Optimum Conditions for Electric Pulse-mediated Gene Transfer to Bacillus subtilis Cells

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It was found that plasmid DNA (pUB 110) can be introduced into not only protoplasts but also intact cells of Bacillus subtilis by electric field pulses. The transformation of B. subtilis using protoplasts results in an efficiency of $2.5 \times 10^6$ transformants per µg of DNA, with a single pulse of 50 µsec with an initial electric field strength of 7 kV/cm. Even transformation of intact B. subtilis cells results in a maximum efficiency of $1.5 \times 10^5$ transformants per µg DNA, with a single pulse of 400 µsec with an initial electric field strength of 16 kV/cm. The cell survival of protoplasts and intact cells was approximately 100% and 30%, respectively, under the conditions found to be optimal for the transformation process. Plasmid DNA isolated from pUB 110 containing transformants was indistinguishable from authentic preparations of pUB 110 on gel electrophoretic analysis.

Electroporation involves the application of high-intensity electric fields of a short duration (pulse length) to reversibly permeabilize biomembranes. This technique is commonly used to transfer DNA into mammalian cells, and applications to plant protoplasts and yeast have been reported. Recently, electrical impulses have also been used for the transformation of prokaryotic cells with plasmid DNA. Shivarova et al. reported that the transformation frequency, $1.1 \times 10^{-3}$, for Bacillus cereus protoplasts in the presence of polyethylene glycol (PEG) is enhanced by the application of high electric field pulses of 14 kV/cm. Taketo has reported the transformation and transfection of Escherichia coli with an electric field strength of 6.25 kV/cm and a 25 µF capacitor, the results of this electroporation being for better than those of CaCl2-dependent transformation and transfection. Chassy and Flickinger reported that the electric transformation of intact Lactobacillus casei cells with plasmid pNZ12DNA results in an efficiency of $1.1 \sim 8.5 \times 10^4$ transformants per µg DNA. Miller et al. also reported that the electrical transformation of intact cells of Campylobacter jejuni results in frequencies of as high as $1.2 \times 10^6$ transformants per µg of DNA. It appeared feasible to use this technique to enhance the genetic transformation of not only protoplasts but also intact cells of B. subtilis.

Using the B. subtilis Marburg 168 strain and plasmid pUB 110 as a model test system, the aim of this study is to determine the optimum conditions for gene transfer. We report the relationship between transformation and cell survival as to electric field strength and pulse duration.

Materials and Methods

Bacterial strain and growth conditions. Bacillus subtilis Marburg 168 strain (trpC, phe-1) was obtained from H. Takahashi (Institute of Applied Microbiology, University of Tokyo).

The strain was subcultured in Pen medium (Difco antibiotic medium No. 3) at 37°C. The DM3 regeneration medium consisted of the following sterile solutions, per liter; 4% agar, 200 ml, 1 m sodium succinate (pH 7.3), 500 ml, 5% Difco casamino acids, 100 ml, 10% Difco yeast extract, 50 ml, 3.5% K2HPO4 and 1.5% KH2PO4, 100 ml, 20% glucose, 25 ml, 1 m MgCl2, 20 ml, and filter-sterilized 2% bovine serum albumin, 5 ml, containing 150 µg/ml
kanamycin was used as the selection medium for transformants obtained from B. subtilis protoplasts. Pen agar medium containing 1.5% agar was used as the selection medium for transformants.

Plasmid DNA. Plasmid pUB 110 obtained from Y. Yoneda (Nippon Gene Co., Ltd.) was prepared by the alkaline extraction method from a plasmid-harboring B. subtilis strain grown in Pen medium containing 5 μg/ml of kanamycin, and it was purified by ethidium bromide cesium chloride equilibrium centrifugation.

