Gene Transfer into Some Species of *Bacillus* by Electroporation

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ABSTRACT

Electroporation was applied to ten spesies of *Bacillus*. It was found that transformation of three strains of *B. licheniformis*, *B. cereus*, and *B. subtilis* with plasmid DNA (pUB110) was possible and resulted in maximum efficiencies of 1.0×10^3, 1.2×10^2, and 9.3×10^4 per μg DNA, respectively, under optimal pulse conditions of an electric field strength of 7 kV/cm and a duration of 500 μs with a single squared pulse at 0 ℃. The transformants were confirmed to harbor the same intact plasmid by agarose gel electrophoresis.

INTRODUCTION

Electroportion was succeefully applied to many systems of mammalian cells and plants protoplasts.[1] Recently gene transfer was obtained with walled systems, such as bacteria and yeasts, and electroporation is, in fact, now routinely used in many bacterial genetics laboratories. The bacilli were the first gram-positive bacteria to be transformed by electroporation. Shivarova et al.[2] used total plasmid DNA from *B. thuringiensis* subsp. *galleriae* harboring pUB110 to transform protoplasts of *B. cereus* by electroporation. We found that plasmid DNA can be introduced into not only protoplsts but also intact cells of *B. subtilis* by electroporation.[3] We also reported that the transfromation efficiency as high as 9.3×10^4 transformants per μg of purified plasmid pUB110 was obtained.[4] *Bacillus* strain produces a large variety of extracellular enzymes, some of which are of industrial

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importance, and offers many potential advantages in the production of cloned gene products.

Electrotransformation methods have now been applied to at least seven species of *Bacillus*. In this study we tried the transformation of not only these spesies but also other species of *Bacillus* by electroportion.

Materials and Methods

Bacterial strains and media. The strain used in this study are described in Table 1. Pen broth[3] was used for culturing bacteria. For plating, Pen broth was solidified with 1.5 % agar. All cultures were incubated at 37 ℃ with flask-shaking. To select for plasmid-containing transformants, kanamycin (Km) for pUB110 plasmid or chloramphenicol (Cm) for pC194 were added to agar plates at concentration of 25 μg/ml and 10 μg/ml, respectively.

Table 1 Ten Species of *Bacillus* Used

Strain	Culture[a] temperature (℃)	Source/ Reference[b]
B. amyloliquefaciens IFO1414	30	IFO
B. brevis IFO3331	30	IFO
B. cereus IFO3466	30	IFO
B. coagulans IFO12583T	37	IFO
B. licheniformis IFO12107	30	IFO
B. megaterium IFO13498	30	IFO
B. natto IFO3335	30	IFO
B. pumilus IFO14367	30	IFO
B. stearothermophilus IAM11001	55	IAM
B. subtilis ISW1214	30	Ref. 3

a) Institute for Fermentation, Osaka
b) Institute of Applied Microbiology, University of Tokyo

Plasmid isolation. Plasmid pUB110 obtained from Y. Yoneda (Nippon Gene Co. Ltd.) was prepared by the alkaline method from a plasmid-haboring *B. subtilis* grown in Pen medium containing 5 μg/

ml of kanamycin, and it was purified by ethidium bromide-cesium chloride equiblirium centrifugation.[3] Plasmid pC194 obtained from T. Tanaka (Tokai University) was prepared as same in plasmid pUB110.

Electroporation procedures. Electroporation was done by a small modification of the method of Kusaoke *et al*.[3] Usually a mixture of 0.5 M sucrose, 0.1 mM $MgCl_2$, and 0.1 mM maleic acid (pH 7.0) was used for washing buffer, and a mixture of 0.3 M of sucrose, 1 mM $CaCl_2$, and 1 mM sodium citrate (pH 7.0) was used as an electroporation-suspension buffer. SMMP medium was used for dilution and expression medium. A high voltage generator (Somatic Hybridizer Shimadzu SSH-1 Type, Shimadzu Corp.) that can supply an electric pulse of a square wave was mainly used. Chamber (electric distance,1 mm; volume, 20 μl) were used to obtain various electric field strengths up to 28 kV/cm at pulse duration of 500 μsec. The electric field pulse was applied as a single pulse. An Electric Gene Transfer Equipment GTE-10 (Shimadzu) was used as a high voltage generator that can supply an electric pulse of a decayed wave.

