DNA Transformation of Bacillus subtilis by Electroporation

HIDEO KUSAOKE

Electroporation was applied to transformation of Bacillus subtilis. Efficient DNA transformation of protoplasts of B. subtilis was achieved by a single voltage pulse at 4−7 kV with a brief period time of 50 μsec. Electrical transformation of B. subtilis protoplasts by plasmid pUB110 DNA and plasmid pHY300pLk DNA results in efficiencies as hige as 2.5×10^4 and 2.0×10^4 transformants, respectively, per μg DNA. Plasmid pUB110 DNA could be also introduced into intact B. subtilis cells by this methods.

INTRODUCTION

Electric pulse−mediated gene transfer, electroporation, is effective for mammalian cells, plant protoplasts, and yeast [1]. Employing electrical pulses for genetic transformation of procaryotic cells has been scarecely reported. Electroporation involves the application of high−intensityelectric fields of short duration to reversibly permeabilize biomembranes. For genetic transformation, the viability of protoplasts as recipient cells is essential for DNA uptake. This method is mostly empirical, and permits uptake of free nucleic acid through the cell membrane.

Recently, several reports are available on this technique of employing pulse of high electric fields for bacteria. Shivarova et al. have reported transformation of B. cereus protoplasts at a frequency of 1.1×10^{-3} in the presence of polyethylene glycol (PEG) by application of high electric field pulses of 14kV/*cm* [1]. Taketo has shown transformation and transfection of Esherichia coli by electric field strength of 6.25kV/*cm* and a 25μF capacitor, and the results of this electroporation was more efficient than Ca^{2+}−dependent transfection and transformation [3]. Miller et al.[4] reported electrical transformation of intact Campylobacter jejuni at frequencies as high as 1.2×10^6 transformants per μg of DNA.

The aim of this study was to transform protoplasts and intact cells of B. subtilis with plasmid DNA.

MATERIAL AND METHODS

Bacterial strain and growth conditions. Bacillus subtilis Marburg 168 strain was obtained form H. Takahashi (Institute of Applied Microbiology, University of tokyo), Bacillus subtilis ISW 1214 was perchased by TAKARA SYUZO Co.,Ltd.

The strain was subcultured in Pen medium (Difco antibiotic medium No.3) at 37℃. The DM 3 regeneration medium consisted of the following sterile solution, per liter; 4% agar, 200mℓ, 1M sodium succinate (pH 7.3), 500mℓ, 5% Difco casamino acids, 100mℓ, 10% Difco yeast extract, 50mℓ, 3.5% K_2HPO_4 and KH_2PO_4, 100mℓ, 20% glucose, 25mℓ, 1M $MgCl_2$, 20mℓ, and filter-sterilized 2% bovine serum albumin, 5mℓ, containing 150 μg/mℓ kanamycin was used as the selection medium for transformants obtained from B. subtilis protoplasts. Pen agar medium comtaining 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 comtainting 5μg/mℓ of kanamycin, and it was purified by ethidiun bromid-cesium chloride equilibrium centrifugation. Plasmid pHY 300pLK was perchased by TAKARA SYUZO Co., Lted.

Preparation of protoplasts. Protoplasts of B. subtilis were prepared by a little modification of the method of Chang and Cohen [5].

Electroporation apparatus. A high voltage generator (Shimazu SSH-1 Type) that can supply electric pulses of square was constructed. Chambers (electric distance, 1 mm; volume, 20μl or 100μl) were used to obtain pulse durations of 50-500μsec and various field strengths up to 28kV/cm at 25℃. The electric field pulse was applied as a single pulse.

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

PEG-induced protoplast transformation was performed as described by Chang and Cohen.[5]

Determination of transformation and cell survival. A cell suspension with DNA was

exposed to a single electric pulse of a certain duration at 25℃. After pulsation, the cells were diluted with growth medium and then cultured at 30℃. Cell survival was expressed as the ratio of living cells (regenerants) counted at 48hr after pulsation to input bacillary cells.

