An Improved Method for DNA Transformation of Intact Cells of *Bacillus subtilis* by Electroporation

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ABSTRACT

An electroporation procedure for the plasmid-mediated genetic transformation of intact cells of Bacillus subtilis was performed. The method was optimized for B. subtilis ISW1214, and the transformation of ISW1214 with plasmid pUB110, using the mixture of 0.3 M sucrose, 1 mM $CaCl_2$ and 1 mM sodium citrate as a buffer for pulsation, results in a maximum efficiency of 2.8×10^4 transformants per μg DNA, with a single pulse duration of 500 μsec with an initial electric field strength of 7 kV/cm. Transformation of ISW1214 occurred at comparable frequencies with pUB110(4.5 kbp), pC194(2.9 kbp), and pE194(3.7 kbp) plasmids. Plasmid DNA isolated from transformants was indistinguishable from authentic preparation of plasmid DNA on gel electrophoretic analysis. The electroporation was simple, rapid, and sampled at 10 μl-small volume of cell suspension($\sim$ 1×10^{10} cells/ml, 4 pg DNA/μl).

INTRODUCTION

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, plant protoplasts and yeast[1,2]. Electrical impulses have also been used for the transformation of procaryotic cells with plasmid DNA[3-17]. Transformation by electroporation is a recent method that has been successfully employed with a number of bacterial species. This technique is less tedious and time-consuming and has proved to be useful in hitherto untransformable species. Gene transfer by electroporation has been widely applied to Gram-positive bacteria[3-13] as well as Gram-negative bacteria[14-17]. Calvin et al.[15] reported that the electric transformation of intact Escherichia coli cells with plasmid pUC18 results in a high efficiency of 3.3×10^8 transformants per μg DNA. Using Lactobacillus casei as Gram-positive bacterium, Chassy et al.[4] reported the transformation of intact cells with an electric field strength of 6.25 kV/cm and a 25 μF capacitor, and the results being the efficiency of as high as 8.5×10^4 per μg of plasmid pLZ15 DNA.

Formerly we succeeded in gene transfer into intact cells as well as protoplasts of Bacillus

subtilis as a Gram-positive bacterium[13]. Even transformation of intact cells resulted in a maximum efficiency of 1.5×10^3 transformants per μg DNA (plasmid pUB110). Bacillus strains offer many potential advantages in the producion of cloned products, which are secrected to the growth medium, and have been widely used for production of industrial enzymes in large-scale fermentation processes. The coming aim of our study is further to determine the optimum conditions for high efficiency and simple protocol gene transfer to B. subtilis cells. We investigated the effects of electric field conditions (pulse field strength, pulse duration, and the number of pulses) and cytophysiologicalconditions (osmotic pressure, ion composition, the temperature of cell suspension during electric treatment). We here describe an improved and simple protocol which makes small scale (10 μl) with high transformation efficiency possible.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacillus subtilis Marburg 168 strain (trpC2, phe-1) was obtained from H. Takahashi (Institute of Applied Microbiology, University of Tokyo). B. subtilis ISW 1214 strain (HsrM1, LeuA8, MetB5) was purchased from TAKARA SHUZO Co., LTD. Both strains were subcultured in Pen assay medium (Difco antibiotic medium No.3) at 37 ℃. Pen assay agar medium containing each antibiotic was used as the selection medium for transformants.

Plasmid DNA. Plasmid pUB110 (4.5 kbp, Km^R) was obtained from Y. Yoneda (Nippon Gene Co. LTD.), pC194 (2.9 kbp, Cml^R) and pE194 (3.7 kbp, Ery^R) were obtained from T. Tanaka (Mitsubishi Kasei Institute of Life Science), and shuttle vector pHY300pLK (4.87 kbp, Ap^R, Tc^R) was purchased from TAKARA SHUZO Co. LTD. Plasmid DNAs were isolated from ISW1214 and Marburg 168 by standard procedures [19] and was purified for use in electroporation by centrifugation to equilibrium in cesium chloride/ethidium bromide density gradients.

