Transformation of *Pseudomonas putida* by Electroporation

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The optimum electrotransformation conditions were determined for *Pseudomonas putida* PpY101 with plasmid pSUP104 (9.5 kb) and pSR134 (18.6 kb). Field strength was a very important parameter for electrotransformation efficiency. Optimum efficiencies (1.1 × 10⁵ transformants/μg DNA) with pSUP104 and pSR134 were obtained at a field strength of 12.5 kV/cm, a time constant of about 4.5 ms (resistance setting of 200 Ω), a supercoiled DNA concentration of 100 ng/ml, and a cell concentration of 10⁷/ml. Because the efficiency obtained is high enough, electrotransformation is useful for the direct cloning of *P. putida* PpY101. No significant relationship between plasmid size and electrotransformation efficiency was observed. These efficiencies were about 4.5 times higher than those using the MgCl₂ method. Under these conditions, electrotransformation efficiencies of relaxed plasmid DNA treated with topoisomerase I and that linearized by EcoRI digestion were high.

Transformation of bacteria with DNA is an important method in genetic analysis studies. In a few bacterial species, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Bacillus subtilis*, *Neisseria gonorrhoeae*, *Azotobacter vinelandii*, and *Methylobacterium organophilum*, natural competence has been described. Transformation methods, such as the CaCl₂ method for *Escherichia coli*, the MgCl₂ method for *Pseudomonas aeruginosa*, and the polyethylene glycol method for *B. subtilis*, have been developed. However, many bacterial species cannot be transformed because methods induce competence have not been established.

The technique of electroporation has been used recently to transform various bacterial species. This method uses an electric current to generate membrane distortions, thereby allowing the uptake of DNA. Electroporation is a simple and easy method compared with chemically induced competence (e.g., the CaCl₂ method), and most gram-negative bacteria can be electrotransformed at high frequencies and efficiencies.

*Pseudomonas* species are known to have the ability to act as scavengers in nature, resulting in the biodegradation and removal of a large number of natural and synthetic compounds. Many studies have been made of their metabolic pathways and gene structure. The development of efficient transformation systems is necessary to facilitate these studies. *Pseudomonas putida* is frequently used as a host strain for genetic engineering. Although a few studies of electrotransformation efficiency for *P. putida* have been reported, there is little understanding of the optimal conditions for electroporation.

In the study reported here we investigated the ability of *P. putida* PpY101 to be electrotransformed with the broad-host-range vector pSUP104 and recombinant plasmid pSR134 encoding a mercury resistance gene, and optimized electroporation conditions for PpY101. Additional experiments were done to analyze the effects of DNA conformation on electrotransformation efficiencies.

**Materials and Methods**

*Bacterial strains and plasmids.* *P. putida* PpY101, *E. coli* HB101, pSUP104, and HB101(pSR134) were used in this study. Plasmid pSUP104 (9.5 kb) is a broad-host-range vector encoding the tetracycline and chloramphenicol resistance genes. Construction of a recombinant plasmid, pSR134 (18.6 kb), by inserting the mercury resistance gene into pSUP104, was described previously.

Large-scale plasmid DNA preparations from *E. coli* hosts were done using standard CaCl₂-ethidium bromide gradient centrifugation. As the final step, samples were dialyzed overnight against a 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) buffer. Some of this buffer was autoclaved and used as a control, instead of DNA samples. The boiling method was used for rapid small-scale isolations of plasmid DNA from *P. putida* cultures.

*Aparatus for electroporation.* For this study, the Gene Pulser electroporation apparatus from Bio-Rad laboratories (Richmond) was used. It was set to discharge a 25 μF capacitor through a sample of cells at an initial voltage of between 0 and 12.5 kV/cm, in conjunction with the Pulse Controller unit (Bio-Rad Laboratories), and a selection of resistors of 100 to 800 Ω in parallel with the sample. The time for a given pulse to decline to 37% from its initial setting is displayed by the apparatus as the time constant (ms). Varying the parallel resistors allowed control of the time constant. The pulses were delivered to sterile, disposable plastic cuvettes with the electrodes 0.2 cm apart.

