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High Frequency Electroporation-Transformation System for Coryneform Bacteria

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전기장 충격법에 의한 코리네형 세균의 고효율 형질전환

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ABSTRACT

Escherichia coli/ *Corynebacterium glutamicum* shuttle vectors, pECCG1 and pECCG2 were constructed by joining a 3.0 kb *C. glutamicum* cryptic plasmid pCB1 and a 3.94 kb *E. coli* plasmid pACYC177. Using the plasmid pECCG1, various parameters involved in electroporation system including electric field strength, resistance, DNA concentration, cell concentration and growth stage were investigated independently and optimized for the high efficiency transformation of coryneform bacteria. Transformation efficiencies of 10^6 transformants / μg of plasmid DNA were achieved with *Corynebacterium glutamicum*.

INTRODUCTION

Coryneform bacteria are microorganisms that have been traditionally extensively used for amino acid production (1). Despite their industrial importance and their role in animal and plant diseases, little effort was dedicated until a few years ago to advance the molecular biology of corynebacteria(2, 24). Extensive "classical" mutagenesis of some corynebacteria (*Corynebacterium glutamicum*, *Brevibacterium lactofermentum*, *Brevibacterium flavum*) was used to obtain improved strains for the production of lysin, glutamic acid, threonine, tryptophan and a variety of other amino acids and flavor enhancing nucleotides(Xanthosine-5'-monophosphate, Inosine-5'-monophosphate, Guanosine-5'-monophosphate)(3).

Recent advances in biotechnology has facilitated the *in vitro* construction of recombinant DNA and its transformation into a suitable host. Currently, it is possible to amplify the genes which are bottlenecks in amino acid biosynthesis pathways, thus to lead to increased production of amino acids. To apply the technique to the improvement of amino acid production, it is basically very important to develop a host-vector system and efficient transformation system. Various PEG mediated protoplast transformation methods were reported(4-9). The system met some difficulties in generating protoplast and regeneration to

viable cells. Coryneformmbacteria are usually more resistant to lysozyme treatment than other gram-positive bacteria and their protoplasts take long time to regenerate to viable cells. So many factors are involved in protoplast transformation system that transformation efficiencies are very variable from experiment to another and from strain to strain.

As an attempt to improve transformation efficiency with *Corynebacterium glutamicum* we employed electroporation system. Electroporation involves the application of a brief and high voltage pulse to a suspension of cells and DNA. The result is transient membrane permeability and the subsequent uptake of DNA. The technique of electroporation was first used to induce cells to fuse via their plasma membrane(10-11). Potter *et al.* (12) modified and extended the system to allow the introduction of exogeneous DNA into a broad spectrum of animal cell types. Electroporation systems have already been applied successfully to the transformation of some gram-positive bacteria including *Lactobacillus*(13), *Streptococcus*(14), *Bacillus*(15) and *Clostridium*(16). Meilhoc *et al.*(17) have achieved transformation efficiencies of a 10^7 transformants/㎍ of plasmid DNA with intact yeast cells by using of electroporation system. Dower *et al.*(15) applied the technique to the transformation of *E. coli* and obtained 10^9 to 10^{10} transformants/㎍ with strains LE392 and DH 5, and plasmids pUC18 and pBR329.

In this report, we describe the construction of hybrid plasmid having the replication origin of *C. glutamicum* and selectable drug resistance marker gene. The optimal conditions of parameters for the efficient and rapid transformation of *C. glutamicum* using electroporation system are also discussed.

MATERIALS AND METHODS

Bacterial strains and plasmids

C. glutamicum ATCC13058 was used as a source of cryptic plasmid pCB1. Plasmid pACYC177(19) was isolated from *E. coli* ATCC 37031. *E. coli* DH5(r_k^-, m_k^+) competent cells were purchased from Bethesda Research laboratories, Inc.. *C. glutamicum* ATCC13032 and JS231 were used as the host strains for the transformation of constructed shuttle vectors.