Buffers. Usually the mixture of 0.5 M sucrose, 0.1 mM $MgCl_2$, and 0.1 mM maleic acid (pH 6.5) was used for washing buffer, and the mixture of 0.3 M sucrose, 1 mM $CaCl_2$, and 1 mM sodium citrate (pH 6.0) was employed as an electroporation-suspension buffer. SMMP medium[18] (3.5% Pen, 17.1 % sucrose, 0.23 % maleic acid, 0.41 % $MgCl_2$) was used for dilution and expression medium.

Electroporation apparatus. A high voltage generator (SHIMAZU SSH-I TYPE) that can supply electric pulse of square waves was constructed. Chambers (elctric distance, 1 mm; volume, 10 μl) were used to obtain pulse durations of 10 to 500 μsec and various field strengths up to 28 KV/cm at 0 - 50

℃. The electric field pulse was applied as a single pulse or as a train of up to 4 successive pulses.

DNA transformation by electroporation. Bacterium was stationary cultured overnight on pen medium at 37 ℃, and a portion of the bacterium was inoculated into 100 ml of pen medium and the cells were grown with shaking at 37 ℃ to OD660 = 0.4～0.5 (～ 1 x 10^8 cells/ml). This culture was centrifuged at 5000 rpm for 10 min and the cells were washed with washing buffer. The cells were resuspended in suspension buffer at a density of about 5 x 10^9 - 8 x 10^{10} cells/ml. The cell suspension was cooled at 0 ℃, and the 196 μl- portion was transferred to a 0.5 ml Eppendorf tube. Four μl of plasmid DNA solution (0.2 μg/μl) were added, followed by careful mixing with a pipette. The cell suspension was incubated at room temperature for 10 min. Ten μl of the cell suspension were placed in a chamber. The chamber was incubated at pulse temperature for 3 min. Soon after, the cells were subjected to electroporation at each pulse and temperature. After the electric treatment, the cells were left for 5 min at room temperature. The cells were pipetted out of the chamber to an Eppendorf tube. The chamber was then washed with SMMP buffer, and the remaining cells in the electrodes were recovered into the same tube. The suspension in the tube was suitably diluted in SMMP medium and then plated on pen agar medium. Prior to plating on media containing antibiotics, the diluted cells were incubated at 30 ℃ for 1.5 hrs to allow the phenotypic expression of antibiotic resistance makers. Plates were incubated at 37 ℃ for 1 to 2 days, the numbers of regenerated colonies were counted. As the control, those of regenerated colonies that had not been subjected to electroporation were similarly counted.

Determination of transformation efficiency, transformation frequency, and cell survival was made as described in our previous paper[13].

RESULTS

Effects of electric field strength on transformation and cell survival.

We first investigated the optimization of electric field strength conditions. The results are shown in Fig. 1. The cell survival with pulsation showed sudden drop for the electric breakdown of cell membranes at electric field strength over 5 kV/cm with the pulse duration of 500 μsec at 0 ℃. The transformation efficiency increased with increasing electric field strength in the range of 0 to 7 kV/cm, but decreased with increasing it in the range of 8 to 20 kV/cm. Maximum transformation efficiency was given as 2.6 x 10^3 transformants per μg plasmid DNA with an electric field strength of 7 kV/cm.

