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Cloning of Pectate Lyase Gene of Alkali-tolerant *Bacillus* sp. YA-14 and Its Expression in *Escherichia coli*

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알카리 내성 *Bacillus* sp. YA-14의 Pectate Lyase 유전자의 클로닝과 발현

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Pectate lyase (PL) was cloned from alkali-tolerant *Bacillus* sp. YA-14 into *Escherichia coli* MB1000 by inserting *Hind*III-generated DNA fragment into the *Hind*III site of pBR322 and then screening recombinant transformant for the ability to hydrolyze sodium polypectate on agar plate. The recombinant plasmid, called pYPC29, was isolated, and the size of the cloned *Hind*III fragment was found to be 1.6 kb. The PL gene was stablely maintained and expressed efficiently in *Escherichia coli*. The PL accumulated largely in the periplasmic space of *Escherichia coli* clones.

Pectic substances are structural polysaccharides (chains of 1,4-linked α -D-galacturonic acid and methoxylated derivatives), occuring mainly in the middle lamella and primary cell wall of higher plants. Pectolysis is an important phenomenon associated with many biological process in which plant material is involved. Pectic enzymes are classified into three main groups-deesterifying enzymes (pectin esterase) and two groups of chain splitting enzymes(hydrolase and lyase). Among them, pectate lyase(PL) cleaves internal glycosidic linkages in pectic substances by β -elimination. The β -eliminative attack of this enzyme on its substances results in formation of products with a double bond between C-4 and C-5 (1,2). Some kinds of PL produced by many bacteria have been studied in detail (3-11). Especially, some species of the genus *Bacillus* are known as endopectate lyase producers (3-6). The alkali-tolerant *Bacillus* sp. YA-14, which was isolated from soil in our laboratory, produces PL as the other *Bacillus* strains do.

The present paper deals with the cloning and expression of PL gene from alkali-tolerant *Bacillus* sp. YA-14 (12) in *E. coli*.

Materials and Methods

Bacterial strains and plasmids

Alkali-tolerant *Bacillus* sp. YA-14 which was isolated from soil (12) was used as a DNA donor to construct a gene library. *E. coli* MB1000(*hsdR hsdM lac trp pyrF*)(13) was used as a host in the cloning experiment and the plasmid pBR322(14) was used as a vector.

DNA isolation

Bacillus sp. YA-14 chromosomal DNA was isolated by the spool method (15). Plasmid DNA was isolated by the modification of the method of Birnboim and Doly (16), and rapid isolation of recombinant plasmids was performed by the miniscreening method (15).

DNA cloning

Bacillus sp. YA-14 chromosomal DNA was digested with restriction enzyme *Hind*III. Plasmid pBR322 DNA was digested with *Hind*III and dephosphorylated with bacterial alkaline phosphatase(BAP). Ligation of pBR322 and *Hind*III-

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digested *Bacillus* sp. YA-14 DNA with T4 DNA ligase was performed at 12 °C for 16 hrs. The ligation mixture was transformed into *E. coli* MB1000 by the procedure of Norgard *et al.* (17), and transformants were selected on LB-ampicilline (50 μg/m*l*) plates. The presence of cloned DNA was verified by transferring clones onto LB-tetracycline(20 μg/m*l*). Clones expressing PL activity were detected by replica plating the bacterial colonies onto YCP plates (2 g of ammonium sulfate, 0.2 g of magnesium sulfate-7H_2O, 3 g of Casamino acids, 2 g of yeast extract, distilled water to 1 liter; the medium was adjusted to pH 7.5 with 0.1N NaOH, 15 g of agar was added, and the medium was autoclaved)(18). The plates were incubated at 37 °C for 16 to 20 hrs, treated with chloroform and lysozyme (17) and incubated at 37 °C for 10 hrs to allow the diffusion of the PL. The plates were flood with 6N-HCl and stood at room temperature. After 5-10 min., clear zones appeared around PL positive clones.