Preparation of protoplasts. Protoplasts of B. subtilis were prepared by a little modification of the method of Chang and Cohen.10) Bacteria were cultured overnight on Pen medium at 37°C, and a portion of the bacteria was inoculated into 100 ml of Pen medium and the cells were grown with shaking at 37°C to OD660 = 0.4 - 0.5 (1 - 2 × 10^9 cells/ml). This culture was centrifuged at 5000 rpm for 10 min and the cells were suspended in 4.5 ml of SMM.0.5 ml of SMMP containing 10 mg of lysozyme was added to the cell suspension, followed by slow shaking at 37°C for 1 hr. After incubation, protoplasts were centrifuged at 3000 rpm for 20 min and used for transformation experiments.

Electroporation apparatus. A high voltage generator (Shimadzu SSH-1 Type) that can supply electric pulses of square waves was constructed. Chambers (electric distance: 1 mm; volume: 20 μl) were used to obtain pulse durations of 10 to 500 μsec and various field strengths up to 28 kV/cm at 25°C. The electric field pulse was applied as a single pulse or as a train of successive pulses up to 4 times.

DNA transformation by electroporation. The whole cells were washed with sterile SMM (0.5 mM sucrose, 0.01 mM malic acid and 0.01 mM magnesium chloride) 3 times and then suspended in 1 ml of SMM. A 198 μl portion of this cell suspension was transferred to a 1.5-ml Eppendorf tube. Two μl of plasmid DNA solution (1.7 μg/μl) was added, followed by careful mixing with a pipette. 20 μl of the cell suspension was placed in a chamber. The cells were subjected to pulse electroporation at 25°C, diluted in liquid SMMP medium and then plated. Prior to plating on media containing antibiotics, the diluted cells were incubated at 30°C for 3 hr to allow the phenotypic expression of antibiotic resistance markers.

PEG-induced protoplast transformation was performed as described by Chang and Cohen.10) Determination of transformation and cell survival. A cell suspension with DNA was exposed to a single electric pulse of a certain duration at 25°C. After pulsation, the cells were diluted with growth medium and then cultured at 30°C. Cell survival was expressed as the ratio of living cells (regenerants) counted at 48 hr after pulsation to input bacillary cells.

To determine transformation, cells were pulsed and then cultured for 3 hr, after which the cells were selected in medium containing kanamycin. The number of kanamycin-resistant colonies was determined after 48 hr. The transformation frequency is the ratio of transformants to regenerants, and transformation efficiency the number of transformants per μg of DNA.

Results

Transformation of B. subtilis with plasmid DNA by electroporation

In preliminary experiments we examined if electroporation can be used to introduce plas-

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**Table I. Transformation of Bacillus subtilis by Electroporation**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Strain preparation</th>
<th>Voltage (kV/cm)</th>
<th>Duration (μsec)</th>
<th>Regenerants (No./ml)</th>
<th>Transformants (No./ml)</th>
<th>Cell survival (%)</th>
<th>Frequency (CFU_{trans} / CFU_{int})</th>
<th>Efficiency (CFU_{obs} / μg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulse</td>
<td>Protoplasts</td>
<td>7</td>
<td>50</td>
<td>2.8 × 10^6</td>
<td>6.8 × 10^5</td>
<td>98</td>
<td>2.4 × 10^{-1}</td>
<td>4.0 × 10^4</td>
</tr>
<tr>
<td>Pulse</td>
<td>Intact cells</td>
<td>16</td>
<td>400</td>
<td>2.5 × 10^6</td>
<td>2.5 × 10^5</td>
<td>28</td>
<td>1.0 × 10^{-4}</td>
<td>1.5 × 10^3</td>
</tr>
<tr>
<td>PEC</td>
<td>Protoplasts</td>
<td>—</td>
<td>—</td>
<td>3.0 × 10^7</td>
<td>3.5 × 10^6</td>
<td>11</td>
<td>1.2 × 10^{-3}</td>
<td>2.1 × 10^4</td>
</tr>
</tbody>
</table>

CFU_{trans} = total colony forming units used in the electroporation or PEG experiments; CFU_{int} = colony forming units recovered after electroporation or PEG treatment; CFU_{obs} = colony forming units observed as antibiotic resistant cells on selective plates.

a The DNA concentration for transformation was 17 μg/ml. The experimental procedures were described under Material and Methods.