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

RESULTS AND DISCUSSION

We succeeded in introducing plasmid DNA (pUB 110) into protoplasts and intact cells of B. subtilis Marburg 168 strain by electroporation in the absence of PEG [6]. Our method involves exposure of protoplasts or intact cell suspension to an electric field strength (4-16kV/*cm*) for a brief period of time (50μsec) in the presence of plasmid DNA. Values are shown as frequency and efficiency of transformation and cell survival (see Table 1). We obtained a high transformation frequency (1.5×10^{-1}) of B. subtilis Marburg 168 protoplasts with plasmid pUB110 DNA in the absence of PEG by electroporation. This value was approximately 30-times higher than that by PEG. Cell survival of protoplasts treated by electroporation was 97% though that treated by PEG 9% and lower. It was found that plasmid DNA can be introduced into around one per seven protoplasts by electroporation. With plasmid DNA pHY300pLk as a shuttle vector for B. subtilis and E. coli, electrical transformation of B. subtilis ISW 1214 resulted in efficiency of 2.0×10^{4} transformants per μg of DNA, similar to that of B. subtilis protoplasts with pUB110 DNA. We also succeeded in introducing plasmid pUB110 DNA into intact cells though electrical transformation results in frequency as low as 1.5×10^{3} transformants per μg of DNA.

We investigated the introduction of plasmid DNA into various intact Bacillus cells. The results are shown in Table 2. We also succeeded in introducing plasmid DNA intovarious Bacillus cells though their electrical transformation results in frequency as low as 1.5×10^{3} transformants per μg of DNA. Even transformation of intact B. stearothermophilus cells which is thermophile strain results in a efficiency of 1.6×10^{2} transformants per μg DNA, with a single pulse of 500μsec with an initial field strength of 6kV/*cm*.

Tabel 1　Transformation of Bacillus subtilis with plasmid DNA by electroporation

Strain and plasmid	Strain preparation	Methods	Voltages KV/cm	% Cell servival $CFU_{rec} \times 100/CFU_{tot}$	Frequency CFU_{obs}/CFU_{tot}	Efficiency $CFU_{obs}/\mu g$ DNA
Bacillus subtilis Marburg 168						
pUB 110	Protoplasts	Electroporation[a)]	7	97	1.5×10^{-1}	2.5×10^{4}
pUB 110	Protoplasts	Electroporation with PEG[b)]	7	2	1.8×10^{-3}	1.2×10^{4}
pUB 110	Protoplasts	PEG[c)]	-	9	4.5×10^{-3}	1.6×10^{4}
pUB 110	Intact cells	Electroporation[d)]	16	11	1.1×10^{-4}	1.5×10^{3}
Bacillus subtilis ISW 1214						
pHY300pLK	Protoplasts	Electroporation[a)]	4	74	1.4×10^{-2}	2.0×10^{4}
pHY300pLK	Protoplasts	PEG[c)]	-	28	1.1×10^{-4}	1.7×10^{4}

CFUtot = total colony forming units used in the electroporation or PEG exeriments, CFUrec = colony forming units recovered after electroporation or PEG, and CFUobs = colony forming untits observed as antibiotic resistant.

Intact cells were treated with and suspended in SMM buffer. Protoplasts were treated with lysozyme in SMMP buffer and suspended in SMM buffer.

a) The DNA concentration for transformation was 17 μg/ $m\ell$. The experimental procedures were described under Matrerial and Methods.

b) The mixture of 1 μl of 1.7 μg/ μl DNA and 300 μl of 40% PEG 6000 were added to 99 μl of protoplasts suspension in SMM buffer, then pulse was field.

c) The mixture of 1 μl of 1.7 μg/ μl DNA, 1 μl of 2×SMMP buffer, and 1.5 $m\ell$ of 40% PEG 6000 were added to 0.5 $m\ell$ of protoplasts suspension in SMMP buffer, and then the mixture was kept for 2hr at room temperature.

d) The mixture of 96 μl of cell suspension in SMM buffer and 4 μl of 1.7 μg/ μl DNA in chamber was subjected to a electric shock under the condition of electric strength of 16kV/ cm.

This indicates that electroporation may be applied to gene transfer into thermophile cells. The cells survival of these intact cells was approximately 60% under the conditions found to be for the transformation process.

