Kor. J. Appl. Microbiol. Bioeng.
Vol. 13, No. 4, 345 - 348 (1985)

Transformation of *Bacillus subtilis* Protoplast by Recombinant Plasmid DNA

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(Received August 17, 1985)

재조합 Plasmid DNA에 의한 *Bacillus subtilis*의 형질전환

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(1985년 8 월 17일 수리)

Recombinant chimeric plasmid constructed with Xba I digested pUB110 and -pE194 was transformed by polyethylene glycol induced protoplast transformation system into *Bacillus subtilis* BR 151 on the mannitol regeneration media, and two genes of antibiotics resistance were expressed simultaneously in the transformant.

Transformation frequency of the recombinant plasmid was 6.5×10^{-5} on the mannitol regeneration agar plate containing neomycin and erythromycin. The replication of recombinant plasmid in the recipient cells was confirmed by the alkaline extraction method and agarose gel electrophoresis.

Despite of transformation system steadily developed for gene transfer in *Bacillus* since the report of Spizizen[1], polyethylene glycol (PEG) induced fusion of bacterial protoplast was firstly reported in *Bacillus megaterium* by Fodor and Alföldi[2] and *Bacillus subtilis* by Schaeffer et al.[3]

From a series of these experiments, Chang and Cohen[4] improved PEG-treated protoplast transformation system by plasmid DNA which is an essential step in most cloning experiments. However protoplast transformation of *Bacillus* has been reported by several researchers[5-9]. Moreover, there was no investigation of protoplast transformation of *B. subtilis* by recombinant chimeric plasmid, and scarcely anybody used the mannitol regeneration agar for transformation of *B. subtilis* protoplasts to avoid inactivation of neomycin by sodium succinate[11].

In the present report, transformation of *B. subtilis* protoplasts by recombinant chimeric plasmid in the mannitol regeneration agar media and the other regeneration media is described. Particulary we would like to describe high frequency of regeneration and transformation by using the mannitol hypertonic regeneration media.

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Materials and Methods

Bacterial strains

Bacillus subtilis IE31 BR151 (*try*C2, *lys*3, *met*B10) and *Bacillus subtilis* IE32 BR151 (*try*C2, *lys*3, *met*B10) were used as the recipient cell in the transformation experiments. Plasmid pUB110 (Neo^R, Km^R) and pE194 (Em^R) were isolated from *Bacillus subtilis* BD366 (pUB110) and *Bacillus subtilis* IE7 (pE194) which were transformed by plasmids from *Staphylococcus aureus*[10].

Media

B. subtilis BD366 (pUB110) and *B. subtilis* IE7 (pE194) were grown in Spizizen's minimal medium with supplemented casein hydrolysate and yeast extract containing 5μg/ml neomycin or 5μg/ml erythromycin. *B. subtilis* BR151 of recipient cells were grown in PAB (Penassay broth,

Difco, Antibiotic media No. 3). After transformation, transformants were plated on TBAB (Tryptose blood agar base, Difco), DMB regeneration plate (DMB) and the mannitol regeneration agar media (MA). DMB hypertonic plate used for regeneration of *Bacillus* was modified from DM3 medium described by Chang and Cohen[4]. DMB contained 8g of agar, 500ml of 1M sodium succinate (pH 7.3), 100ml of 5% casein hydrolysate, 50ml of 10% yeast extract, 100ml of 3.5% K_2HPO_4 −1.5% KH_2PO_4, 10ml of 50% glucose, 20ml of 1M $MgCl_2$, 5ml of filtered 2% bovin serum albumin and 5μg/ml erythromycin in 1000ml. The mannitol hypertonic regeneration plate for protoplast regeneration was prepared by the method of Chen and Spizizen.[11] It contained 2g of casamino acid, 2g of yeast extract, 3.2g of agar, 280ml of 1M mannitol, 8g of gelatin, 4ml of 50% glucose, 8ml of 1M $MgCl_2$, 40ml of 5% K_2HPO_4, 60ml of distilled water and 5μg/ml of neomycin.

Preparation of plasmid DNA

Plasmid pUB110 and plasmid pE194 were isolated by the method of Gryczan *et al.*[10] After phenol treatment of cleared lysate, the ccc-DNA was purified by two cycles of CsCl-EtBr equilibrium gradient ultracentrifugation with 36K for 50hrs at 25°C using Sw 56 rotor.

Rapid small scale plasmid extraction was carried out as described by Birnboim and Doly[12]. Electrophoretic analysis of the plasmids was accomplished on 0.8% vertical agarose gels in Tris-phosphate buffer (pH 7.8) at 100V for 2hrs.

Protoplast formation

B. subtilis BR151s were cultured overnight in 40ml of PAB at 30°C. This precultured cells were transferred to 50ml of PAB to give klett 7 and incubated with shaking at 37°C for 1.5hrs to klett 70 ($1\times10^8-5\times10^8$ cell per ml on TBAB). After observation of cell growth, the cultured cells were harvested by centrifugation (5000rpm. Sorvall SS-34 Rotor) for 15min at RT. Cell pellet was resuspended in 5ml of SMMP containing 5mg/ml of lysozyme, and incubated for 3hrs at 37°C with gentle shaking. After microscopical observation, protoplasts were centrifuged at 4500rpm for 15min and washed two times with 5ml of SMMP. For transformation experiments, protoplasts were resuspended in 5ml of SMMP.