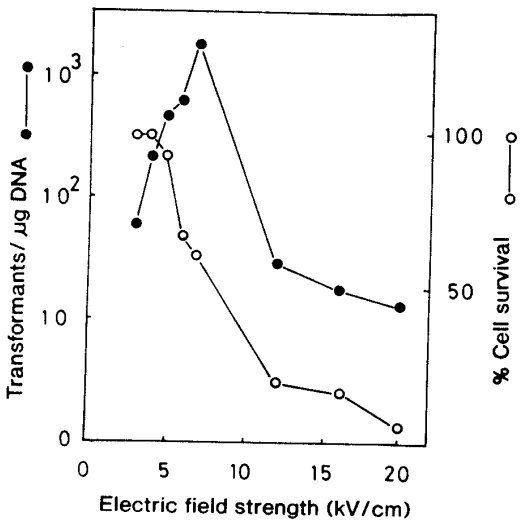

Fig. 1. Effects of The Electric Field Strength on Transformation Efficiency and Cell Survival with B. subtilis ISW 1214.
Cells suspension of B. subtilis ISW1214 (5×10^9/ml) in buffer containing 0.5 M sucrose, 0.1 mM maleic acid and 0.1 mM $MgCl_2$ were mixed with 4 μg/ml of pUB110 and subjected to electropoaration at the indicated electric field strength at a pulse duration of 500 μsec, at pulsation temperature of 0 ℃, and at pulsation of one time.

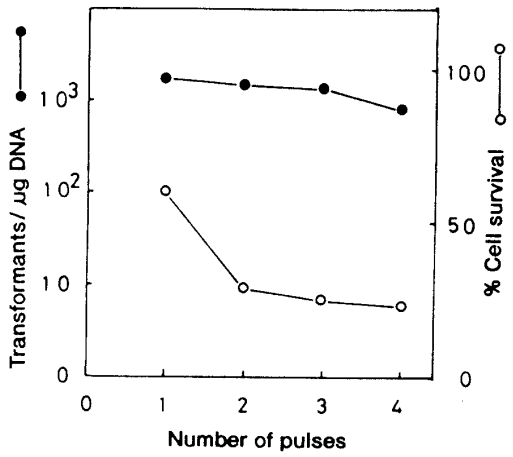

Fig. 2. Effect of The Number of Successive Pulse on Transformation Efficiency and Cell Survival with B. subtilis ISW1214.
Cells with plasmid DNA were exposed in buffer and subjected to repeated electric pulses of a duration of 500 μsec and a field strength of 7 kV/cm, at 3 min intervals. The other conditions were the same as in Fig. 1.

The influence of the number of pulses used for transformation by electroporation is illustrated in Fig. 2. The cell survival showed the decrease with increasing number of pulses in the range of 0 to 4 times. The transformation efficiency as well as the cell survival also gradually decreased with increasing number of pulses.

The effects of the pulse duration are shown in Fig. 3. The cell survival decreased with increasing pulse duration in the range of 0 to 500 μsec. However, the highest transformation efficiency, in this experiment, was found at 500 μsec, which is the maximum pulse duration for the equipment used here. The data suggests that increased pulse times would yield higher transformation efficiencies.

Effect of pulse temperature on transformation efficiency and cell survivsal. Fig. 4 shows the effect of the various temperature of cell suspension in chamber during pulsation on transformation efficiency and cell survival, using the mixture of 0.3 M sucrose, 1 mM sodium citrate, and 1 mM $CaCl_2$ as a buffer of cell suspension, at fixed field strength of 7 kV/cm and pulse duration of 500 μsec. The cell survival was gradually decreased with increasing pulse temperature in the range of 0 to 50 ℃. The transformation efficiency exponentially increased with respect to pulse temperature in the range of 0 to 30 ℃, but pulse temperature over 30 ℃ showed sudden decrease of the efficiency. The highest transformation efficiency, 2.8×10^4 transformants

per μg DNA in this experiment, was found at 30 ℃.

Effect of the composition of the cell suspension buffer on transformation. The effects of the osmotic pressure of the suspension buffer are shown in Fig. 5. The cell suspension was pulsated with the electric field strength of 7 kV/cm and pulse duration of 500 μsec at 30 ℃ with various sucrose concentration of 0 to 1 M. The transformation efficiency reached a maximum at a sucrose concenration of 0.3 M, and then gradually declined toward the higher concentration. Next we examined the optimization of various salts in buffer on transformation. As shown in Table I, comparatively efficient salts in buffer with 0.3 M sucrose and 1 mM sodium citrate were $CaCl_2$, sodium bicarbonate, and sodium acetate. Using 1 mM $CaCl_2$, the maximum efficiency and the cell survival were 2.8×10^4 per μg DNA and 66 %, respectively. Even to addition of lysozyme or sodium dodecylsulfate as bacteria-lysis agents in cell suspension medium, high transformation was not obtained (data not shown). Fig. 6 shows the effect of concentration of $CaCl_2$ on transformation and cell survival. The experiment was done with buffer containing 0.3 M sucrose and 1 mM sodium citrate with various concentration of $CaCl_2$ in the range of 0 to 2 mM. The cell survival was gradually increased, and the transformation efficiency was enhanced with the increase of the concenration of $CaCl_2$ up to 1 mM. But it was slightly reduced at more than 1 mM of $CaCl_2$. The transformation efficiency was reached a maximum of 2.4×10^4 transformants per μg DNA, when $CaCl_2$ concentration was 1 mM.

