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Transformation and Mutation of *Bacillus licheniformis* 9945a Producing γ-Poly(glutamic acid)

Wan-Seok Chung and Young Hwan Ko*

Department of Food Science & Engineering, College of Engineering, Cheju National University, Cheju 690-756, Korea

Abstract : *Bacillus licheniformis* 9945a releases a natural γ-poly(glutamic acid)(γ-PGA) into fermentation broth and shows a mucoid phenotype on the solid agar medium. Transformation of mucoid cells of *Bacillus* species has not been simple and straightforward. The transpositional activity of Tn10 in *B. licheniformis* also has not been known either. Thus, a spontaneous non-mucoid derivative of the *B. licheniformis* was obtained first. Shuttle vector pHV1248 containing mini-Tn10 was introduced into the non-mucoid derivative by the method of protoplast transformation. The resulting transformant was reverted to the wild mucoid phenotype, and then mutated randomly with the mini-transposon by heat induction. Auxotrophs requiring arginine, lysine, or tryptophan were isolated by replica plating method. Southern blotting and DNA-DNA hybridzation analysis showed that these auxotrophs were generated by mini-Tn10 insertion into the chromosomal DNA. This method of transformation and mutation using pHV1248 would be useful for the generation of diverse mutants of *B. licheniformis* 9945a.(Received January 24, 1997; accepted March 10, 1997)

Introduction

Recent interests in biodegradable polymers have brought γ-poly(glutamic acid)(γ-PGA) into attention. *Bacillus licheniformis* 9945a is one of the bacterial strains that produce γ-PGA.[1,2] γ-PGA is an unusual natural polyamide and differs from the most protein in that the glutamate repeat units are covalently linked between the α-amino and γ-carboxylic functional groups(Fig. 1). The molecular weight of γ-PGA decreases over cultivation time, which limits its utility. The slow reduction of average molecular weight over the cultivation time turned out to be due to enzymatic degradation by the producer itself.[1] Thus, γ-PGA depolymerase-deficient mutants are needed for prevention of enzymatic degradation and production of high quality γ-PGA.

Even though several strategies and methods for transformation of *Bacillus* species by plasmid DNA have been known to be successful,[3-7] transformation of mucoid cells of *B. licheniformis* was reported not to be simple and easy.[8-11] In addition, not a single report about transformation with foreign plasmid DNA of *B. licheniformis* 9945a which synthesizes highly viscous extracellular polymer has been found. Transformation of *B. licheniformis* 9945a with plasmid DNA harboring mini-transposon is necessary as a preliminary step for transpositional mutation of the strain. Plasmid pHV1248 has a Tn10 derivative that is active in *B. subtilis*.[12] Even if the mini-Tn10 has been known to transpose randomly in *B. subtilis*, it has not been known whether its transposition occurs in *B. licheniformis* ATCC9945a. Insertional mutagenesis of the *B. licheniformis* 9945a with mini-Tn10 is the early steps leading to the final isolation of γ-PGA depolymerase-deficient mutants of the strain.

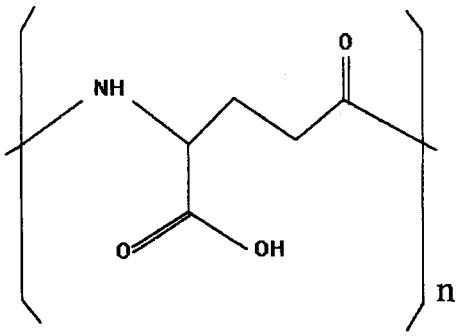

Fig. 1. Structure of γ-poly(glutamic acid) or γ-PGA.

We report here transformation of *B. licheniformis* 9945a with plasmid pHV1248 and its subsequent transpositional mutation with mini-Tn10. Auxotrophs requiring arginine, lysine, or tryptophan were isolated from the mutant populations.

