Isolation and Characterization of γ-PGA Depolymerase-deficient Mutants of *Bacillus licheniformis* : Mutation of *Bacillus licheniformis* with mini-Tn10

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ABSTRACT

Bacillus licheniformis releases a natural γ-poly (glutamic acid) (γ-PGA) into the fermentation broth. However, the molecular weight of γ-PGA decreases over cultivation time, which limits its utility. This study has been designed to overcome the degradation of naturally-produced γ-PGA during fermentation. We report the early steps, mutation of *Bacillus licheniformis* with mini-Tn10, leading to the final isolation and characterization of γ-PGA depolymerase-deficient mutants of *Bacillus licheniformis*. Shuttle vector pHV1248 containing mini-Tn10 was introduced into *Bacillus licheniformis* using the method of protoplast transformation and the transformed cells were mutated randomly with the mini-transposon by heat induction. Auxotrophs requiring arginine, lysine, or tryptophan were isolated, which resulted from mini-Tn10 insertion into chromosomal DNA.

INTRODUCTION

Recent interests in biodegradable polymers have brought γ-poly(glutamic acid)(γ-PGA) into attention. *Bacillus licheniformis* ATCC9945a is one of the bacterial strains that produce γ-PGA. 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). Its molecular weight ranges from one million to hundred thousands depending upon culture condition and age. The slow reduction of average molecular weight over the cultivation time was observed and turned out to be due to enzymatic degradation by the producer itself. Thus, γ-PGA depolymerase-deficient mutants are needed for prevention of enzymatic degradation and production of high quality γ-PGA.

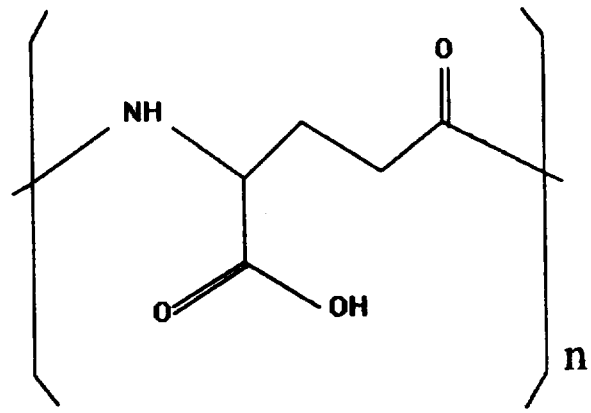

Fig. 1. Structure of γ-Poly(glutamic acid) or γ-PGA

Several strategies and methods for transformation of *Bacillus* species by plasmid DNA have been known to be successful. But not a single report about transformation of *Bacillus licheniformis* ATCC9945a which synthesizes highly viscous extracellular polymer was found. Even though transformation of mucous cells of *Bacillus subtilis* was reported not to be simple, transfomation of *Bacillus licheniformis* ATCC9945a with mini-transposon harboring plasmid was necessary as a preliminary step for transpositional mutation of the strain. Random transpositional insertion is expected to generate γ-PGA depolymerase- deficient mutants.

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

MATERIALS & METHODS

Bacterial strain, plasmid and medium

Bacillus licheniformis ATCC9945a was obtained from the American Type Culture Collection(ATCC), and *Escherichia coli* cells harboring plasmid DNA pHV1248(Petit, et al., 1990) were kindly provided by Bacillus Genetic Stock Center, The Ohio State University. This plasmid has resistance genes to ampicillin, chloramphenicol, and erythromycin(Fig. 2). Plate Count Agar(Difco, USA) for isolation of spontaneous mutant of *B. licheniformis* lacking γ-PGA and LB for *E. coli* growth and plasmid isolation were used respectively.

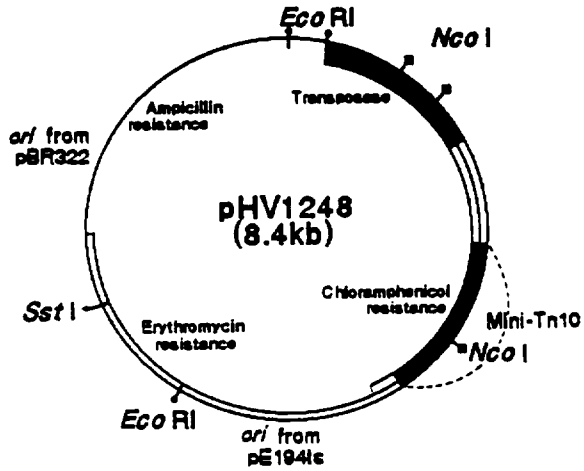

Fig. 2. Structure of pHV1248

DNA handling

Methods and procedures for handling plasmid and chromosomal DNA's were not different from common, general ones that are published elsewhere.

