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Cloning and Expression in *Escherichia coli* of a Bacteriolytic Enzyme Gene from Alkalophilic *Bacillus* sp.

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The gene encoding the bacteriolytic enzyme cell wall peptidoglycan hydrolase from alkalophilic *Bacillus* sp. was cloned in *E. coli* using pBR322 as a vector. A recombinant plasmid, designated pYTR451, was isolated and the size of the cloned *Hin*dIII fragment was found to be 4.8 Kb. The cell wall hydrolysis activity of an extract of the *E. coli* harboring the recombinant plasmid pYTR 451 was detected by SDS- polyacrylamide gel containing 0.2% (w/v) purified cell wall of *Bacillus* sp. The molecular weight of the enzyme was estimated to be about 27,000 corresponding to the molecular weight of the *Bacillus* sp. bacteriolytic enzyme. The recombinant plasmid was found to contain the fragment originated from *Bacillus* sp. YJ-451 chromosomal DNA by Southern hybridization.

Bacteriolysis can be induced by various method, including the action of antibiotics, proteins and enzymes (21, 7). Bacteriolytic enzyme is a peptidoglycan hydrolase which catalyzes the hydrolysis of cell wall peptidoglycan with a result of the lysis of the bacterial cells. Some peptidoglycan hydrolases have been characterized and classified as N-acetylmuramidase (lysozyme), N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase, endopeptidase, and transglycosylase (7). Bacteriolytic enzymes has been used to study the cell wall structure of microorganisms (7) and to investigate microbial spoilage prevention because of its antimicrobial potential (11). It is also used for the isolation of cytosolic fractions by removal of cell walls. Although numerous enzymes originated from bacterial species have been described (7), only a few genes encoding bacterolytic enzymes have been cloned and expressed in *E. coli* (6, 9, 12, 17, 18).

We have newly isolated alkalophilic *Bacillus* sp. YJ-451 which produces a bacteriolytic enzyme from soil collected at several areas in Korea and described the properties of the enzyme and its lytic action against *Bacillus* sp.(13). In this report we describe the cloning and expression of a gene encoding bacteriolytic enzyme from *Bacillus* sp. YJ-451.

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MATERIALS AND METHODS

Bacterial Strains and Plasmid

Bacillus sp. YJ-451 was used as the source of chromosomal DNA. *E. coli* HB101 (*supE44 hsd20 recA13 ara⁻14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*) (3) was used as the host strain. *Bacillus* sp. YC-335 (29) was used as the substrate for testing bacteriolytic activity. *E. coli* was grown at 37℃ with shaking in Luria Broth (10g of Bacto-Tryptone [Difco laboratories, Detroit, Mich.], 5g of Yeast extract [Difco], 5g of NaCl per liter, pH 7.2) and *Bacillus* sp. YJ-451 and *Bacillus* sp. YC-335 were grown in a medium containing 10g of soluble starch, 5g of yeast extract [Difco], 5g of polypeptone, 1g of K_2HPO_4, 0.2g of $MgSO_4 \cdot 7H_2O$ and 10g of Na_2CO_3 per liter (pH 10.2). Sodium carbonate was sterilized separately. pBR322 (Ap^r, Tc^r) (2) was used as vector. Antibiotics were used, when required, at following concentration; ampicillin, 50 μg/ml, and tetracycline, 15 μg/ml.

Preparation of DNA

Chromosomal DNA from *Bacillus* sp. YJ-451 was iso-

lated by the method of Rodriguez and Tait (19). Plasmid DNA was purified by the method of Tanaka and Weisblum (22). The rapid preparation of plasmid DNA was performed by alkaline-SDS method (1).

DNA Cloning

Bacillus sp. YJ-451 DNA was partially digested with *Hin*dIII, and ligated pBR322 which was digested with *Hin*dIII and phospholylated using calf intestine phosphatase. Transformation of *E. coli* was performed using competent cells prepared by the calcium chloride heat shock procedure. The ampicillin resistant and tetracycline sensitive transformants were selected on an agar plate. Colonies expressing bacteriolytic activity were detected by replica plating the transformants on to an LB agar medium containing 0.5% (w/v) autoclaved, lyophilized *Bacillus* sp. YC-335 cells. The plates were incubated at 37℃ for 2 to 3 hours and then overlaid with soft agar containing D-cycloserine (3 mg/5 ml). The bacteriolytic positive clone was selected by a clear zone in the agar medium after 16 to 18 hours incubation.

Preparation of Cell Wall from *Bacillus* sp.

The cell wall of *Bacillus* sp. was prepared by treatment with SDS followed by Triton X-100, as described by Potvin *et al.* (17).