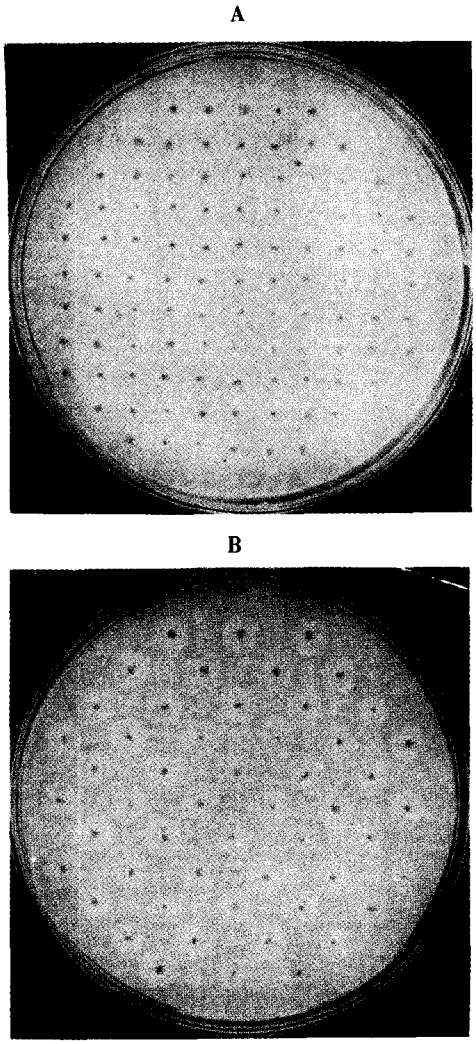

Fig. 1. Screening of *E. coli* MB1000 containing recombinant pectate lyase (PL) gene by 6 N-HCl precipitating method.

A: PL positive clone screening after Ap^r, Tc^s selection.
B: Screening by retransformation of PL positive hybrid plasmid.

Nick translation and hybridization

Probe DNA was labeled with biotin-labeled dUTP by using the BRL nick translation system. DNA transfer to a nitrocellulose filter and DNA-DNA hybridization were performed by the method of Southern (19). The technique used for DNA detection was described by Leary *et al.* (20).

Localization of PL in *E. coli*

Fractionation of extracellular, periplasmic and intracellular PL in *E. coli* was performed by the procedure of Reverchon *et al.* (21).

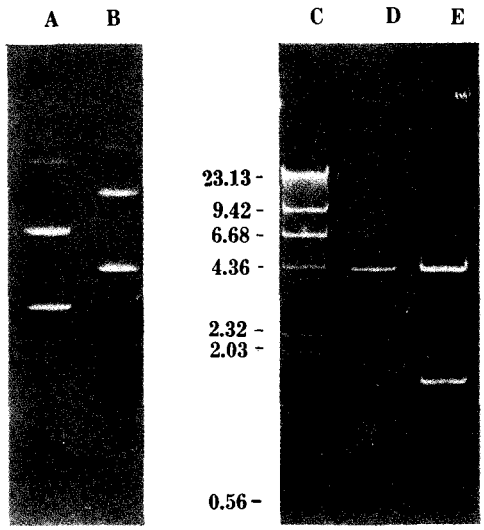

Fig. 2. Agarose gel elecrophoresis of the recombinant plasmid DNA containing PL gene of *Bacillus* sp. YA-14.

lane A; pBR322
lane B; recombinant plasmid DNA
lane C; λ DNA digested with *Hin*dIII as a molecular weight marker (kb)
lane D; pBR322 digested with *Hin*dIII
lane E; recombinant plasmid DNA digested with *Hin*dIII

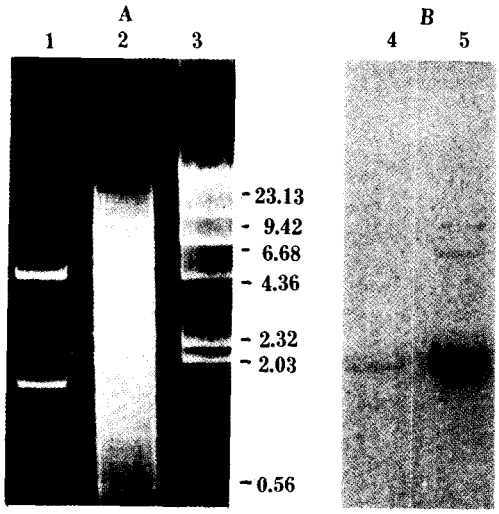

Fig. 3. Southern hybridization of the *Hin*dIII-digested *Bacillus* sp. YA-14 chromosomal DNA and recombinant plasmid pYPC29. The 1.6 kb *Hin*dIII-digested DNA fragment of pYPC29 was used as a probe.

A. lane 1; pYPC29 digested with *Hin*dIII
lane 2; *Bacillus* sp. YA-14 chromosomal DNA digested with *Hin*dIII
lane 3: λ DNA digested with *Hin*dIII as a molecular weight marker (kb)

B. Hybridization patterns
lane 4: *Bacillus* sp. YA-14 chromosonal DNA digested with *Hin*dIII
lane 5: pYPC29 digested with *Hin*dIII

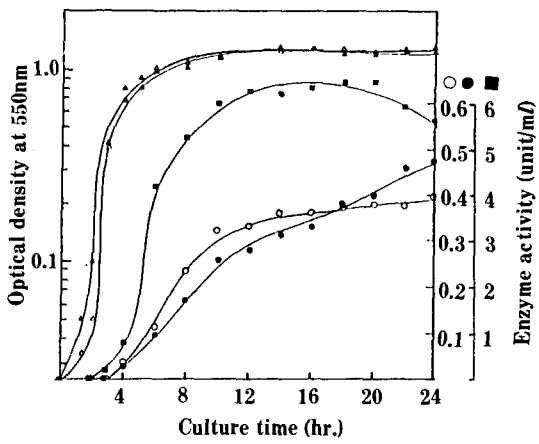

Fig. 4. Growth and enzyme production by *Bacillus* sp. YA-14 and *E. coli* MB1000 (pYPC29).