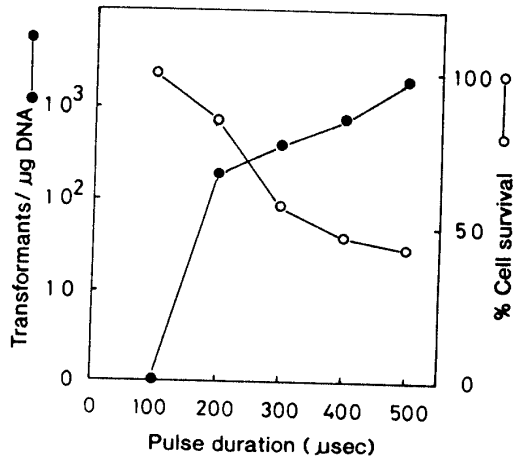

Fig. 3. Effects of The Pulse Duration on Transformation Efficiency and Cell Survival with B. subtilis ISW1214. Cells with plasmid DNA were suspended in buffer and exposed to a single pulse of various durations. Other conditions were the same in Fig. 1.

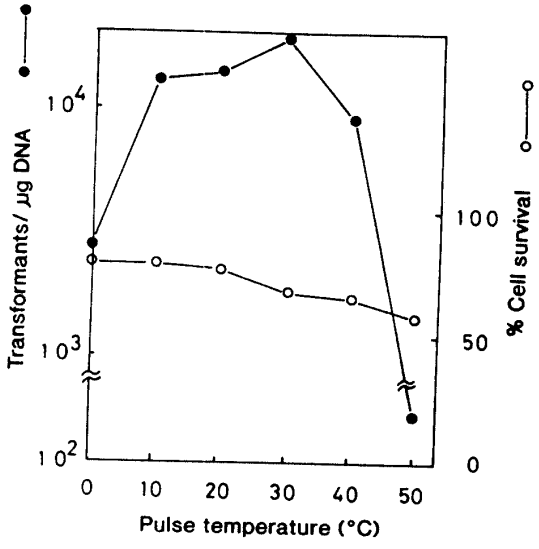

Fig. 4 Effects of Pulse Temperature on Transformation Efficiency and Cell Survival. Cells with plasmid DNA were suspended in buffer containing 0.3 M sucrose, 1 mM $CaCl_2$, and sodium citrate and subjected to electroporation under the conditions of the same as in Fig. 1.

TABLE I. EFFECTS OF SALTS IN BUFFER ON TRANSFORMATION OF B. subtilis ISW1214 WITH PLASMID pUB110

Buffer	Cell survival (%)	Frequency	Efficiency (Transfromsnts/μg DNA)
A	45	1.1×10^{-5}	7.5×10^{3}
B	80	7.0×10^{-6}	5.6×10^{3}
C	41	2.7×10^{-6}	1.4×10^{3}
D	79	1.4×10^{-6}	1.2×10^{3}
E	52	3.5×10^{-6}	3.4×10^{3}
F	91	3.4×10^{-6}	4.1×10^{3}
G	66	2.0×10^{-4}	2.8×10^{4}