Detection of Bacteriolytic Activity in SDS-Polyacrylamide Gel

SDS-PAGE gel containing 0.2% (w/v) purified cell wall of *Bacillus* sp. YC-335 was used for detection of cell wall hydrolysis activity of the recombinant clones, as described by Jayaswal *et al.* (12).

Southern Hybridization

Probe DNA was prepared by a random primer extension labeling system with [α-^{32}P]dCTP. DNA transfer to a nitrocellulose filter and hybridization were performed by the method of Southern (20).

RESULTS

Cloning of the Bacteriolytic Enzyme Gene

*Hin*dIII generated chromosomal DNA from *Bacillus* sp. YJ-451 was ligated with pBR322, which had been linearized with *Hin*dIII. The hybridized plasmid was transformed into *E. coli* HB101 and the transformants were selected on LB-ampicillin plates. Among 4,900 transformants selected as Ap^r Tc^s strains, one clone was selected which poduced a clear zone, indicating the presence of bacteriolytic activity (Fig. 1A) and the recombinant plasmid, designated pYTR451, was used for retransformation of *E. coli* HB101 to verify the presence of the bacteriolytic enzyme gene. All transformants had bacteriolytic activity (Fig. 1B). The cell wall hydrolysis activity of recombinant *E. coli* haboring recombinant plasmid pYTR451 exhibiting a clear zone was also detected by analysis of sonicated *E. coli* extracts on SDS-PAGE containing 0.2% (w/v) purified cell wall of *Bacillus* sp. YC-335. Recombinant *E. coli* showed a clear zone by a

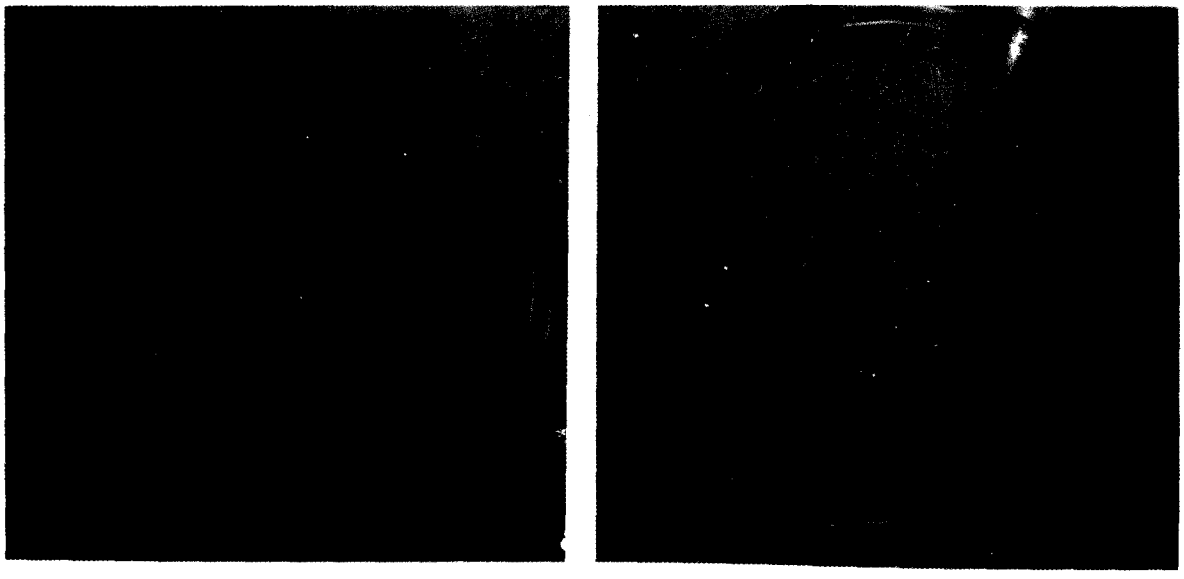

Fig. 1. The transformants showing bacteriolytic activity.

The transformants which were transferred onto LB agar medium containing 0.5% (w/v) autoclaved, lyophilized *Bacillus* sp. YC-335 cells were incubated at 37℃ for 2 to 3 h, and the plates were overlaid with soft agar containing D-cycloserine. After 16 to 18 h, lytic activity was shown by formation of a clear zone around colonies. (A) bacteriolytic enzyme positive clone; (B) retransformation of recombinant plasmid pYTR451.