Media and bacterial cultivation

LB medium (1% Tryptone, 0.5% Yeast extract, 1% NaCl, pH 7.0) and LB agar(1.8%) were used for the routine growth of bacteria. *E. coli* and *C. glutamicum* were grown at 37℃ and 30℃, respectively. TYG medium (1% Tryptone, 1% Yeast extract, 0.5% Glucose, 0.5% NaCl, pH7.0) was used for the cultivation of *C. glutamicum* to isolate plasmid DNA. For the protoplast transformation and electroporation-transformation, *C. glutamicum* grown in glycine medium (LB medium supplemented with 2% glycine and 2% glucose)was used.

Plasmid DNA preparations and DNA manipulations

Large-scale plasmid preparation from *E. coli* and *C. glutamicum* was carried out by the alkaline lysis method of Birnboim(20) and further purified with CsCl-ethidium bromide density gradient centrifugation(21). For the rapid screening of transformants via plasmid isolation, we followed th method of Kieser(22). Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. Inc.. Digestion and ligation of DNAs were performd by following the instructions of the enzyme suppliers.

Transformation of *E. coli* strains with plasmid DNAs or cross ligated DNAs were performed as described by Morrison(23). Transformants were screened on LB plate with an appropriate concentration of antibiotic. PEG-meadiated protoplast transformation of *C. glutamicum* was carried out by the method of Yoshihama(9).

Electroporation equipment and electroporation procedure

Electroporation was carried out using a Gene-Pulser system (Bio-Rad Laboratories, Richmond, CA). The system is composed of Gene-Pulser apparatus, pulse controller unit and gene pulser cuvettes of 0.2cm electrode gap.

C. glutamicum was grown to an A_{600} of 2.0 at 30℃ with shaking 260 rpm in 10ml of glycine medium. After chilling on ice for 30 min the cultures were harvested and washed twice with 100ml of cold 1mM HEPES. The cells were finally resuspened in 4ml of solution of 10% glycerol and 1mM HEPES. Cuvettes and sliding holder were chilled on ice for 5 min prior to use. 40㎕ of cell suspension and 2㎕ of DNA dissolved in TE buffer(10mM Tris, 1 mM EDTA, pH 8.0) were mixed in cold cuvette and

pulsed once. Immediately 1ml of LB medium was added to the cuvette. The cells were shaken at 30℃ for 1 hr for the transformed gene to be expressed and then plated on LB agar containing 50㎍/ml of kanamycin.

RESULTS

Construction of *E. coli*/*C. glutamicum* shuttle vectors

To develop a cloning system for *C. glutamicum* it is necessary to construct a replicable plasmid containing a selectable genetic marker which is expressed in that strain. Cryptic plasmid pCB1 isolated from *C.glutamicum* ATCC 13058 was chosen as a replicon of corynebacterium because of its small size(3.0 kb) and high copy number(24). As a selectable genetic marker, kanamycin resistance determinant was thought to be most desirable because it is one of the drug marker genes which were reported to express very efficiently in *C. glutamicum*(25). As a source of kanamycin resistant determinant, plasmid pUB110 from *Bacillus subtilis* and plasmid pACYC177 from *E. coli* were considered. The sizes of pUB110 and pACYC177 are 4.4kb and 3.94kb, respectively. Finally, pACYC177 was used to develop shuttle vectors because of its small size and convenience of DNA manipulation in *E. coli* system.

Brief schemes for the construction of shuttle vectors and restriction sites of plasmid pCB1 and pACYC177 are presented in Fig. 1. Plasmid pCB1 was linearized by digestion with *Bcl*I and ligated with *Bam*HI-digested plasmid pACYC177. The ligates were transformed into *E. coli* DH5 competent cells. The transformants were screened on LB agar plate containing 50㎍/ml of kanamycin. Finally, we selected two types of plasmids which differ in orientation. The orientation was checked by digesting the plasmids with restriction enzyme *Hind*Ⅲ. The two different-oriented plasmids were named pECCG1 and pECCG2, respectively.

Electrophoretic picture of intact plasmid pECCG1, its *Eco*RI digest and *Hind*Ⅲ digest is shown in Fig. 2.