Key words : *Bacillus licheniformis*, γ-poly(glutamic acid), transformation, mini-Tn10, mutation
*Corresponding author

Materials and Methods

Bacterial strain, plasmid and medium

B. licheniformis 9945a was obtained from the American Type Culture Collection, and *Escherichia coli* cells harboring plasmid pHV1248[12] were kindly provided by Bacillus Genetic Stock Center, The Ohio State University. The plasmid has resistance genes to ampicillin, chloramphenicol, and erythromycin(Fig. 2). Plate Count Agar(PCA, Difco, USA) for the isolation of a spontaneous derivative of *B. licheniformis* 9945a lacking γ-PGA and LB[13] for *E. coli* growth and plasmid isolation were used, respectively. Antibiotics were added to the medium whenever necessary. The working concentrations of antibiotics were 50 μg/m*l* for ampicillin, 10 μg/m*l* for chloramphenicol, and 5 μg/m*l* for erythromycin.

DNA handling

Basic protocols and procedures for handling DNAs were not different from ordinary ones that were published elsewhere.[13,14] For plasmid DNA isolation, rapid boiling method[15,16] was used. Chromosomal DNA was separated on agarose gel by in-well-lysis technique[17] for Southern blotting and DNA-DNA hybridization. Probe DNA was labeled with biotinylated dUTP.[18]

Transformation

Cells of *B. licheniformis* 9945a were grown with shaking at 37℃ in LB.[13] Aliquots were withdrawn from the early stationary phase culture and spreaded on PCA plates after dilution with sterile distilled water. The plates were incubated at 37℃ for a day, and non-mucoid tiny colonies occuring spontaneously were selected and purified. One of the spontaneous mutants that lacked capsular γ-PGA was used as a recipient cell. Its cell wall was removed by lysozyme treatment. Plasmid pHV1248 harboring mini-Tn10[12] was introduced into the resulting protoplasts according to the previously reported procedures.[4] Bacterial colonies resistant to both erythromycin and chloramphenicol were selected and examined for the presence of the plasmid inside the cell. The cells harboring the plasmid pHV1248 were grown with shaking at 30℃ in LB[13] containing both antibiotics until middle exponential phase, and then aliquots of the culture were spreaded on LB agar plates containing the same antibiotics. Large mucoid colonies that reverted spontaneously to wild phenotype after incubation for a day at 30℃ were isolated. One of the revertants was used as a parent strain for transpositional mutation. Both electroporation[19-21] and natural competency induction[3,5-7,22] were also tested for the transformation of *B. licheniformis* 9945a by pHV 1248.

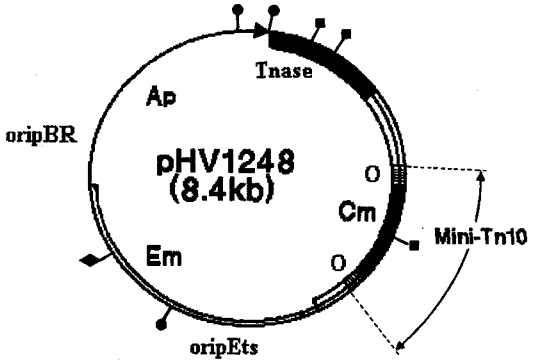

Fig. 2. Structure of plasmid pHV1248. Restriction sites are indicated by circle(*Eco*RI), rectangle(*Nco*I), and diamond(*Sst*I). oripBR, *ori* from pBR322; oripEts, *ori* from pE194*ts*; Ap, ampicillin resistance; Em, erythromycin resistance; Cm, chloramphenicol resistance; Tnase, transposase; O, outside ends of Tn10.