*Preparation of cells and electroporation conditions.* *P. putida* was grown to mid-log phase (A₆₀₀ = 0.26–0.38) at 30°C with shaking in Luria-Bertani (LB) medium, prepared with 5 g instead of 10 g of NaCl per liter. The culture was immediately placed on ice and cells were harvested by centrifugation. The cells were then washed twice with sterile 300 mM sucrose and resuspended in the same solution. Some of the cell suspension was removed and the viable cells counted before the discharge. Samples of 100 μl were placed in a prechilled sterile electroporation cuvette, and plasmid DNA samples were added. The same volume of buffer was added to a control cuvette. Cuvettes were kept on ice for 10 min and then placed in the electroporation apparatus. The electrical setting in all experiments other than initial optimization experiments were as follows: set voltage, 2.5 kV (12.5 kV/cm); capacitor, 25 μF; and pulse controller parallel resistor, 200 Ω. Immediately after discharge, 900 μl of LB medium was added directly to the cuvettes. The samples were transferred to test tubes and incubated at 30°C for 2 h with shaking (140 rpm). In experiments with pSUP104 and pSR134, cells were plated onto LB agar containing 25 μg/ml...
tetracycline and 10 μg/ml HgCl₂, respectively. Samples were plated onto LB agar without antibiotics, to count the viable cells. All plates were incubated for 2 days at 30°C. Results were expressed as either electrotransformation efficiency (number of transformants/μg DNA) or as electrotransformation frequency (percentage of post-pulse viable cells transformed).

Transformation of *P. putida* PpY101 by chemical treatment. To compare the difference in transformation efficiency between electroporation and the chemical method, transformation of *P. putida* PpY101 was done as described previously, with the following modification. The cells were grown and harvested the same as for electroporation. The cells were washed with 0.1 M MgCl₂ and 0.15 M MgCl₂, and were suspended in 1/10 of the original volume of 0.15 M MgCl₂. A plasmid DNA sample was then added to 100 μl of cell suspension and incubated on ice for 30 min. The mixture was then heat-pulsed at 42°C for 2 min, 900 μl of LB broth was added, and the sample was incubated at 30°C for 2 h with shaking (140 rpm). Detection of transformants was done by the same method as for electroporation.

**DNA manipulations.** To determine whether supercoiled plasmid DNA was required for electrottransformation of *P. putida* PpY101, pSUP104 DNA was linearized by digestion with EcoRI (Takara Shuzo Co., Kyoto) or relaxed by treatment with DNA topoisomerase I (Takara Shuzo Co., Kyoto). Samples were then extracted with phenol to inactivate enzymes. DNA concentrations and conformations were then measured based on absorbance at 260 nm and agarose gel electrophoresis.

**Results**

**Optimization experiments for the electroporation of *P. putida* PpY101**

An initial experiment was done to determine the optimum electroporation conditions for *P. putida* PpY101 with pSUP104 and recombinant pSR134. The following parameters were varied: field strengths, time constant, DNA concentration, and cell concentration.

Figure 1 shows the effects of field strengths on electrotransformation efficiency. The voltage applied varied between 0 and 2.5 kV (field strength, 0 to 12.5 kV/cm). No transformants were detected below a field strength of 2 kV/cm, but the viable cell count after electroporation was affected by a field strength of over 2 kV/cm. Field strength at 12.5 kV/cm resulted in the highest electrotransformation efficiency, although the survival of *P. putida* was only 0.88% with both plasmids. In the following experiments, the field strength was set at the maximum of 12.5 kV/cm.

We tested the effects of the time constant on electrotransformation efficiency (Fig. 2). Although the resistor setting was varied between 100 and 600 Ω, there was little effect on efficiency. The viable cell count decreased with increasing time constant, and only 0.2% and 0.48% of the cells were viable at 14.9 ms (pSUP104) and 14.8 ms (pSR134), respectively. Further experiments were done at a resistance setting of 200 Ω (predicted time constant = 4.5 ms).

Figure 3 shows the effects of the DNA concentration on the electrotransformation efficiency and frequency. The electrotransformation efficiency decreased as the DNA concentration increased, while the electrotransformation frequency increased with increasing DNA concentration.