Cells were treated with plasmid DNA to electroporation in the following buffer; A, 0.3 sucrose, 1 mM sodium citrate, and 1 mM sodium bicarbonate; B, 0.3 M sucrose, 1 mM sodium citrate, and 1 mM sodium acetate; C, 0.3 M sucrose, 1 mM sodium citrate, and 1 mM $BaCl_2$; D, 0.3 M sucrose, 1 mM sodium citrate, 1 mM $MgCl_2$, and 1 mM $CaCl_2$; E, 0.3 M sucrose, 1 mM sodium citrate, and $MgCl_2$; F, 0.3 M sucrose, 1 mM maleic acid and 1 mM $MgCl_2$; G, 0.3 M sucrose, 1 mM sodium citrate, and 1 mM $CaCl_2$. Each value of pH from buffer A to G was adjusted to 6.0. The other pulsation standard conditions were the same as those in Fig. 5.

TABLE II. TRANSFORMATION OF B. subtilis ISW1214 WITH VARIOUS PLASMID DNAs

Plasmid	Pulsation temperature (℃)	Efficiency (Transformants/μg DNA)
pUB110	20	1.5×10^{4}
	30	2.8×10^{4}
pC194	20	1.0×10^{4}
	30	1.1×10^{4}

Cells with various plasmid DNAs were suspended in buffer G indicated in Table I and subjected to electroporation under the standard condition(7 kV/cm, 500 μsec) at a single pulse and at pulsation temperature of 30 ℃.

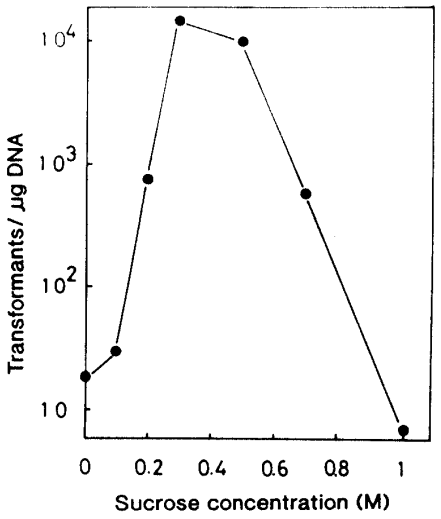

Fig. 5. Effects of Sucrose Concentration on Transformation Efficiency.
Sucrose was added to the buffer containing 1 mM $CaCl_2$ and 1 mM sodium citratea at the condition indicated and the suspension was subjected to electroporation under the standard conditions (7 kV/cm, 500 μsec) at a single pulse and at pulsation temperatrure of 30 ℃.

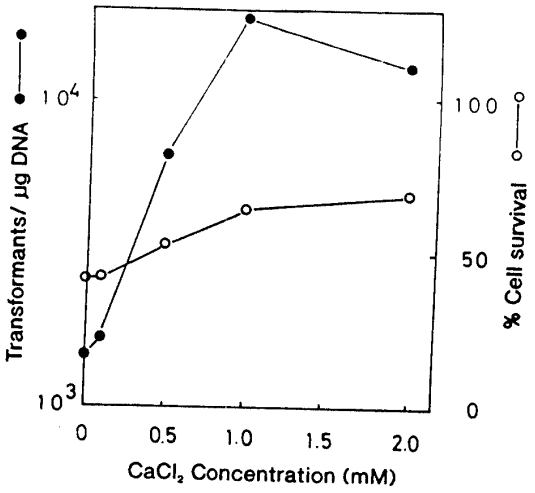

Fig. 6. Effects of Concentration of $CaCl_2$ on Transformation Efficiency and Cell Survival.
$CaCl_2$ was added to the buffer containing 0.3 M sucrose and 1 mM sodium citrate at the concentration indicated and the cell suspension with plasmid DNA was subjected to electroporation under the standard condition (7 kV/cm, 500 μsec) at a single pulse and at pulsation temperature of 30 ℃.