b A mixture of 1 μl of 1.7 μg/μl DNA, 1 μl of 2 × SMMP buffer and 0.5 ml of a protoplast suspension in SMMP buffer was added to 1.5 ml of 40% PEG 6000, and then the mixture was kept for 2 min at room temperature, diluted in the liquid medium and then plated on medium containing antibiotic.
mid DNA molecules into B. subtilis protoplasts. Electrical transformation of B. subtilis protoplasts results in a frequency and a cell survival higher than those with PEG (Table I). Even a single pulse of 400 μsec with intact cells gave a transformation efficiency of $1.5 \times 10^3$ transformants per μg DNA. Plasmid DNA was also introduced into protoplasts at a higher survival rate of 98% than into intact cells at one of 28%.

**Effects of electric field strength and pulse duration on cell survival**

The cell survival of protoplasts with pulsation was almost the same with electric field strengths in the range of 1 ~ 7 kV/cm with the pulse duration of 50 μsec, there being a high cell survival of ca. 100% (Fig. 1). But, the cells survival of intact cells with pulsation decreased sharply with increasing electric field strength above than 12 kV/cm with the pulse duration of 50 μsec (Fig. 1). Knowing the field strength for a given duration at which cell survival begins to decrease should be useful for gene-transfer. When the duration was greater than 200 μsec, the cell survival of protoplasts decreased a little with increasing duration (Fig. 2). The cell survival of intact cells, however, showed no change even with pulsation of field strengths in the range of 100 to 500 μsec. The electric field strength and duration are effective factors for the electric breakdown of various cell membranes. As shown above, we analyzed the effects of various field strengths and durations on cell survival when the field strength was less than 28 kV/cm and the duration less than 500 μsec.

**Effects of electric field strength and duration on transformation**

To determine the optimum electric pulse for gene transfer, we suspended cells (ca. 1 × 10⁷/ml) in SMM and then exposed them to pulses of different field strength and duration combinations. At a fixed pulse duration of 50 μsec, the transformation frequency with both protoplasts and intact cells increased with increasing field strength (Fig. 3). The transformation of intact cells with plasmid DNA was possible with pulses of high electric field strength, i.e., greater than 16 kV/cm. At a fixed field strength of 16 kV/cm, the transformation efficiency for intact cells increased with increasing pulse duration until 400 μsec (Fig. 4). For the transformation of intact cells, both a high field strength and a long duration were effective. At a given field strength of 7 kV/cm, longer pulses than 200 μsec slightly decreased the transformation efficiency for protoplasts. The combination of a higher field strength with short pulses was very effective for the transformation of protoplasts, because the number of viable cells almost
Fig. 3. Effect of the Field Strength on Transformation.
Electric pulses of different field strengths, with a fixed duration of 50 μsec, were applied to the cell suspension (10⁷/ml) mixed with DNA (17 μg/ml), and the number of transformed cells was determined as described in the text. (○), intact cells; (●), protoplasts.

Fig. 4. Effect of the Pulse Duration on Transformation.
The electric field strength was fixed at 16 kV/cm for intact cells (○) and at 7 kV/cm for protoplasts (●). The other conditions were the same as in Fig. 3.

did not decrease at all with increasing field strength.

We obtained a high transformation frequency (ca. 10⁻¹ with protoplasts and 10⁻⁴ with intact cells) with different field strength and duration combination under the optimum conditions. This shows that the optimum conditions for effective transformation on a viable cells basis can be obtained with the field strength (16~28 kV/cm with intact cells and 5~7 kV/cm with protoplasts) and the pulse duration (300~400 μsec with intact cells and 50~200 μsec with protoplasts).

Other factors affecting electroporation-mediated transformation and cell survival
The transformation and cell survival of B. subtilis protoplasts with repeated pulsation were investigated under the pulse-conditions of an electric field strength of 7 kV/cm and a pulse duration of 50 μsec. Even repeated pulsation up to 4-times did not affect transformation or cell survival, there being constant values of ca. 10⁴/μg DNA efficiency and ca. 100% cell survival (Fig. 5). This shows that a single pulse is very effective for transformation.