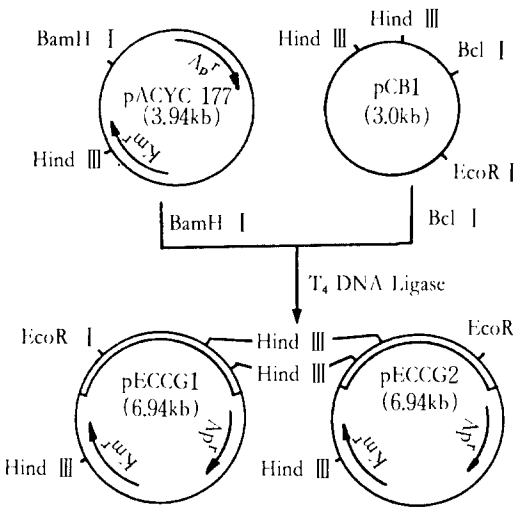

Fig. 1. Schematic diagram for the construction of shuttle vectors between *E. coli* and *C. glutamicum*.

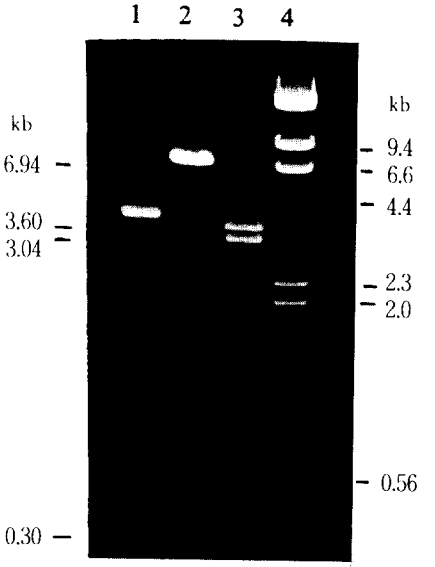

Fig. 2. Electrophoretic picture of pECCG1.
1. Intact plasmid pECCG1
2. pECCG1 digested by *Eco*RI
3. pECCG1 digested by *Hind*Ⅲ
4. λ DNA digested by *Hind*Ⅲ

Protoplast transformation of *C. glutamicum*

The chimeric plasmids isolated from *E. coli* DH5 were used to transform *C. glutamicum* via PEG-mediated

Table 1. Minimal medium composition for *C. glutamicum*

Components	per Liter
$MgSO_4$ $7H_2O$	0.4 g
$FeSO_4$ $7H_2O$	20mg
$MnSO_4$ $4H_2O$	20mg
NaCl	50mg
Glucose	10 g
$(NH_4)_2SO_4$	10 g
K_2HPO_4	1 g
Urea	2 g
d-Biotin	0.1mg
Thiamine-HCl	0.1mg
L-leucine	0.1 g

* The pH was adjusted to 7.0 with 2N HaOH.

protoplast transformation system reported by Yoshihama (9). At first, wild-type *C. glutamicum* ATCC 13032 was used as a host strain but a contamination problem by some unknown microorganisms was observed. To solve this problem, the host strain was switched from wild-type ATCC13032 to JS231 which derived from ATCC13032 and needs leucine for growth. By using two kinds of minimal plates, one was supplemented with leucine and the other was not, it was possible to tell the true transformants from contaminants. The minimal medium composition in shown in Table 1. Both transformants of plasmid pECCG1 and pECCG2 were obtained by using JS231 as a host strain. The transformants were confirmed by analyzing the size and some of their restriction sites.

Optimization of conditions for *C. glutamicum* electroporation

Several results have been reported on the applicaton of electroporation system to the bacterial transformation including both gram-positive and gram-negative bacterial. We tried to apply this system to the transformation of *C. glutamicum* JS231 using *C. glutamicum*/*E. coli* shuttle vector pECCG1 isolated from JS231. Several factors which were thought to be most important for the efficient transformation were studied independently. All the following experiments were performed by using *C. glutamicum* JS231 grown in glycine medium for about 24 hr because cells grown in glycine media showed 10−100 times higher transformation efficiencies than those grown in the medium without glycne. In most of experiments, 3×10^8 cells of JS231 and 0.1μg of plasmid DNA were used per transformation, unless otherwise stated.