![Fig. 1. Effects of Field Strength on Electrotransformation Efficiency (●) and Post-pulse Cell Survival (□) of *P. putida* PpY101 with Plasmid pSR134 (A) and pSUP104 (B).](image1)

The pulse controller resistor was set at 200 Ω, and the actual time constant remained fairly stable for each voltage setting (mean ± 4.5 ± 0.2). Cell and DNA concentrations were set at 10⁵ CFU/ml and 1 μg/ml, respectively.

![Fig. 2. Effects of Time Constant on Electrotransformation Efficiency (●) and Post-pulse Cell Survival (□) of *P. putida* PpY101 with Plasmid pSR134 (A) and pSUP104 (B).](image2)

Field strength was set at 12.5 kV/cm. Cell and DNA concentrations were set at 10⁵ CFU/ml and 1 μg/ml, respectively.

![Fig. 3. Effects of DNA Concentration on Electrotransformation Efficiency (●) and Frequency (○) of *P. putida* PpY101 with Plasmid pSR134 (A) and pSUP104 (B).](image3)

Field strength and parallel resistor were set at 12.5 kV/cm and 200 Ω, respectively. Cell concentration was set at 10⁵ CFU/ml.
Electrotransformation of *P. putida*

Fig. 4. Effects of Cell Concentration on Electrotransformation Efficiency of *P. putida* PpY101 with Plasmid pSR134 (A) and pSUP104 (B).

Field strength and parallel resistor were set at 12.5 kV/cm and 200 Ω, respectively. DNA concentrations was set at 1 μg/ml.

Table Transformation of *Pseudomonas putida* PpY101 with Plasmid pSUP104 and pSR134 by Electroporation and Chemical Treatment

<table>
<thead>
<tr>
<th>Methods</th>
<th>Transformation efficiency (transformants/μg DNA)</th>
<th>pSR134b</th>
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<tbody>
<tr>
<td></td>
<td>Supercycled</td>
<td>Relaxed</td>
</tr>
<tr>
<td>Electroperoration</td>
<td>$1.1 \times 10^3$</td>
<td>$1.1 \times 10^3$</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>$2.4 \times 10^4$</td>
<td>$2.8 \times 10^4$</td>
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* These transformation efficiencies were for the one that had given the best results in several experiments.

pSUP104, respectively.

No difference in transformation efficiencies between pSUP104 (9.5 kb) and pSR134 (18.6 kb) was observed with either method.

**Discussion**

We defined the optimum parameters for the transformation of *P. putida* PpY101 using electroporation. Only two parameters, field strength and DNA concentration, were studied to determine optimum conditions for electrotransformation of *P. putida*. Because it was reported that use of cells at the mid-log phase yielded the maximum electrotransformation efficiency for gram-negative bacteria or in a generalized protocol, we used the mid-log phase cells for optimization experiments of *P. putida* PpY101. Our optimization experiments showed that the most important factor in increasing electrotransformation efficiency was the field strength. Other authors reported the same results using other bacteria. It has been demonstrated that high field strength is required for most bacterial strains to obtain optimal electrotransformation efficiency.

As *P. aeruginosa* was electrotransformed there was little effect of the time constant, ranging from 3.3 to 12.1 ms, on the electrotransformation efficiency. In contrast, another species, *Pseudomonas elodea*, was optimally electrotransformed at a time constant of 7.5–9.0 ms. We observed no significant influence of the time constant on the electrotransformation efficiencies between 2.3 and 12 ms (100 to 600 Ω), but efficiencies decreased at about 15 ms (800 Ω). Effects of the time constant differed among *Pseudomonas* species. From this study it appears that the decrease of efficiency is the result of the excessive number of cells killed by the long pulse.