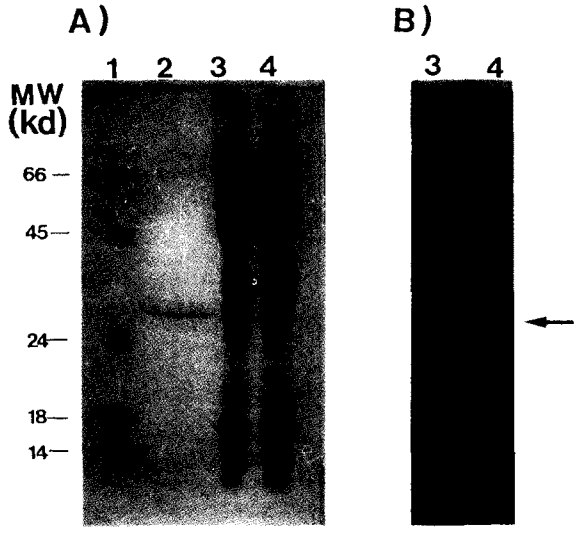

Fig. 2. Detection of bacterial cell wall hydrolysis activity in recombinant *E. coli* by SDS-PAGE (12% polyacrylamide gel containing 0.2% purified *Bacillus* sp. cell walls as substrate).

After electrophoresis, proteins were renatured by treatment with 25mM Tris-HCl (pH 8.0) containing 1% Triton X-100. (A) Coomassie brilliant blue staining; (B) methylene blue (1% methylene blue in 0.01% KOH) staining. Lanes: 1, protein standard markers 2, *Bacillus* sp. YJ-451 bacteriolytic enzyme 3, *E. coli* HB101 (pBR322) 4, *E. coli* HB101 (pYTR451).

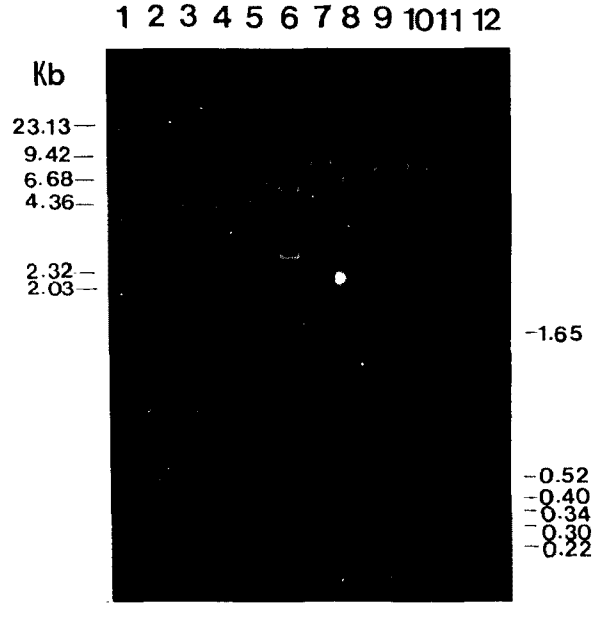

Fig. 3. Agarose gel electrophoretic analysis of restriction fragments of pYTR451.

Lane: 1, λDNA digested with *Hin*dIII as a markers 2, intact pYTR451 3, *Hin*dIII 4, *Bam*HI 5, *Pvu*II 6, *Ava*I 7, *Xho*I 8, *Cla*I 9, *Sal*I 10, *Kpn*I 11, *Pst*I 12, pBR322 digested with *Hin*fI as a markers. Kb, Kilobases.

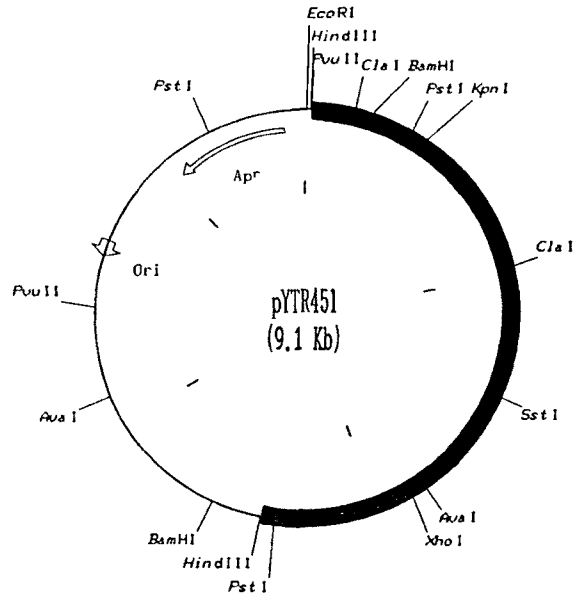

Fig. 4. Endonuclease cleavage map of pYTR451 DNA. Thick line denotes the insert. Kb, Kilobases.