Resistance: In *E. coli* system, highest transformation frequency was reported at the conditions of 12.5kV/cm, 25μF and 200 ohms(18). We determined to start at more stringent condition than that of *E. coli* because *C. glutamicum* has a more rigid cell wall than *E. coli*. Electric field strength and capacitance were set at 12.5kV/cm and 25μF, respectively and resistances ranging from 100 ohms to 1000 ohms were tested. The results are shown in Fig. 3. The number of transformants increased with increasing resistance up to 600 ohms and decreased abruptly thereafter. Cell viability decreased inversely with increasing resistance. These indicate that the optimal resistance value exists and it was 600 ohms. Decrease of the transformants at the resistance higher than 600 ohms seemed to result from decrease in cell viability.

Electric field strength: Now that optimal resistance was determined at the conditions of 12.5kV/cm and 25 μF, the effect of varying electric field strength was exam-

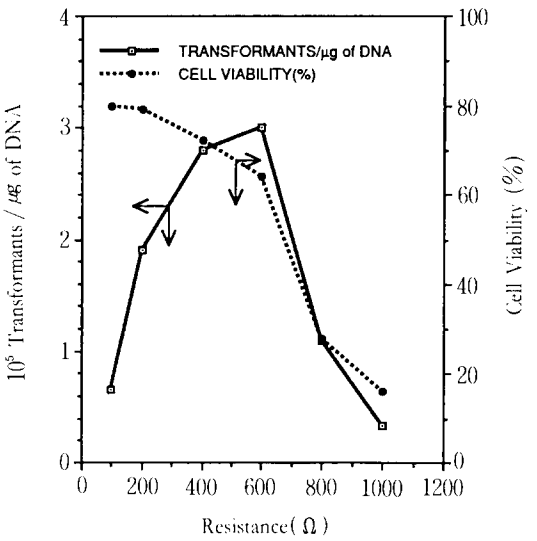

Fig. 3. Effect of resistance variation on the transformation efficiency and cell viability of *C. glutamicum* JS231.

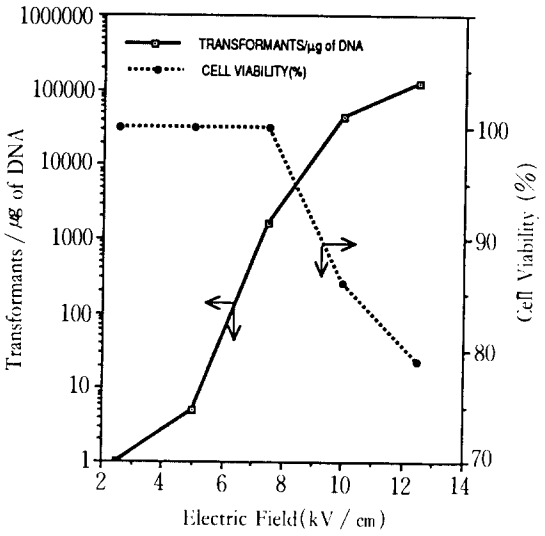

Fig. 4. Effect of electric field strength on the transformation efficiency of *C. glutamicum* JS231.

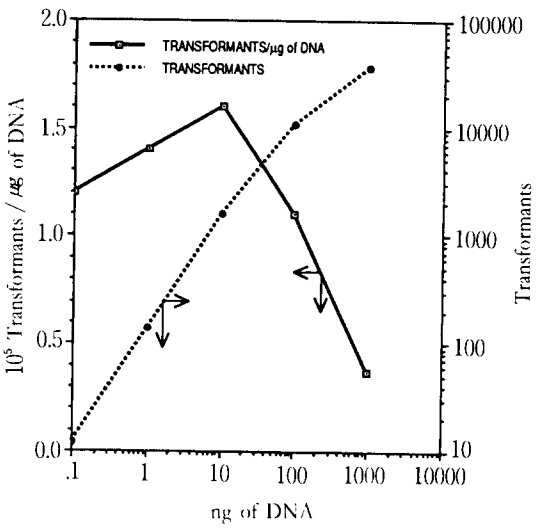

Fig. 5. Effect of increasing concentration of DNA on the transformation efficiency of *C. glutamicum* JS231.

ined with resistance set at 600 ohms. As presented in Fig. 4, transformants were increased in parallel with increasing electric field strength up to 12.5 kV / cm. Transformation efficiency reached maximum at 12.5 kV / cm which was the same value set at the previous experiment. Cell viability was maintained 100% up to 7.5 kV / cm and fell down thereafter. As the data illustrate, higher transformation efficiencies could be achieved at field strength higher than 12.5kV / cm. But we could not do the test at field strength higher than 12.5 kV / cm due to instrument limitation. These results suggest that electric field strength and resistance have somewhat complementary effect on transformation efficiencies of *C. glutamicum*.