Transpositional mutation

The transformant with pHV1248 was grown with shaking in LB[13] containing chloramphenicol until middle exponential phase at 30℃. The incubation temperature was shifted to 51℃ and the cells were further incubated for 2~3 hrs. Aliquots of the culture were spreaded on LB agar plates containing chloramphenicol and the plates were incubated at 51℃.[12] Then, theoretically, only the cells which have mini-Tn10 inserted into the chromosomal DNA grow due to the temperature-sensitive replicon from a plasmid pE194.[23]

Auxotrophs were scored out of the resultant bacterial colonies by replica plating on glucose minimal medium (K_2HPO_4, 0.8 g; NaH_2PO_4, 0.2 g; $CaSO_4 \cdot 2H_2O$, 0.05 g; $FeSO_4 \cdot 7H_2O$, 0.01 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $(NH_4)_2SO_4$, 1.0 g; glucose, 10.0 g; agar, 12.0 g; distilled water, 1000 m*l*; pH, 6.8)[24] and their requirements for specific amino acids were determined by the method of Holliday.[25]

Results and Discussion

Transformation

Transformation of *Bacillus* species with plasmid or chromosomal DNA has been known to be quite successful. Diverse methods such as electroporation,[19-21] induction of natural competency[3,5-7,22] and use of protoplast[4] have been developed for the transformation. Electroporation did not work for the transformation of *B. licheniformis* 9945a with plasmid pHV1248, however. Various electroporation conditions set by combination of capacity(21 μF), voltage(1.0~2.5 KV/2 mm) and resistance(200~600 ohm) did not make any differences even though cell viability changed. Some of the double antibiotic, erythromycin and chloramphenicol, resistant colonies obtained thereafter did not reveal any plasmid DNA on agarose gel electrophoresis. The transformation by inducing natural competency was not successful either(Table 1). It only generated the double

Table 1. Transformation of *Bacillus licheniformis* with plasmid DNA pHV1248

Recipient	Methods of transformation	Transformants/μg DNA
Mucoid wild type	Protoplast	Not detected
	Electroporation	Not detected
	Natural competency	Not detected
Non-mucoid derivative	Protoplast	5×10^2

antibiotic resistant colonies that contained no plasmid DNA. The failure of transformation could be due to thick cell wall, capsular viscous polymer or both. Supposing the transformation was successful, the absence of plasmid DNA could be due to the possibility that plasmid DNA introduced into the recipient cell multimerized[26] or integrated into chromosomal DNA.[6]

Spontaneous mutants, lacking capsular polymer, of *B. licheniformis* 9945a were isolated by repeated plating on PCA medium since mucoid cells were not transformable with plasmid DNA even by the use of protoplast. Most of them were resistant to lysozyme; lysozyme treatment did make any visible protoplasts under a microscope. One of the mutants liable to lysozyme attack was selected. The mutant was unstable. Its reversion frequency to wild phenotype was about 10^{-3}, which was quite higher than usual spontaneous mutation. The mechanism for spontaneous mutation back and forth has been unknown. The mutant strain liable to lysozyme attack was used as a recipient of plasmid pHV1248 in the protoplast transformation by the procedure of Chang and Cohen[4](Table 1). The observed efficiency of transformation was 5×10^2/μg DNA. The possible unstability of introduced plasmid DNA might have contributed to the low efficiency. Transformants showing double antibiotic resistance were purified and confirmed by the presence of plasmid DNA inside the cells(Fig. 3). *B. licheniformis*(pHV1248) in lanes B and C had plasmid DNA which migrated almost the same distance as that from *E. coli*(pHV1248) in lane E. Two bands of plasmid DNA in each lane C and E indicate different forms of plasmid DNA molecules. When plasmid DNA from the transformant, *B. licheniformis*(pHV1248), was compared with donor plasmid from *E. coli*(pHV1248) after digestion with restriction enzyme on 0.8% agarose gel, DNA fragments with the same migration distances were resolved(Fig. 4). Thus, plasmid pHV1248 was successfully introduced into *B. licheniformis* 9945a by the method of protoplast transformation.