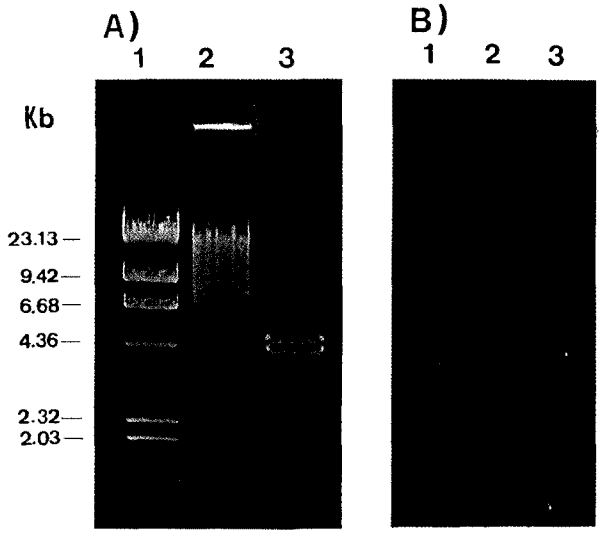

Fig. 5. Southern hybridization of *Bacillus* sp. YJ-451 chromosomal DNA and plasmid pYTR451 DNA.

The DNA digested with *Hin*dIII was electrophoretically separated and transferred onto nitrocellulose paper. After hybridization with ^{32}P-labeled 4.8 Kb *Hin*dIII fragment of pYTR451, the nitrocellulose paper was treated as described in Materials and Methods. (A) Ethidium bromide stained gel; (B) Corresponding southern hybridization autoradiogram. Lanes: 1, λDNA digested with *Hin*dIII as a control 2, *Bacillus* sp. YJ-451 DNA digested with *Hin*dIII 3, pYTR 451 DNA digested with *Hin*dIII.

polypeptide of apparent M.W. 27,000 Da, corresponding to the molecular weight of *Bacillus* sp. YJ-451 bacteriolytic enzyme (Fig. 2). This suggests that the *E. coli* haboring recombinant plasmid has a cell wall hydrolase activity, as well as *Bacillus* sp. YJ-451.

Restriction Analysis of Plasmid pYTR451

Plasmid analysis showed that the recombinant plasmid pYTR451 contained a 4.8 Kb insert. Various restriction enzymes were used to generate a physical map of the 4.8 Kb insert (Fig. 3). The recombinant plasmid pYTR 451 had single site for *Bam*HI, *Pvu*II, *Ava*I, *Xho*I, *Sst*I, *Kpn*I, and two *Pst*I, *Cla*I sites in 4.8 Kb inserted fragment (Fig. 4).

Southern Hybridization

To confirm the origin of the inserted fragment of pYTR-451 to be the chromosomal DNA of *Bcillus* sp.YJ-451, hybridization experiment was performed. The 4.8 Kb *Hin*dIII fragment from pYTR451 was labeled with [α-^{32}P]dCTP, as a hybridization probe. Hybridization of the ^{32}P-labeled 4.8 Kb *Hin*dIII fragment probe with the chromosomal DNA of *Bacillus* sp. YJ-451 demonstrated that the inserted fragment of pYTR451 originated from *Bacillus* sp. YJ-451 DNA (Fig. 5).

DISCUSSION

We cloned a chromosomal DNA fragment of *Bacillus* sp. YJ-451 which encoded a bacteriolytic enzyme using pBR322 vector in *E. coli*. The bacteriolytic positive clone was selected by a clear zone appearance on LB agar medium containing 0.5% (w/v) autoclaved, lyophilized *Bacillus* sp. cells treated with D-cycloserine. The cell wall hydrolysis activity of the *E. coli* harboring recombinant plasmid was identified by a lysis zone produced on SDS polyacrylamide gel containing 0.2% (w/v) purified cell walls of *Bacillus* sp. as substrate. The recombinant *E. coli* showed lytic activity corresponding to the molecular weight of the 27 KDa bacteriolytic enzyme from *Bacillus* sp. YJ-451. This technique can serve as a preliminary procedure for identifying the activity of cell wall hydrolase, but there are two limitations. First, only cell walls of gram positive bacteria can be incorporated in large amounts into denaturing gel. Second, only monomeric cell wall hydrolase, which is able to renature after denaturing electrophoresis, can be detected (15). The retransformation of the recombinant plasmid into *E. coli* resulted in the production of bacteriolytic enzyme by all transformants. The bacteriolytic enzyme gene can be stably maintained and expressed in *E. coli*. However, unlike *Bacillus* sp. YJ-451 which releases bacteriolytic activity extracellularly, bacteriolytic activity in *E. coli* could only be detected after D-cycloserine treatment.

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