Transformation of Bacillus subtilis with various plasmid DNAs. The plasmid DNAs pUB110, pC194, pE194, and pHY300pLK were transformed into B. subtilis ISW1214 cells (Table II). Transformation efficiency of B. subtilis was 2.8×10^4 per μg DNA at pUB110, 1.1×10^4 at pC194, 1.7×10^3 at pE194 and 2.6×10^2 at pHY300pLK, respectiviely. From these results, gene transfer into ISW1214 found that plasmids pUB110 and pC194 effectiviely were transformed into cells and that these plasmids were about 10 to 10^2 times as high as plasmids pE194 and pHY300pLK. Plasmid DNAs were isolated from transformants and analyzed by agarose gel electrophoresis (Fig. 7). The electrophoresis mobility of plasmid DNAs isolated from ISW1214 transformants was identical to those of authentic preparation isolated from B. subtilis. Twenty transfromants with each plamid DNA were examined, and all yielded a plasmid profile identical to that in native B. subtilis strain. The restriction endonuclease cutting patterns observed were also identical (data not shown).

Transformation of various Bacillus cells with plasmid pUB110. Transfer of pUB110 into several Bacillus cells by electroporation was evaluated (Table III). Intact cells of B. subtilis Marburg 168 strain were transformed at efficiency of 1.5×10^3 per μg DNA, using a buffer of 0.5 M sucrose, 0.01 mM maleic acid, and 0.01 mM $MgCl_2$, as shown in our previouspaper[13]. Under the optimum pulse-conditions obtained in this study, trsansformation of ISW1214 with plasmid pUB110 resulted in an efficiency of 2.8×10^4 transformants per μg DNA. Transformation of B. cereus results in efficiencies as low as 1.2×10^2 transformants per μg DNA[20]. Using thermophilic bacterium, B.

TABLE III. TRANSFORMATION OF SEVERAL BACILLUS STRAINS WITH PLASMID DNA

strain	Cells[a)]	DNA	Pulse conditions[b)]	Buffer[c)]	Cell survival (%)	Frequency	Efficiency (transformants/μg DNA)	Source or reference
B. cereus 9592	Pro.	pUB110	14 kv/cm, 5μsec, RT	A	-	1.1×10^{-3}	8.8×10^{3}	(3)
B. cereus 569	In.	pC194	6.25kv/cm, 25μF, 0℃	B	46	2.0×10^{-5}	-	(11)
B. cereus F4165/75	In.	pGK12	6.25kv/cm, 25μF, 0℃	C	-	-	6.5×10^{1}	(8)
B. brevis HPD31	In.	pBAM101	6.25kv/cm, 25μF, 0℃	D	35	-	1.3×10^{4}	(12)
B. subtilis Marburg 168	Pro.	pUB110	7 kV/cm, 50 μsec, 0 ℃	E	98	2.4×10^{-1}	4.0×10^{4}	Our previous study(13)
B. subtilis Marburg 168	In.	pUB110	16 kV/cm, 400 μsec, 25 ℃	E	28	1.0×10^{-4}	1.5×10^{3}	Our previous study(13)
B. subtilis ISW1214	In.	pUB110	7 kV/cm, 500 μsec, 30 ℃	F	66	2.0×10^{-4}	2.8×10^{4}	This study
B. subtilis ISW1214	In.	pC194	7 kv/cm, 500 μsec, 30 ℃	F	60	1.5×10^{-4}	1.3×10^{4}	This study
B. cereus	In.	pUB110	12 kV/cm, 500 μsec, 0 ℃	C	20	3.5×10^{-6}	1.2×10^{2}	Submitted (20)

a) Cells for electroporation indicate Pro., Protoplasts and In., intact cells.

b) Each value indicates electric field strength, pulse duration, and pulation temperature, respectively.

c) Buffers used indicate A, 40 % polyethyleneglycol; B, 10mM N-2-hydroxylethylpiperazine-N'-2-ethanesulfuric acid (HEPES), pH 7.0; C, 272 mM sucrose, 1 mM $MgCl_2$, 7 mM potassium phosphate, pH 7.4; D, 15 % glycerol, 272 mM sucrose, 16 mM HEPES, 1 mM $CaCl_2$; E, 0.5 M sucrose, 0.01 mM Maleic acid, and 0.01 mM $MgCl_2$ (pH 6.5); F, 0.3 M sucrose, 0.1 mM sodium citrate, and 0.1 mM $CaCl_2$ (pH 6.0); and G, 0.3 M sucrose, 0.1 mM maleic acid, and 0.1 mM $MgCl_2$ (pH 7.0).

stearothermophilus, gene transformation was tried, however, we didn't succeed in gene transfer intothermophilic intact cells, under the conditions investigated in this study (data not shown).

