.25 kV/cm (0.4-cm gap), and a 50-μF external capacitor was used. The time constants for each data point are indicated. Transformation frequency values are expressed as in Fig. 1; the mean time constant (±SD) was 20.6 ± 0.9 msec.

shown), although the inhibitory effect of $MnCl_2$ was about 50-fold greater than that of $MgCl_2$ or $CaCl_2$.

DNA Dosage. The effect of plasmid DNA concentration on the total number of transformants obtained by the electroporation of identical quantities of cells was examined (Fig. 3). The DNA dose–response curve was linear over a 500-fold range of concentration, and saturation was not observed within the range of concentrations tested. Thus, the number of transformants per μg remained at a nearly constant value of $6.1 \pm 2.2 \times 10^5$ (5.25 kV/cm, 30.4 ± 0.5 msec) over the DNA concentration range of 20 ng/ml to 10 μg/ml. The electroporation system appeared to be quite sensitive, and transformants were easily obtained with very small amounts of DNA. The range of concentrations tested corresponds to ratios of plasmid molecules to viable cells from approximately 0.33:1 to 170:1.

Restriction/Modification. As shown in Table 2, the source of plasmid DNA had a profound effect on the efficiency of *Campylobacter* transformation. We have not detected transformation of *C. jejuni* C31 with pILL512 DNA isolated from *E. coli* HB101 [*hsdS20* (r_B^-, m_B^-); ref. 15], although the same DNA preparation was capable of efficiently transforming HB101 by $CaCl_2$-mediated transformation (Table 2) and electroporation (data not shown). DNA isolated from *C. jejuni* C31, however, served as an efficient substrate for transformation of HB101 and C31. These observations suggest that *C. jejuni* strain C31 contains a DNA restriction/modification system capable of restricting heterologous DNA and modifying endogenous DNA. Plasmid DNA extracted from *C. jejuni* C31 transformants obtained under a variety of electroporation conditions was analyzed by restriction enzyme digestion and showed no detectable deletions or rearrangements (data not shown).

It has been observed that pILL512 DNA isolated from *C. jejuni* C31 transconjugants is resistant to digestion with *Eco*RI (14), indicating that the *Eco*RI recognition sequence is modified in *C. jejuni*. We therefore tested whether methylation of the internal adenine residues of the unique *Eco*RI sites in pILL512 molecules isolated from *E. coli* HB101 was sufficient to allow transformation of *C. jejuni* C31. pILL512 DNA was methylated using *Eco*RI methylase and *S*-adenosylmethionine. Although the resulting DNA was modified to completion as shown by resistance to *Eco*RI digestion *in vitro*, no transformants were observed. It therefore appears that complete *Eco*RI sites are not the only DNA sequences affected by the C31 restriction/modification system or that the *Campylobacter* enzyme is unaffected by the type of adenine methylation tested.

Table 2. Restriction and modification of plasmid DNA by *C. jejuni* strain C31

pILL512 DNA source	Recipient strain	Transformation efficiency, no. of transformants per μg
HB101	HB101	3.5×10^6
HB101	C31	<75*
C31	HB101	4.2×10^6
C31	C31	2.5×10^5

pILL512 DNA was isolated from *E. coli* HB101 and *C. jejuni* C31. Plasmid DNA from either source was then used to transform HB101 by a standard $CaCl_2$ protocol (20) or C31 by using electroporation (5.25 kV/cm, 24 msec). Selection was for kanamycin resistance. All transformations were repeated several times and similar results were observed.

*Repeated attempts to obtain *C. jejuni* C31 transformants with plasmid DNA isolated from *E. coli* HB101 have been unsuccessful. Seventy-five transformants per μg was the lower limit of detection.

In addition to *C. jejuni*, we have also obtained efficient transformation of *C. coli* strain INN183-83 with pILL512 DNA isolated from *C. jejuni* C31. Although *C. coli* and *C. jejuni* are distantly related and share only about 35% DNA sequence homology (21), there does not seem to be a DNA restriction barrier operating between these species. Further research is necessary to determine the extent of variation in the efficiency of transformation by electroporation among different *Campylobacter* sp. strains.

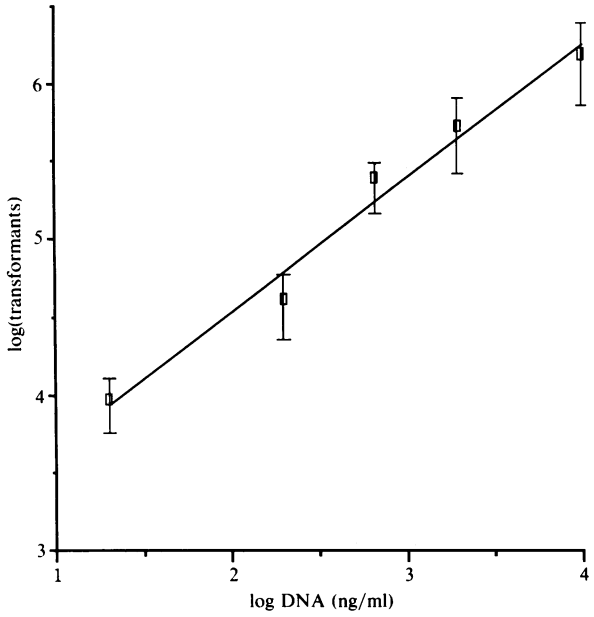

FIG. 3. Dependency of transformation efficiency on DNA concentration. Variable amounts of DNA (in a constant volume of 5 μl in 10 mM Tris·HCl/1 mM EDTA, pH 8.0) were added to the cell samples. DNA concentrations ranging from 0.02 μg/ml to 10 μg/ml were tested. The electric field strength was constant at 5.25 kV/cm (0.4-cm gap), and the 50-μF external capacitor was used. The mean time constant (±SD) for all points was 30.4 ± 0.5 msec. Values are as described in the legend to Fig. 1.