Transformation

PEG-induced protoplast transformation was performed as described by Chang and Cohen[4]. 0.5ml of protoplast suspension was added to the Corex tube containing plasmid DNA and equal volume of 2×SMM, and mixed gently. Subsequently 1.5ml of PEG (40% wt/vol., MW 6000) was added into the tube and swirled for 2min at room temperature, and diluted with 5ml of SMMP (SMM: 0.5M sucrose. 0.02M maleate, 0.02M $MgCl_2$; SMMP: 2×SMM and 4×PAB) and the protoplasts were harvested by centrifugation. The harvested protoplasts were suspended in 1ml of SMMP, and shaked for 2hrs at 30°C. The transformed protoplasts were diluted with SMMP appropriately, and plated on DMB plate and mannitol agar with or without antibiotics.

Digestion of plasmids

Purified pUB110 and pE194 were linealized with XbaI under the buffering conditions recommended by the supplier (BRL). Plasmid pUB110 was digested with EcoRI. Digestions were terminated by heat treatment and phenol extraction.

Ligation of digested plasmids

Ligation of digested pUB110 and pE194 with XbaI and of digested pUB110 with EcoRI were performed overnight at 4°C in 66mM Tris-HCl (pH 7.6), 6.6mM $MgCl_2$, 10mM dithiothreitol, 0.4mM ATP and 10U T_4 DNA ligase of BRL.

Results and Discussion

Ligation of digested plasmids

Electrophoretic patterns of ligated recombinant plasmids from pUB110 and pE194 linealized with XbaI are shown Fig. 1. We found 5 main bands of recombinant plasmids and several thin bands. Because of nontreat of alkaline

Fig. 1. Agrose gel electrophoresis of ligated recomibinant plasmids.

Lane 1: pUB110 digested with Xba I, 2: pE194 digested with Xba I, 3: ligated recombinant plasmids, 4: ϕ29/EcoRI, 5: *E. coli* V517.

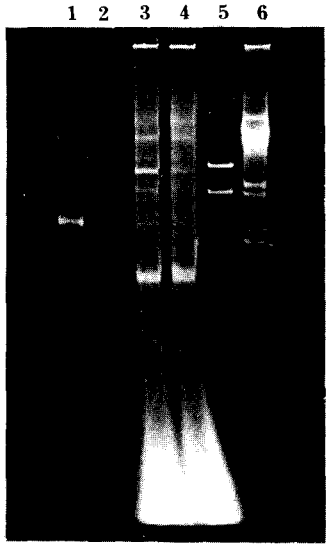

Fig. 2. Agrose gel electrophorsis of replicated plasmid isolated from transformants.

Lane 1:pUB110, 2:pE194, 3, 4:replicated plasmids from transformants, 5:ϕ29/EcoRI, 6:*E. coli* V517.

phosphatase, several recombinant plasmids which were recombinated with digested pUB110 and pE194, or selfligation to monomer and dimer were constructed. Also one of the five main bands is presumed to pBD9 (5.4M dalton) of Gryczan[13].

Frequency of regeneration

Monomeric ccc-plasmid transformation of *B. subtilis* is very inefficient compared with oligomeric plasmid. However, protoplasts regeneration system of *B. subtilis* can be transformed wih monomeric ccc-DNA at 10^5 fold compared with competent system.

In order to investigate regeneration frequency of recipient protoplast on different kinds of hypertonic regeneration media, DMB plate and the mannitol agar plate were used as regeneration media for *B. subtilis* BR151 (IE31) and *B. subtilis* BR151 (IE32).

When $1.0 \times 10^8 - 5.0 \times 10^8$ /ml of fresh cells were protoplasted by lysozyme, remaining survivors on TBAB plate were generally $1.0 \times 10^4 - 4.0 \times 10^4$ cells. As results of regeneration frequency in Table 1, the protoplast of *B. subtilis* IE32 BR151 was regenerated more efficiently on the mannitol agar plate. But the frequency of *B. subtilis* IE32 BR151 protoplast on DMB plate was more effective than on the mannitol agar plate. Although the regeneration frequency of 12.8% on the mannitol agar plate is lower than 10-25% of *B. subtilis*[4], this frequency is higher than the 3-9% of *B. thuringiensis* 17A[7] and the $1.0 \times 10^{-4} - 6.2 \times 10^{-3}$ of several *Bacillus* species[14].

Table 1. Frequency regeneration.