The bacterium was grown in Pen medium with shaking at 37℃ to OD_{660} = 0.4 ~ 0.5 (~1 x 10^8 cells/ml), and the culture was centrifuged at 5000 rpm for 10 min, and then the cells were washed with washing buffer. The cells were suspended in suspension buffer at a density of about 1.0 x 10^{10}- 6.0 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 (*ca*. 0.2 μl/ μl) were added and mixed with a pipette. Twenty μl of the cell suspension were placed in a chamber, and electroporated at 0 ℃. After the electric treatment, the cells were pipetted out of the chamber to an Eppemdorf tube, and the suspension in the tube was suitably diluted in SMMP medium, and then placed on Pen agar medium. Before plating on media containing antibiotics, the diluted cells were incubated at 37 ℃ for 24 to 48 h, the number of regenerated colonies was

counted.

Measurement of transformation efficiency and cell survival. The measurement of transformation efficiency and cell survival was done as described in our previous paper.[3)]

Results and Discussion

Growth curve of some species of Bacillus. Figures 1 and 2 show the growth curves *Bacillus* species. Culture was made with 100 ml-flask shaking at the temperature shown in Table 1. All strains showed a typical growth curve of bacteria. Seven strains except for *B. coagulans* and *B. megaterium* indicated the same growth curve patterns which reached at stationary phase at *ca.* 7 - 8 h. However the culture of *B. coagulans* reached the stationary phase at *ca.* 15 h and was longer than that of eight other strains. Two strains of *B. coagulans* and *B. megaterium* had lower growth potency than that of other strains, showing that OD_{660} at stationary

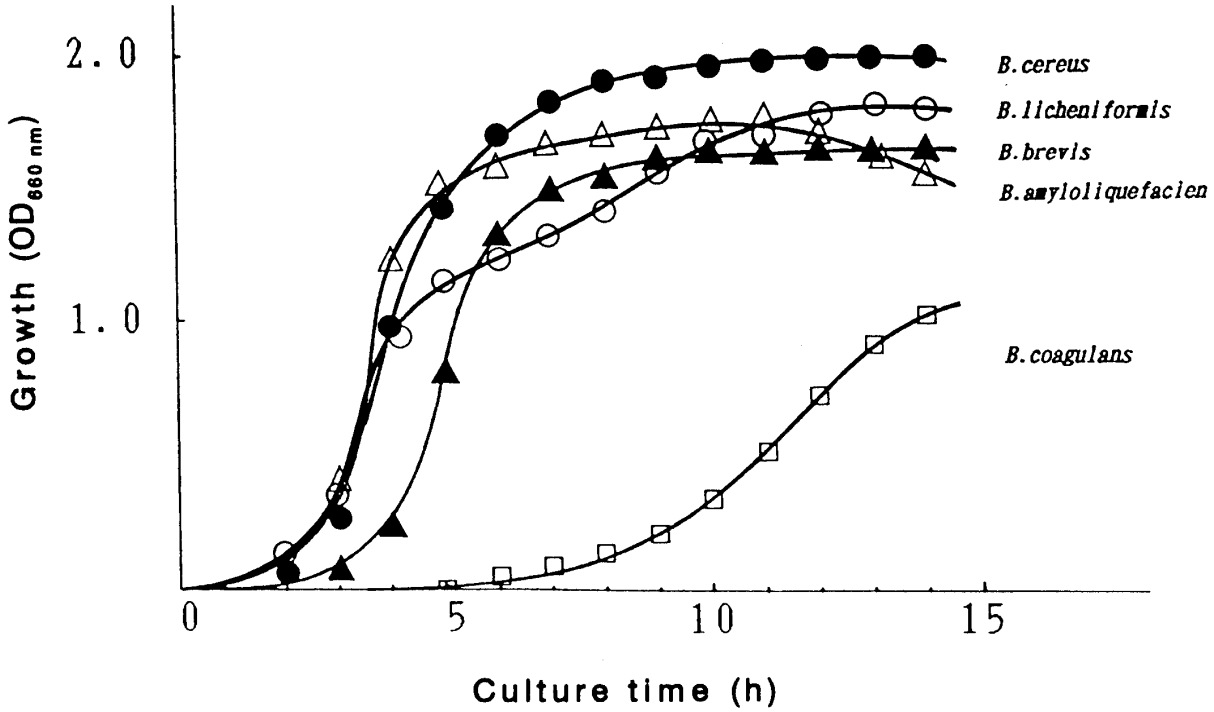

Fig. 1 Growth Curves of Five Species of Bacillus.