DISCUSSION

The results we have presented show that intact bacterial cells can be efficiently transformed by electroporation. Electroporation is rapid, easy to perform, and requires minimal sample preparation. Since this technique has been successfully applied to mammalian cells, plant protoplasts, yeast, and, now, bacterial cells, it most likely depends on conserved physical properties of biomembranes. In addition, the transformation frequency of highly competent *Bacillus cereus* protoplasts with a *Bacillus thuringiensis* plasmid has been shown to be moderately increased by application of an electric pulse (22). Electroporation may therefore be a general method that will be useful for introducing DNA into many bacterial species in addition to *C. jejuni* and *C. coli* (see below).

The amplitude (electric field strength) and duration (time constant) of the discharge waveform are important, and optimal values may depend on the bacterial species and strain being tested. Within the ranges that we have examined, the strength of the initial electric field has a greater effect on transformation efficiency than does the time constant. For field strengths between 5 kV/cm and 13 kV/cm, a 2-fold increase in voltage results in a >1000-fold increase in the number of transformants per μg. At 5.25 kV/cm, increasing the time constant from 10 msec to 20 msec results in a <100-fold elevation in efficiency.

Theoretical arguments suggest that pore formation is due to a transmembrane potential that develops as a result of electric field-induced accumulation of ions on the surfaces of membranes (1, 23, 24). The magnitude of the external field

required to generate a transmembrane potential sufficient for pore formation is inversely related to the size of the cell and also depends on cell shape. In general, electroporation of eukaryotic cells requires lower electric field strengths than we have employed for bacterial transformation, and it is likely that this is due in part to differences in cell size.

Our results indicate that pulse amplitude and duration have compensatory effects. For example, an efficiency of $\approx 5.0 \times 10^5$ transformants per μg may be obtained with an electric field strength of 5.25 kV/cm and a time constant of 21 msec (Fig. 1*B*) or a field strength of 10.3 kV/cm and a time constant of 2.4 msec (Fig. 1*A*). This observation affords some flexibility in designing electroporation equipment and protocols; however, it also appears that there are threshold values of pulse amplitude and duration below which transformation occurs rarely, if at all. An electric field strength of $\approx$5 kV/cm with a time constant of about 3 msec are minimal conditions required for detectable transformation of *C. jejuni*.

Campylobacter cells are remarkably resistant to damage by electrical impulses. Statistically significant lethality was not observed, even at the highest voltages tested (12 kV/cm, 2.1 msec). These observations are in contrast to those obtained with mammalian cells (25), carrot protoplasts (6), yeast cells (9), and *E. coli* (W.J.D., unpublished data), for which decreased survival accompanies increased electroporation efficiency. Our results indicate that death of a significant proportion of the cell population is not a prerequisite for efficient transformation and that the magnitude of lethality depends on the particular cell type.

Growth stage may also affect transformation efficiencies, although to a lesser extent than the electrical parameters surveyed. In our system, chloride salts of some divalent cations have a detrimental effect, with $MnCl_2$ causing $\approx$50-fold greater inhibition than $CaCl_2$ or $MgCl_2$. $MgCl_2$ has also been shown to decrease and, at high concentrations, prevent gene transfer into mouse lyoma cells (1), although maximum electroporation of carrot protoplasts occurs in the presence of 4 mM $CaCl_2$ (6).

Restriction/modification systems can severely inhibit the transformation of bacteria with heterologous DNA. Our results show that *C. jejuni* possesses a DNA restriction system that is capable of decreasing the efficiency of transformation of unmodified plasmid DNA by at least 4 orders of magnitude. The ability to isolate the pILL512 shuttle vector from *C. jejuni* was essential to the development of our protocol. In efforts to develop electroporation systems for other bacteria, it is important if possible to begin with modified DNA purified from the same bacterial strain, especially during the initial stages of optimization when electroporation efficiencies may be low.

Electroporation can be used to develop a genomic cloning system that will allow the establishment of gene banks containing *Campylobacter* sequences in *Campylobacter* hosts. We have begun preliminary investigations of the general utility of electroporation with development of protocols for efficient introduction of plasmid DNA into *E. coli* (W.J.D. and J.F.M., unpublished data) and *Actinobacillus pleuropneumonia* (G. Lalonde and J.F.M., unpublished data). As a result of our experience with *Campylobacter* and other organisms, we are optimistic that electroporation can successfully be applied to many bacterial species of medical, veterinary, and industrial importance, for which genetic transformation systems are either inefficient or nonexistent.

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