	B. subtilis BR151 (IE31)	*B. subtilis* BR151 (IE32)
DMB	8.8×10^{-2}	8.2×10^{-2}
MA	8.6×10^{-2}	1.28×10^{-1}

$$\frac{\text{cfu on DMB or MA after regeneration}}{\text{cfu on TBAB before protoplasting}}$$

Transforamtion frequency of plasmids

Undigested plasmid pUB110 and pE194 were transformed in *B. subtilis* by protoplast transformation system which involved PEG induction of DNA uptake in protoplast and sequenetial regeneration of cell wall on different regeneration media. Because of neomycin inactivation by sodium succinate of DMB media, the mannitol regeneration agar was used for pUB110 transformation. The mannitol regeneration agar worked with high frequency for pUB110 transformation. Transformation frequency of pUB110 (Neo^R) was 15.8% for *B. subtili* IE32 BR151 on the mannitol agar containing 5μg/ml of neomycin and pE194 (Em^R) was 19% for *B. subtilis* IE31 BR151 on DMB agar containing 5μg/ml of erythromycin (Table 2). These show the pUB110 transformed *B. sutbilis* IE32 BR151 more effectively than *B. subtilis* IE31 BR151. On the other hand *B. subtilis* IE31 BR151 was more effectively transformed by pE194. The transformation frequencies of 15.8% with pUB110, 19.0% with pE194 were higher than those of other protoplast transformation systems of *Bacillus*.

Several results repoted that the transformation frequency of *B. thuringiensis*[9] protoplast by pUB110 was 1.57×10^{-6}, and of *B. megaterium*[6] protoplast by pUB110 was $1 \times 10^{-6} - 2 \times 10^{-5}$ with low efficiency. Its low frequency is due to impossiblility of direct regeneration in regeneration media containing neomycin or kanamycin. But others reported that the portoplasts of several *Bacillus* species[14] were transformed by

Table 2. Transformation frequency of plasmid.

	B. subtilis BR151 (IE31)	*B. subtilis* BR151 (IE32)
pUB110	11.3×10^{-2}	15.8×10^{-2}
pE194	19.0×10^{-2}	12.9×10^{-2}

1) DNA : 3.33μg

2) $\frac{\text{cfu on MA/Neo or DMB/Em}}{\text{cfu on MA or DMB}}$

Table 3. Transformation frequency of recombinant plasmid.

Plasmid	Selection	Frequency*
Recombinant plasmid	Neo	1.09×10^{-3}
(pUB110+pE194)	Em	2.16×10^{-3}
	Neo+Em	6.5×10^{-5}
Religated pUB110	Neo	6.4×10^{-3}

*cfu on MA/antibiotic / cfu on MA

pTP4 in the range of 1.3×10^{-2} to 7.1×10^{-1}, and *B. subtilis* was transformed by pC194 and pUB110 in the range of 1.0×10^{-1} to 4.0×10^{-1}

Transformation frequency of recombinat plasmids

The recombinant chimeric plasmid was constructed using XbaI digested-pUB110 and -pE194, then ligated with T_4 DNA ligase. Transformation of *B. subtilis* IE32 BR151 protoplasts by the recombinant plasmids was accomplished using the mannitol regeneration agar containing 5μg/ml of neomycin and erythromycin respectively. Also reconstructed pUB110 digested with EcoRI was transformed by the protoplast transformation system on the mannitol regeneration agar containing 5μg/ml of neomycin.

Expression of two kinds of antibiotics resistance genes from different donor strain was demonstrated in *B. subtilis* IE32 BR151. The transformation frequency of recombinant pUB110-pE194 chimeric plasmid was 1.09×10^{-3} on the mannitol agar with neomycin and 2.16×10^{-3} on the mannitol agar with erythromycin (Table 3). But the transformation frequency of recombinant chimeric plasmid was decreased to 6.5×10^{-5} on the mannitol agar with neomycin and erythromycin. Furthmore, after subculture on the mannitol agar with neomycin and the mannitol agar with erythromycin alternately, the frequency was reduced to 6.0×10^{-6}.

Confirmation of recombinant plasmid

To confirm the replication of recombinant chimeric plasmid in recipient cells, the plasmids were isolated from the transformants by Birnboim's rapid alkaline extraction procedure[12]. Electrophoretic mobilities of recombinant plasmids isolated from transformants are shown in Fig. 2. We suppose that a upper big band is recombinant chimeric plasmid (5.4M dalton) of pUB110-pE194 and a bottom big one is religated pE194. These results mean the recombinant plasmids were replicated compatibly in the recipient cell.

요 약

Mannitol hypertonic regeneration media를 사용하는 PEG-induced protoplast transformation system을 이용해서 pUB110과 pE194의 recombinant plasmid로 *B. subtilis* BR151을 transformation 시킴으로써 두 plasmid에서 유래되는 각각의 Neo^R와 Em^R을 동일한 recipient cell 내에서 동시에 발현시킬 수 있었다. Neomycin과 erythromycin을 함께 함유하는 mannitol regeneration media상에서 recombinant plasmid의 transformation frequency는 6.5×10^{-5}이었다. 한편 transformant cell 내에서 recombinant plasmid의 replication이 agarose gel electrophoresis로 확인되었다.

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