△—△; Growth of *Bacillus* sp. YA-14
▲—▲; Growth of *E. coli* MB1000 (pYPC29)
○—○; The extracellular PL acitivity of *Bacillus* sp. YA-14
●—●; The extracellular PL activity of *E. coli* MB1000 (pYPC29)
■—■; The intracellular PL activity of *E. coli* MB1000 (pYPC29)

Assay of PL activity

PL activity was assayed on the basis of the enzymatic production of unsaturated products which have an absorption maximum at 232 nm. Reaction mixture contained 1.3 m*l* of appropriately diluted enzyme, 0.5 m*l* of 0.2 M Tris-HCl(pH 8.5), 0.2 m*l* of 3 mM $CaCl_2$, and to start the reaction, 1 m*l* of 1 % (wt/vol) sodium polypectate(98% pure; Sigma). Absorbancy changes at 232 nm were measured in the Hitachi Model 200-20 spectrophotometer. One unit of PL activity was defined as the change of the absorbancy per min under the conditions outlined above.

Results and Discussion

Cloning of *Bacillus* sp. YA-14 PL gene in *E. coli*

A gene library of *Bacillus* sp. YA-14 DNA was constructed in *E. coli* MB1000 and 4,500 Ap^r Tc^s transformants were selected. These recombinant clones were done replica plating onto the YCP plates to search for PL positive transformants. After that, only one colony showed a clear zone on the YCP plate (Fig. 1, A).

The hybrid plasmid, named pYPC29, was reintroduced into the original *E. coli* MB1000 to verify the presence of PL gene on the plasmid. All of these transformants showed PL activity on the YCP plates (Fig. 1, B). The clone, as well as the donor strain, did not show hydrolase activity but showed PL activity (data not shown).

Characterization of the recombinant plasmid

The recombinant plasmid, pYPC29, was digested with *Hin*dIII and analyzed on agarose gel electrophoresis (Fig. 2). The pYPC29 contained a 1.6 kb *Hin*dIII insert. Southern hybridization of the biotin-labeled 1.6 kb-*Hin*dIII insert of pYPC29 was performed with the chromosomal DNA of *Bacillus* sp. YA-14. The result demonstrated that the inserted fragment originated from this chromosome (Fig. 3).

Expression and localization of the PL in *E. coli*

The level of synthesis of the PL, produced by the donor strain or PL positive clone, was deter-

Table 1. Localization of PL in *E. coli* MB1000 (pYPC29), and *Bacillus* sp. YA-14.

Strain (Total activity, U/ml)	% of total activity of PL		
	Extracellular	Periplasmic	Cytoplasmic
pYPC29 (4.34)	9.0	72.4	18.2
YA-14 (0.23)	100.0	—	0.0

mined (Fig. 4). Both strain produced PL efficiently after the late logarithmic phase of growth. Especially, *E. coli* harboring pYPC29 produced very high level of PL in the intracellular fraction which was the supernatant after ultra-sonication. Direct comparison of the PL activities between the donor strain and *E. coli* harboring pYPC29 may not be valid, but the latter produced much more PL than the former did. About 72% of the total PL activity in *E. coli* harboring pYPC29 was localized in the periplasmic fraction, and about 9% was excreted into the medium (Table 1).

From this result it could be expected that the PL gene had the signal peptide that was also recognized by *E. coli*. This result is the same as that found in the expressions of PL genes of *Erwinia* strains in *E. coli*.

요 약

토양으로부터 분리한 알카리내성 *Bacillus* sp. YA-14의 pectate lyase(PL) 유전자를 *E. coli*에 cloning하여 제조한 재조합 plasmid pYPC29는 삽입된 1.6kb 단편내에 PL 유전자를 함유하고 있었으며, 이 외래 DNA가 *Bacillus* sp. YA-14의 chromosomal DNA에서 유래된 것임을 Southern hybridization을 통하여 확인하였다. 재조합 plasmid pYPC29는 *E. coli*내에서 안정하게 존재하였으며 이를 함유한 재조합체의 전체 PL 활성 중 약 70%가 periplasmic space에 존재하였다.

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