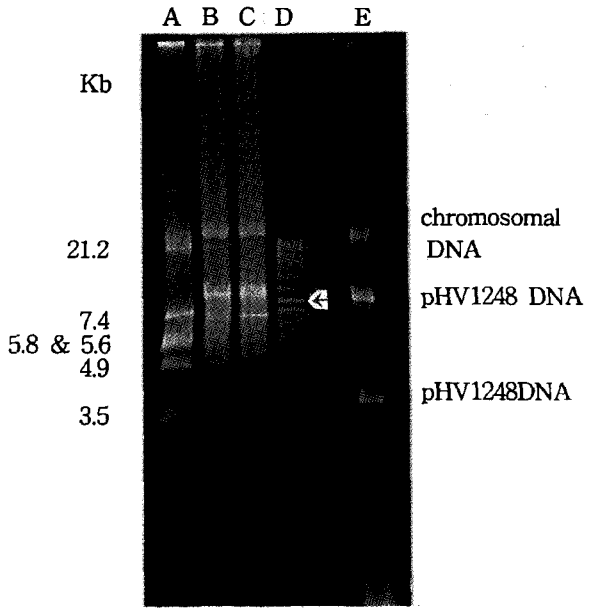

Fig. 3. Identification of plasmid DNA in transformants by agarose-gel electrophoresis. An arrow marker in lane D indicates 8.4 Kb DNA fragment. A, λDNA cut with *Eco*RI; B, *B. licheniformis*(pHV1248); C, *B. licheniformis*(pHV1248); D, DNA M.W. marker; E, *E. coli* (pHV1248).

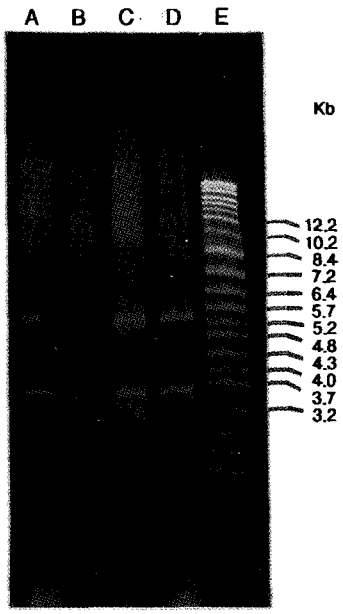

Fig. 4. Analysis of plasmid DNA from transformants after restriction digestion on agarose gel. DNAs from *E. coli* and *B. licheniformis* were digested with *Eco*RI separately. A, *E. coli*(pHV1248); B, *B. licheniformis*(pHV1248); C, *B. licheniformis*(pHV1248); D, *E. coli* (pHV1248); E, DNA M.W. marker.

Transpositional mutation

Using *B. licheniformis*(pHV1248) as a parent strain, isolation of random mutants was attempted. Transpositional random insertion of mini-Tn10 into the chromosomal DNA was induced by shift-up of the incubation temperature and the transpositional frequency was 2×10^{-5}(Table 2). When the parent cells resistant to both chloramphenicol and erythromycin are exposed to higher temperature, transpositional mutants resistant to chloramphenicol but sensitive to erythromycin can result from silmutaneous plasmid DNA loss and mini-Tn10 inserton into the chromosome.[12]

Table 2. Transpositional mutation in the transformant of *Bacillus licheniformis*

Plasmid (transposon)	Cm^r & Em^r cell before heat induction	Cm^r & Em^s cell after heat induction	Transpositonal frequency	Auxotroph* requiring casamino acid	Proportion of auxotroph
pHV1248 (mini-Tn10)	5×10^8/ml	1×10^4/ml	2×10^{-5}	20/ml	0.2%

Abbreviations: Cm^r, chloramphenicol resistant; Em^r/Em^s, erythromycin resistant/sensitive. *Auxotrophs requiring arginine, lysine, or tryptophan were included.