Transformation

A spontaneous mutant of *B. licheniformis* ATCC9945a that lacks capsular γ-PGA was isolated first and its cell wall was removed by lysozyme treatment. Plasmid pHV1248 harboring mini-Tn10(Petit, et al., 1990) was introduced into the protoplast according to the previously reported procedures(Chang and Cohen, 1979). Bacterial colonies resistant to both erythromycin and chloramphenicol were selected and examined for the presence of the plasmid inside the cell. Other transformation protocols were also investigated.

Transpositional mutation

The transformant with pHV1248 was grown until middle exponential phase in the presence of chloramphenicol at 30℃. The incubation temperature was shifted to 51℃ and the cells were further incubated for 2−3 hrs. Aliquots of culture were spreaded on complex medium plates containing chloramphenicol and the plates were incubated at 51℃(see Reference 4 for details). Auxotrophs requiring specific amino acids were scored out of the resultant bacterial colonies by replica plating on glucose minimal medium.

RESULTS & DISCUSSION

Transformation of *Bacillus* species with plasmid or chromosomal DNA has been quite successful. Diverse methods has also been applied to the transformation ; electroporation, induction of natural competency, and use of protoplast. Electroporation did not work for the transformation of *B. licheniformis* ATCC 9945a with plasmid pHV1248, however. Various electroporation conditions set by combination of capacity, voltage and resistance did not make any difference even though cell viability changed. Some of the double antibiotic (erythromycin and chloramphenicol) resistance colonies obtained thereafter did not reveal any plsmid DNA on agarose gel electrophoresis. It could be due to thick cell wall, capsular viscous polymer or both. The transformation by inducing natural competency neither worked but only generated double antibiotic resistance colonies that contained no plasmid DNA. Capsular γ-PGA might contributed to the double antibiotic resistance of the cells(refer to Table 1).

Spontaneous mutants, lacking capsular polymer, of *B. licheniformis* ATCC9945a were isolated by repeated plating on PCA medium. Some of them were resistant to lysozyme ; lysozyme treatment did make any visible protoplast under microscope. One of the muatants liable to lysozyme attack was purified and used for transformation with plasmid pHV1248 by the protoplast transformation procedure of Chang and Cohen(1979). The transformation was confirmed by the presence of plasmid DNA inside the recipient cell(Fig. 3). The transformant was reverted spontaneously to the origianl γ-PGA producing strain. The mechanism for spontaneous mutation back and forth has been unknown.

Table 1. Transformation of *Bacillus licheniformis* by 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

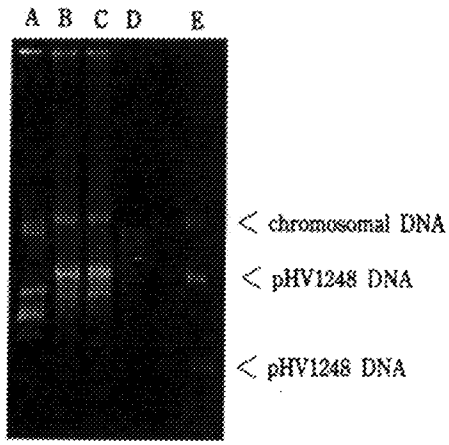

Fig. 3. Identification of plasmid DNA in transformants by agarose-gel electrophoresis
A, λDNA cut with EcoRI ;
B, plasmid DNA from a transformant ;
C, plasmid DNA from a transformant ;
D, DNA M.W. marker ;
E, plasmid pHV1248 DNA from a *E. coli*

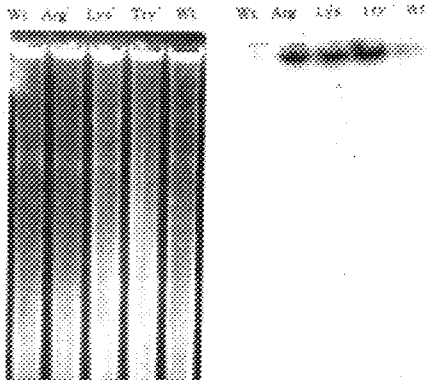

Fig. 4. Southern blotting and DNA-DNA hybridization using a donor plasmid DNA as a probe. DNAs were separated on agarose gel by in-well-lysis technique.

Transpositional random insertion of mini-Tn10 was induced by shift-up of the incubation temperature. Auxotrophs requiring arginine, lysine, or tryptophan were isolated(Table 2). Those auxotrophs lost plasmid DNA at high temperature due to the temperature-sensitive replicon pE194 within the plasmid pHV 1248. They resulted from mini-Tn10 insertion into chromosomal DNA according to the Southern blotting and DNA-DNA hybridization(Fig. 4).

ACKNOWLEDGEMENT

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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	Transpositional frequency	Auxotroph* requiring casamino acid	Proportion of auxotroph
pHV1248(mini-Tn10)	5×10^8/ml	1×10^6/ml	2×10^{-3}	20/ml	0.2(%)

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