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Nucleotide Sequence of a Bacteriolytic Enzyme Gene from Alkalophilic *Bacillus* sp.

JUNG, MYEONG-HO, SEUNG-HO OHK, DO-YOUNG YUM, IN-SOO KONG[1], DONG-HOON BAI[2] AND JU-HYUN YU*

Department of Food Engineering, College of Engineering, Yonsei University, Seoul 120-749, Korea
[1]Department of Biotechnology and Bioengineering, National Fisheries University of Pusan, Pusan 608-737, Korea
[2]Department of Food Engneering, College of Engineering, Dankook University, Chunan 330-714, Korea

The nucleotide sequence of *Bacillus* sp. bacteriolytic enzyme gene, *lyt*P and its flanking regions were determined. A unique open reading frame for a protein of Mw. 27,000, and a putative terminator sequence, were found behind a concensus ribosome binding site located 8 nt upstream from ATG start codon. The primary amino acid sequence deduced from nucleotide sequence revealed a putative protein of 255 amino acid residues with an Mw. of 27,420. No significant homology could be found between the amino acid sequence of *Bacillus* sp. bacteriolytic enzyme and that of other cell wall hydrolases.

INTRODUCTION

Bacteria possesses enzymes that can degrade their own cell walls (27) and the cell walls of various microorganisms (22, 23). In most cases, a bacterial cell wall is hydrolyzed by enzymes active on peptidoglycan. Bacteriolytic enzymes that catalyze the lysis of bacterial cell walls have been characterized as N-acetylmuramidase (lysozyme), N-acetylglucosaminidase, N-acetylmuramyl-L-alanine amidase, endopeptidase, or transglycosylase (9). Bacteriolytic enzymes are interesting because of their latent antimicrobial activity (11). Several bacteriolytic enzymes have been detected because of the ready availability of *Micrococus lysodeikticus* which is highly sensitive to bacteriolysis of live or dead cells, provoked by dissolution of cell walls (12).

We isolated several alkalophilic *Bacillus* strains from soil under alkaline conditions (13, 30). These strains produced various useful enzymes (4, 5, 31, 32), and the properties and expression of gene codings for these enzymes were investigated (14, 16, 18, 33, 34). And we identified an alkalophilic *Bacillus* sp. produces a novel bacteriolytic enzyme from soil (13) and cloned a gene encoding the bacteriolytic enzyme from a genomic bank and expressed in *Escherichia coli* (29).

In this report, we report the nucleotide sequence of bacteriolytic enzyme gene *lyt*P, and its putative amino acid sequence.

MATERIALS AND METHOD

Bacterias and Plasmids

E. coli HB101 (*supE44, hsd20, recA13, ara*$^{-}$*14, proA 2, lacY1, galK2, rpsL20, xyl*$^{-}$*5, mtl*$^{-}$*1*) (3) was used as a host for the cloning experiment, and *E. coli* JM109 (*rK*$^{-}$, *mK*$^{-}$, *recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1,* λ-, Δ(*lac-proAB*)/*F'*, *traD36, proAB, lacIq ZΔM 15*) (28) was used to propagate recombinant M13mp19 phages for nucleotide sequence analysis. pBR322 (Ap^{r}, Tc^{r}) (2) was used as vector. Plasmid pYTR451 was recombinant plasmid harboring bacteriolytic enzyme gene.

DNA Isolation

Plasmid DNA was purified by the method of Tanaka and Weisblum (25). The rapid preparation of plasmid DNA from *E. coli* was performed by using alkaline SDS extraction of cell lysates (1). Bacteriophage RF DNA was isolated from *E. coli* by the method of Messing *et. al.*(15).

*Corresponding author
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Construction of Overlapping Deletion Subclones

Overlapping deletions were generated with Cyclonel Biosystem as described by Dale *et. al.*(6). To subclone into M13mp19, plasmid pYTR453 was digested with *Cla*I. A 1.6 Kb DNA fragment was ligated with *Acc*I-digested M13mp19 in both directions. The 1.6 Kb insert DNA fragment was deleted with exonuclease activity of T_4 DNA polymerase from the 3'-end of the inserted DNA, tailed with polyA, ligated and transformed into JM109. Single strand DNAs were isolated from the transformed white plaque, electrophoresed and fractionated in size.