Plasmid DNA was isolated from transformants by electroporation and then analyzed by agarose gel electrophoresis. The electrophoretic mobility of plasmid DNA isolated fromintact B. subtilis Marburg 168 transformants was identical to that of authentic preparations isolated from B. subtilis (see Fig. 1). That from B. subtilis ISW 1214 was also identical to that of authentic preparations isolated from B. subtilis (see Fig. 2). The restriction endonuclease cutting patterns observed with Eco RI were also identical (data not shown).

Tabel 2 Transformation of intact Bacillus subtilis cells with plasmid DNA

Strain	Plasmid	Pulse conditions		Efficiency	Cell survival (%)
		Voltages (kV/cm)	Duration (μsec)	$CFU_{obs}/\mu g$ DNA	$CFU_{rec} \times 100/CFU_{tot}$
Bacillus cereus	pUB 110	7	500	1.8×10^2	60
Bacillus stearothermophilus	pUB 110	6	500	1.6×10^2	63
Bacillus subtilis ISW 1214	pHY300pLK	6	500	2.3×10	63

The electroporation conditions were the same as in caption d of Table 1.

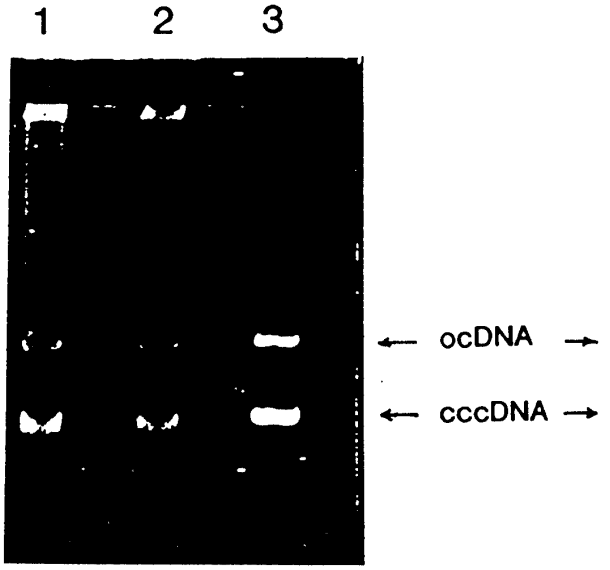

Fig. 1 Agarose gel electrophoresis of pUB 110 isolated from intact B. subtilis Marburg 168.

Lane 1 and 2, pUB 110 isolated from B. subtilis transformed to kanamycin resistance by electroporation with pulses of a field strength of 16kV/cm (lane 1) and 28kv/cm (lane 2) with a duration of 50μsec; lane 3, pUB 110 isolated from B. subtilis.

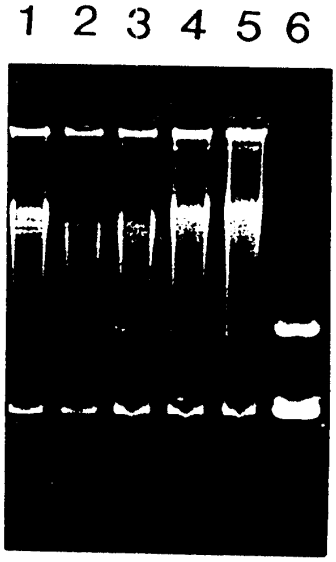

Fig. 2 Agarose gel electrophoresis of pUB 110 isolated from intact B. subtilis ISW 1214.

Lane 1−5, pUB 110 isolated from B. subtilis transformed to kanamycin−resistance with pulses of a field strength of 7kV/cm with a duration of 500μsec; lane 6, pUB 110 isolated from B. subtilis.

The results we have presented show not only that intact bacterial cells as well as bacterial protoplasts can be efficiently transformed by electroporation, but also that

this method is rapid, easy to perform, and requres minimal sample preparation. Electroporation can be used to develop a genomic cloning system that will allow the establishment of gene banks containing Bacillus sequences in Bacillus hosts. As a result of our experimentwith B. subtilis and other microorganisms, we believe that electroporation can successfully be applied to many bacterial species. We will report the detailed electrical pulse conditions for DNA transformation and cell survival of various Bacillus strains elsewhere.

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