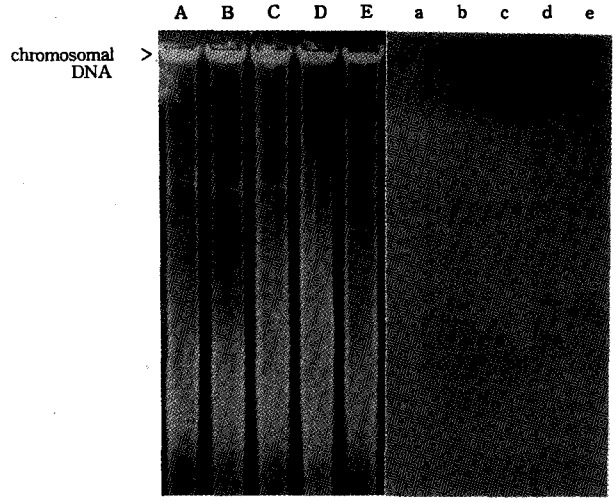

Fig. 5. Southern blotting and DNA-DNA hybridization using a donor pHV1248 DNA as a probe. Chromosomal DNA was separated on agarose gel by in-well-lysis technique. The probe DNA was labeled with biotinylated dUTP. A/a, *B. licheniformis* ATCC9945a(wild type); B/b, *B. licheniformis(arg⁻)*; C/c, *B. licheniformis(lys⁻)*; D/d, *B. licheniformis(trp⁻)*; E/e, *B. licheniformis* ATCC9945a(wild type).

Auxotrophs requiring casamino acid were detected among the transpositional mutants at the ratio of 0.2% (Table 2). Determination of their requirements for specific amino acid by the method of Holiday[25] revealed three different auxotrophs; *B. licheniformis(arg-)*, *B. licheniformis(lys-)* and *B. licheniformis(trp-)*. Southern blotting after separation of chromosomal DNA on agarose gel by in-well lysis technique[17] and DNA-DNA hybridization using biotinylated pHV1248 DNA as a probe[18] showed homologous DNA fragments on the top of the gel where large molecular weight chromosomal DNA existed(Fig. 5). Those three auxotrophs lost plasmid DNA and resulted from mini-Tn10 insertion into chromosomal DNA. This study will be useful in obtaining diverse mutants of *B. licheniformis* 9945a.

Acknowledgements

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γ-Poly(glutamic acid) 생산성 균주 *Bacillus licheniformis* 9945a의 형질전환 및 돌연변이 유도

정완석, 고영환* (제주대학교 식품공학과)

초록 : *Bacillus licheniformis* 9945a는 액체배양시 γ-poly(glutamic acid)를 균체외로 분비하며, 한천배지에 고체배양시는 점액질의 군락을 나타낸다. 점액질의 *Bacillus*속 세균의 형질전환은 그리 단순하지 않은 것으로 알려져 있으며, *B. licheniformis*에서의 transposon Tn10의 활성여부도 알려져 있지 않다. 그래서 점액질을 분비하지 않는, *B. licheniformis*의 자연발생적 변이주를 우선 분리하였다. Mini-Tn10을 함유한 plasmid pHV1248을 protoplast transformation법에 준해서 이 변이주에 도입하여 형질전환체를 분리하였다. pHV1248을 함유한 형질전환체를 점액성의 야생형질로 복귀시킨 후에, 가열처리함으로써 무작위 돌연변이를 유도하였다. Arginine, lysine 또는 tryptophan을 생육인자로 요구하는 돌연변이주들이 replica plating method에 의해서 분리되었고, 이들 영양요구성 변이주는 mini-Tn10이 염색체 DNA상에 삽입됨으로써 생겨났음이 Southern blotting과 DNA-DNA 혼성화 실험으로 증명되었다. 이러한 pHV1248을 이용한 형질전환 및 돌연변이 유도방법은 *Bacillus licheniformis* 9945a의 다양한 변이체를 얻는데 유용할 것이다.

*연락저자