Various sizes of plasmids have been introduced into bacteria via electroporation. Powell et al. reported no relationship between plasmid size (4.4–26.5 kb) and efficiency of *Streptococcus lactis*. In contrast, Monteiro et al. noted that the number of electrotransfomants decreased exponentially with the increase of the size of plasmids (10.2–25 kb). In this study we observed no significant difference in electrotransformation efficiencies between pSUP104 (9.5 kb) and pSR134 (18.6 kb). There are about $9.5 \times 10^8$ molecules of pSUP104 and $4.9 \times 10^8$ molecules of pSR134 in 10 ng of each plasmid DNA. These numbers of plasmid molecules may be sufficient for electrotransformation of *P. putida* PpY101.

It was reported that there was a linear relationship between DNA concentration and electrotransformation frequency for *P. aeruginosa* and *Campylobacter jejuni*. We also observed that this relationship was linear over a wide range, up to 1000 times the DNA concentration.

There are a few reports of the effect of cell concentration on electrotransformation efficiency. Fiedler and Wirth observed that an optimal concentration of *Enterococcus faecalis* was about $5 \times 10^7$ protoplasts/ml, while Monteiro et al. reported that increase of the cell concentration of *P. elodea* up to $4 \times 10^{10}$ CFU/ml led to an increase in the electrotransformation efficiency. We observed that the optimal concentration of *P. putida* PpY101 for electrotransformation was $10^9$ CFU/ml, although the reason is not clear.

concentration increased. However, a higher frequency was obtained at high DNA concentrations. It seems likely that the decrease of electrotransformation efficiency is due to excessive numbers of DNA molecules unconnected with recipient cells.

Figure 4 shows the effects of the recipient cell number on the electrotransformation efficiency. The highest electrotransformation efficiency was obtained at $10^9$ CFU/ml of cell number before the pulse.

**Effects of plasmid conformation and size on transformation efficiency**

We determined whether supercoiled plasmid DNA was required for electrotransformation of *P. putida* PpY101 with pSUP104 (Table). Equivalent amount of supercoiled, relaxed circular, and linearized forms of pSUP104 DNA were used for electroporation and chemical treatment. No significant difference was observed for transformation efficiency between supercoiled DNA and relaxed DNA in either method. When linearized DNA was used to electroporatm, 3% of transformants with supercoiled DNA was detected. In all cases, transformation efficiencies by the MgCl₂ method were lower than by electroporation. The chemical method gave 23% and 22% of transformants obtained by electroporation with supercoiled pSR134 and

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Because the mechanisms of electrotransformation are not explained, in most cases optimization of the procedure for electrotransformation with bacteria is empirical. Recently, Eynard et al. reported kinetics studies of electrotransformation for E. coli. They observed by video monitoring that the field pulse caused bacteria to orient themselves parallel to the field lines. It would seem that the electrical characteristics of the bacterial cell surface might account for the difference in the optimal parameters for electrotransformation among bacterial strains.

Transformation of bacteria with linearized plasmid DNA molecules occurs fortuitously whenever recombinant ligation mixtures from cloning experiments are used as the transforming DNA, because linear molecules will be present even when the ligation conditions are adjusted to favor the formation of the desired circular recombinant (vector-insert) DNA. We determined whether supercoiled plasmid DNA was necessary for the electrotransformation of P. putida PpY101. There are no reports on the effects of DNA conformations on the electrotransformation efficiency for P. putida. For another bacterium it was reported that electrotransformation with the linear DNA showed 0 to 0.2% of the efficiency of the supercoiled form. In our study the electrotransformation efficiencies for P. putida PpY101 using linearized and relaxed DNA were fairly high.

There are many reports on the electrotransformation efficiency in various kinds of bacteria. In E. coli, electrotransformation efficiencies were between 10⁸ and 10¹⁰ transformants/µg DNA. In other gram-negative bacteria, they have been varied and lower than in E. coli. These ranged from 3 x 10⁴ to 9 x 10⁴ transformants/µg DNA. In this study, the electrotransformation efficiencies obtained for P. putida PpY101 (1.1 x 10⁵ transformants/µg DNA) were higher, enough to allow genetic studies. In addition, the electrotransformation had about 4.5 times higher transformation efficiencies compared with the MgCl₂ method. Thus it can be seen that electroporation, which is a substitute for chemical methods, will be an effective tool for the genetic engineering of P. putida PpY101.

References