DNA concentration: The same numbers of *C. glutamicum* JS231 cells grown in glycine medium were transformed with increasing concentration of plasmid pECCG1 from 0.1ng to 1μg in 42μl of the suspension of cells and DNA. As shown in Fig. 5, the number of transformants increased linearly with increasing concentration of plasmid DNA, when log-log scale was used. The transformation efficiency was highest when 10ng of plasmid DNA was used. It is interesting that there existed a point of optimal DNA concentration for the highest transformation efficiency. Meanwhile, when protoplast transformation system was used, the optimal DNA concentration was not observed but transformation efficiencies only decreased with increasing concentration of DNA.

Cell concentration: 3×10^8 cells were used per transformation in all the previous experiments. Optimal cell concentration was examined for higher transformation efficiencies. Number of cells tested per transformation ranged from 0.75×10^8 to 2.4×10^9. Other parameters were adjusted to the results of the previous experiments *i. e.*, 12.5kV / cm, 600 ohms and 25μF. As shown in Fig. 6, optimum cell number per transformation proved to be 6×10^8 and it showed 4×10^5 transformants per μg of plasmid DNA. But there was no significant difference in the transformation efficiencies in the range of cell numbers between 1.5×10^8 and 6×10^8, but sharp decrease in transformation efficiencies was observed in cell numbers deviating that range.

Cell harvest stage: Effect of cell harvest stage during the cell growth in glycine media on the transformation efficiencies was studied. The results were illustrated in Fig. 7. Transformation efficiency was highest when the cells were harvested at early log stage, *i. e.*, an A_{600} of 1.38. When cells were harvested at an A_{600} of 2.0, the transformation efficiency decreased to less than a quarter

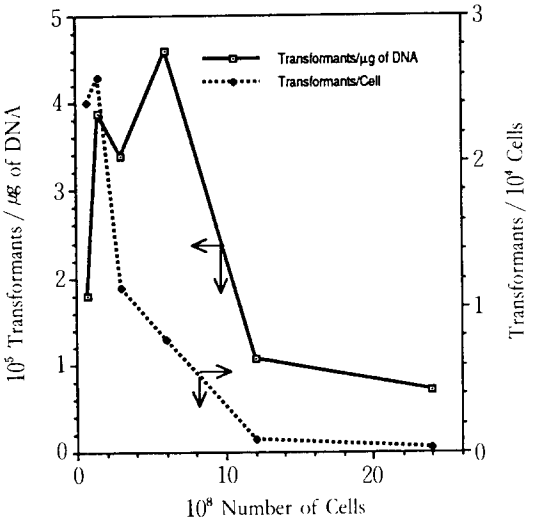

Fig. 6. Effect of cell concentration on the transformation efficiency of *C. glutamicum* JS231.

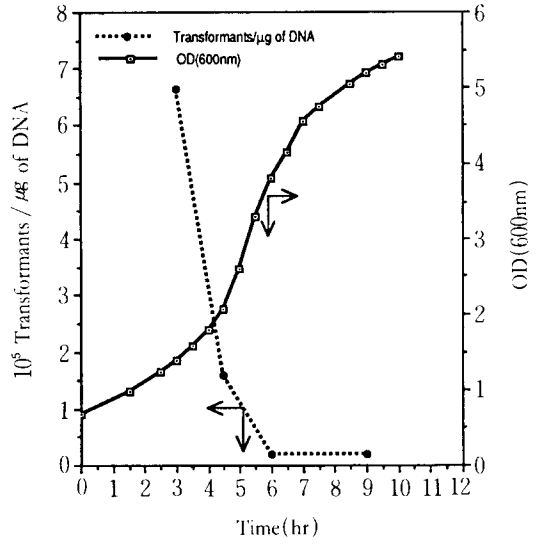

Fig. 7. Effect of cell harvest stage during the cell growth on the transformation efficiency of *C. glutamicum* JS231.