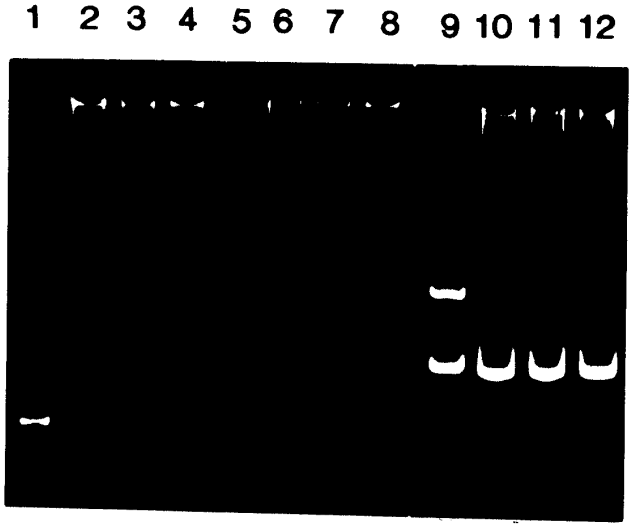

Fig. 7. Detection of Plasmid DNAs by Agarose Gel Electrophoresis. Plasmid DNAs were isolated from the B. subtilis ISW1214 and then electrophoresed in 0.7 % agarose gel. Lanes: 1, B. subtilis harboring-plasmid pC194; 2-4, plasmid DNAs isolated from ISW1214 transformed with pC194; Lane 5, B. subtilis harboring-plasmid pE194; 6-8, plasmid DNAs isolated from ISW1214 transformed with pE194; 9, B. subtilis harboring-plasmid pUB110; 10-12, plasmid DNAs isolated from ISW1214 transformed with pUB110.

DISCUSSION

The results we have presented show that B. subtilis intact cells can be effectively transformed by electroporation. Electroporation is simple, easy to perform, and requires minimal sample preparation. Now, the transformation frequency for ISW1214 intact cells with plasmid DNAs has been shown to be moderately increased by application of an electric pulse. We here reported the application to gene transfer in ISW1214 using a square wave pulse generator. Cell survival on pulsation of intact cells depended largely on the field strength and the pulse duration. Electric field strength of 7 kV/cm, that gave high transformation efficiency, may represent irreversible damage to cells, and higher electric field strength seems to cause the io n flux for membrane breakdown on pulsation. The electric field strength and pulse duration of the d ischarge waveform are important, and optimum values may depend on the bacterial species and strain tested. Within the ranges we examined, the strength of the initial electric field, pulse duration, and pulse temperature have a great effect on the transformation efficiency, though number of pulse is uneffective on it.

Intact B. subtilis cells are also effectively transformed on the use of $CaCl_2$ as a salt in buffer. The use of $CaCl_2$, in comparison with that of $MgCl_2$, results in a ca. 10-fold increase in the efficiency for intact cells. This indicates that Ca++ ions make DNA insersion into cells easier than Mg++ ions. Another way to obtain efficient transformation is to use a wide DNA concentration range of 0.1 μg/ml to 100 μg/ml, showing that the DNA dose-response curve was linear (data not shown). Our electroporation method can be applied to various plasmid DNAs and several intact cells of Bacillus strain (Table III). Using the mixture of 0.3 M sucrose, 1 mM sodium citrate and 1 mM $CaCl_2$ as a buffer, electric pulses for the transformation of intact cell under the

pulsation conditions of a combination of a field strength of 7 kV/cm, a pulse duration of 500 μsec, and pulsation of one time are more effective than those of other workers[3,11,12].

Electroporation will be broadly applicable to many species of bacteria, and will dramaticaly 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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