For the present electroporation system, the relationship between the yield of the transformants from protoplasts and the concentration of plasmid pUB 110 DNA was investigated. The results are shown in Fig. 6. The yield of the transformants was directly proportional to the concentration of input DNA over a wide range (10~170 μg/ml). Our results indicate that electric pulse-medi-
Fig. 6. Effect of the DNA Concentration on Electroporation-mediated Transformation.

Pulses of a fixed field strength of 7 kV/cm with a duration of 100 μsec were applied with different DNA concentrations. The experimental conditions were the same as in Fig. 5.

Isolation of plasmid DNA from transformants

Plasmid DNA was isolated from B. subtilis transformed to kanamycin-resistance by electroporation and then analyzed by agarose gel electrophoresis (Fig. 7). The electrophoretic mobility of plasmid DNA isolated from B. subtilis transformants was identical to that of authentic preparations isolated from B. subtilis. All the transformants (one hundred fifty strains) analyzed had electrophoretically the same plasmid pattern characteristics as the native B. subtilis strain. The restriction endonuclease cutting patterns observed with Eco RI were also identical (data not shown).

Discussion

The results we have presented show that not only protoplasts but also intact bacterial cells can be 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 the conserved physical properties of biomembranes. In addition, the transformation frequency for highly competent B. subtilis protoplasts with plasmid DNA has been shown to be moderately increased by application of an electric pulse.12) Electroporation may therefore effectively be a general method that is useful for introducing DNA into intact B. subtilis cells in addition to protoplasts.

We here reported the application to gene transfer in B. subtilis using a square wave pulse generator. Cell survival on pulsation of intact cells depended largely on the field strength, and not the pulse duration. Field strengths from 10 to 25 kV/cm, that gave ca. 30% cell survival, may represent the field strength range that does irreversible damage to cells, and a high field strength seems to cause the ion flux for membrane breakdown on pulsation.

Cell survival in the case of protoplasts depended on the pulse duration, and not the field...
strength. With a field strength of less than 7 kV/cm, there was 100\% cell survival on pulsation of protoplasts at the fixed pulse duration of 50 \( \mu \)sec, and in cells exposed to these conditions the damage to the membrane may be repaired during culturing in SMMP medium for 3 hr at 30\°C.

The electric field strength and pulse duration of the discharge waveform are important, and optimal values may depend on the bacterial species and strains being tested. Within the ranges we examined, the strength of the initial electric field has a greater effect on the transformation efficiency than the pulse duration does. In the case of transformation of protoplasts with plasmid DNA with field strengths between 4 and 7 kV/cm, ca. 2-fold increase in voltage results in a ca. 1000-fold increase in the number of transformants per \( \mu \)g DNA.

Intact \textit{B. subtilis} cells are also effectively transformed on elevation of the electric field strength up to 28 kV/cm. At 16 kV/cm, increasing the pulse duration from 200 \( \mu \)sec to 400 \( \mu \)sec results in a ca. 10-fold increase in the efficiency for intact cells.

When various numbers of pulses were successively applied, the number of transformants did not appreciably change with the number of pulses. This shows that the permeable pores for the introduction of plasmid DNA into \textit{B. subtilis} cells can be formed by only a single pulse.

Another way to obtain efficient transformation is to use a high concentration of DNA. There is a linear relationship between the total number of transformants and the concentration of plasmid DNA. In this case, the cell survival can be kept high, \textit{i.e.}, more than 90\% (data not shown).

Our electroporation method is the simplest reported so far, requiring only a single pulse for cells in SMM medium at 25\°C. Electric pulses under the pulsation condition of a combination of a field strength of 7 kV/cm and a pulse duration of 50 \( \mu \)sec and of one of a field strength of 16 kV/cm and a pulse duration of 400 \( \mu \)sec are effective for the transformation of protoplasts and intact cells, respectively, and are easily obtained (Table I). Electroporation will be broadly applicable to many species of bacteria, and will dramatically decrease the difficulty of research on less well-studied and exotic strains. As advances in electroporation technology are made, the technique may also replace well-established methods of bacterial transformation.

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\textbf{References}