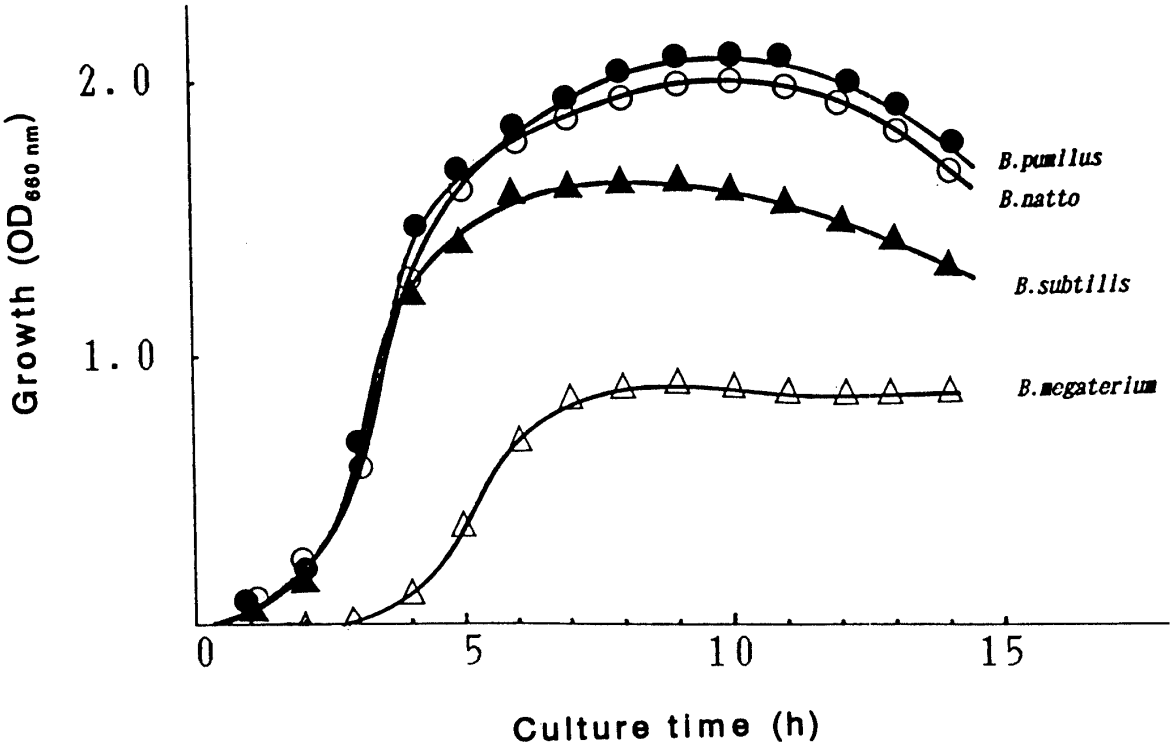

Fig. 2 Growth Curves of Four Species of *Bacillus*.

phase of these strains was *ca.* 1.0, compared with values of OD_{660} of *ca.* 2.0 in other seven strains. Therefore by using the strain which has the higher growth potency, gene transfer may be mediated by electroportion.

Transforamtion of ten species of *Bacillus* with plasmid pUB110 by electroporation. In our previous paper[4], transformation of intact *B. subtilis* ISW1214 resulted in a maximum efficiency of 9.3 x 10^4 transformants per μg DNA (pUB110), using a cell concentration of 7.6 x 10^{10} cell/ml and DNA concentration of 4 μg/ml in buffer containing 0.3 M sucrose, 1 mM $CaCl_2$, and 1 mM sodium citrate (pH 7.0) under optimal pulse conditions of an electric field strength of 7 kV/cm and a duratioin of 500 μs with a single squared pulse at 0 ℃.