of the transformation efficiency of the cells harvested at an A_{600} of 1.38. In conclusion, cells must be harvested

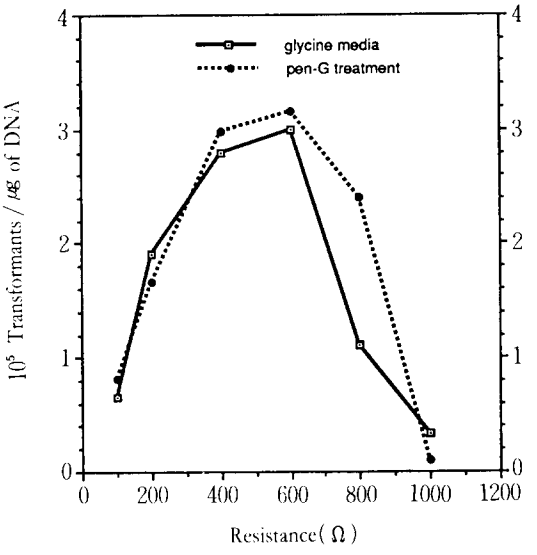

Fig. 8. Comparison of effects of glycine and penicillin-G in culture media on the transformation efficiency of *C. glutamicum* JS231 following resistance.

in early log stage to achieve a high transformation efficiency.

Effect of penicillin-G in the culture medium: Transformation efficiencies of the cells grown in the presence of penicillin-G were studied to compare with those of cells grown in glycine media. Cells were grown in TYG medium containing 0.3 unit / ml of penicillin-G. As shown in Fig. 8, there was no significant difference in the tendency of transformation efficiencies.

DISCUSSION

All the results presented here suggest that cells of *C. glutamicum* grown in glycine medium can be efficiently transformed by electroporation. The method proved to have several advantages when compared with PEG-mediated protoplast transformation, *i. e.*, 100 fold higher transformation efficiencies, simple procedure, rapid and reliable results and applicability to frozen cell stocks. When we tried protoplast transformation with *Corynebacterium glutamicum* we only achieved transformation efficiencies of 10^4 transformants / μg of plasmid DNA.

Several factors influenced the number of transformants obtained in electroporation system. The addition of glycine (2%) to the growth medium showed 10- to 100-fold enhancement of transformation efficiencies. Harvesting cells in early exponential phase was also a very important prerequisite for efficient electrotransformation. In fact, when cells harvested at A_{600} lower than 1.0 were used, transformation efficiencies of 10^6 transformants / ㎍ DNA could be obtained. Cells prepared for electroporation must be suspended completely, otherwise arcing occurs and the number of transformants decreases significantly. When electroporations were performed correctly at the conditions of 25㎌, 12.5kV / ㎝ and 600 ohms, RC time constants, where R is resistance and C is capacitance, were between 13.5 to 15.0 msec. We also made an attempt to pulse the mixture of cells and DNA more than once to see if the number of transformants increases, but the number of transformants did not increase at all.

ABBREVIATIONS

PEG: Polyethylene glycol, Ap^r: Ampicillin resistant, Km^r: Kanamycin resistant, pen-G: Penicillin G, kb:kilo base pairs, LB: Luria Bertani, A: Absorbance

요 약

대장균과 코리네형 세균간의 shuttle vector pECCG1과 pECCG2를 제작하고, plasmid pECCG1과 glycine 배지에서 배양한 *Corynebacterium glutamicum*을 사용하여 전기장 충격법에 의한 형질전환에 있어서 여러 조건을 조사한 결과 세포 현탁액 40㎕와 DNA 2㎕의 혼합액 사용시 저항 600 ohms, 전기장의 세기 12.5 kV / ㎝, DNA양 10ng, 세포수 4.5×10^8와 세포회수 시기를 1.0이하의 A_{600}으로 했을때 10^6transformants / ㎍ of DNA의 형질전환 효율을 보였다.

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