In this study, using aquared and decayed waves as a high voltage electric pulse, eight strains of *Bacillus* were electroporated with field strength between 5 kV/cm and 13 kV/cm (see Table 2). Only *B. licheniformis* was transformed with pUB110 by electroportion. A transformation efficiency as high as 1.0 x 10^2 transformants per

μg of plasmid DNA was obtained for *B. licheniformis*, using a single squared wave pulse of 7 kV/cm at duration of 500 μs. Electroportion also could transform *B. licheniformis* with a decayed wave pulse, having the same value of transformation efficiency with a squared wave pulse. The cell survival showed the values between 51 % and 67 %, indicating the higher cell survival than those of *B. subtilis* and *B. cereus*.

The transformation efficiency of *B. licheniformis* and *B. cereus* was lower values of about 10^2 order than that of *B. subtilis*. This indicated that it is difficult to form the pore through the cell membrane and the cell wall in the case of *B. cereus* and *B. licheniformis*. Transformation of seven strains of *Bacillus* except for *B. cereus*, *B. licheniformis*, *B. stearothermophilus* and *B. subtilis* was impossible in this study. In these strains, it is seemed that gene transfer into cells through the cell surface by pore formation with electric pulse did not resulted. Even if plasmid DNA can be transferred in these cells, it is presumed that the transformants was not obtained by a reason why the replication of DNA by the methylase reaction of plasmid will be protected and consequently the cells can not be grown. As shown in Figures 1 and 2, though strains of *B. pumilus*, *B. natto*, *B.* brevis, and *B. amyloliquefaciens* showed the high grow potency, the cell growth did not affect on transformation.

We tried the transformation of bacilli strains with plasmid pC194, unfortunately transformation in all strains used did not succeed. Considerable reseach on transformation is focused on ascertaining conditions required for specific strains of interest. Most of this effoft is directed at an empirical search for satisfactory, or optimal conditions for a selected strain.

The findings may do little to shed insight into the mechanism of bacterial electroporation or the underlying cases for inability to electroportion. Systematic mechanistic studies are needed in order to explain the factors that negatively affect the success of electroporation of gram-positives. We will study the mechanisms of electroporation of *Bacillus* with DNA in detail later.

Table 2 Transformation of Various Strains of *Bacillus* with plasmid DNA

Strain	Pulse conditions		Survival (%)	Efficiency (No././ μg DNA)	Source/ Reference
	Pulse wave	Field strength (kV/cm)			
B. amyloliquefaciens	Squared	5	94	0	This work
	Squared	7	34	0	
	Decayed	5	63	0	
	Decayed	7	32	0	
	Decayed	10	5	0	
B. brevis	Squared	5	-	0	This work
	Squared	7	-	0	
	Decayed	5	-	0	
	Decayed	7	-	0	
B. cereus	Decayed	5	57	0	This work
	Decayed	7	50	1.2×10^2	
B. coagulans	Squared	5	62	0	This work
	Squared	7	43	0	
	Decayed	5	70	0	
	Decayed	7	49	0	
B. licheniformis	Squared	7	67	1.0×10^3	This work
	Decayed	5	78	4.9×10^2	
	Decayed	7	51	6.2×10^2	
B. megaterium	Squared	5	60	0	This work
	Decayed	5	59	0	
	Decayed	*7*	*83*	*0*	
B. natto	Squared	5	86	0	This work
	Squared	7	74	0	
	Decayed	5	94	0	
	Decayed	7	80	0	
	Decayed	10	30	0	
B. pumillus	Squared	5	80	0	This work
	Squared	7	50	0	
	Decayed	7	88	0	
	Decayed	10	12	0	
	Decayed	13	2	0	
B. stearothermophilus	Squared	20	46	1.2×10^2	To be published
	Decayed	10	70	2.3×10^2	
B. subtilis	Squared	7	60	9.3×10^4	Ref. 3
	Decayed	7	66	2.8×10^4	

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References

1) D. C. Chang, B. M. Chassy, J. A. Saunders, and A. E. Sowers, in "Guid to Electroporationand Electrofusion, "Academic Press, Inc., New York, 1992, pp209-264.

2) N. Shivarova, W. Forster, H. E. Jacob, and R. Grivarova, *Z. All. Mikrobiol.*, **23** 595-599(1983).

3) H. Kusaoke, Y. Hayashi, Y. Kadowaki, and H. Kimoto, *Agric. Biol. Chem.*, **53**, 2441-2445(1989).

4) M. Ohse, K. Takahashi, Y. Kadowaki, and H. Kusaoke, *Biosci. Biotech. Biochem.*, **59**, 1433-1437 (1995).

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