DNA Sequencing

DNA sequencing was done by Sanger's' dideoxy chain termination procedure (21) with a modified T7 polymerase (Sequenase, US Biochemical, Cleveland, OH). Recombinant M13 phage growth, DNA preparation and DNA chain termination sequencing with [α-^{35}S]dATPαS were performed according to the protocols of U.S. Biochemicals. Electrophoresis was carried out on a 6% polyacrylamide/8M urea gel. Sequence data were analyzed by the Pustell DNA analysis program (IBI, New Haven, CT).

Enzyme and Reagent

All restriction endonuclease and T_4 DNA ligase were purchased from Promega Biotec. (Madison, Wis.) and New England Biolabs and were used as recommended by suppliers. Cyclonel Biosystem was obtained from International Biotechnologies, Inc.. Isopropyl-β-D-thiogalacto-pyranoside (IPTG), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were purchased from Sigma Chemical co. (St. Louis, MO.).

Nucleotide Sequence Accession Number

The DNA sequence data here have been submitted to EMBL and were assigned the accession number X 60071.

RESULTS AND DISCUSSION

Subcloning of Bacteriolytic Enzyme Gene

A 1.6 Kb *Cla*I fragment from plasmid pYTR451 was ligated into the *Cla*I site of pBR322 vector in both orientations. The ligation mixtures were used to transform competent *E. coli* HB101, and the activity of the transformants was checked by using replica colonies on LB agar. The LB agar contains 0.5% (w/v) autoclaved, lyophilized *Bacillus* sp. cells and was overlaid with soft agar containing D-cycloserine (13). The clones were scored as lysis positive if a clear zone appeared around the colony. The strain bearing plasmid pYTR452 which was cloned in the opposite orientation, showed lytic activity, whereas the strain bearing plasmid pYTR452-N did not. And the strain bearing a plasmid pYTR451-N, which contained 2.9 Kb *Cla*I/*Hin*dIII fragment of pYTR451 also did not show lytic activity. This suggested that the structural gene of bacteriolytic enzyme was located within the 1.6 Kb *Cla*I fragment without a promoter sequence since only clone (pYTR452) using P1 promoter (24) of the region coding for Tc resistance in pBR322 vector, was able to express bacteriolytic enzyme. Plasmid pYTR 453 was constructed by inserting a 0.7 Kb *Him*dIII/*Kpn*I fragment of pYTR451 into *Him*dIII/*Kpn*I cut pYTR452 to include a promoter region. The restriction map and the extent of the subclones are illustrated in Fig. 1.

Nucleotide Sequence of the *Bacillus* sp. Bacteriolytic Enzyme

The DNA fragment coding bacteriolytic enzyme gene inserted in recombinant plasmid pYTR453, was sequenced by following the strategy summarized in Fig. 2. The nucleotide sequences of the 1.6 Kb *Cla*I fragment from pYTR453 were determined by dideoxy chain termination sequencing method using a series of overlapping M13mp19 recombinant clones carrying bacteriolytic enzyme gene with progressive delection from the 3' end. Such nested sets of delected fragments were created by using CYCLONE technique. The 0.7 Kb *Hin*dIII/*Kpn*I fragment of plasmid pYTR453 was cloned in M13mp19 and the nucleotide sequence of 300 bp *Hin*dIII/*Cla*I fragment was determined. The nucleotide sequence and the predicted amino acid sequence of the bacteriolytic enzyme are shown in Fig. 3. The amino acid were analyzed with a computer program (IBI). An open reading frame of 255 amino acid residues extended from an AUG initiation codon to an UAA termination codon. The coding sequence corresponding to a bacteriolytic enzyme protein with an estimated molecular weight of

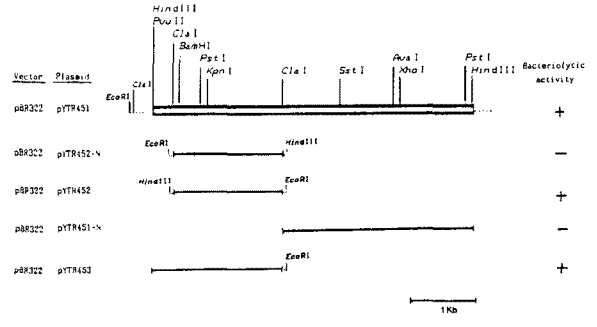

Fig. 1. Restriction endonuclease map of the 4.8 Kb chromosomal DNA fragment inserted into the *Hin*dIII site of pBR322.

The subclones were constructed using pBR322 vector as described in Results and Discussion. Each clones was tested for lytic activity. Symbols: +, lysis; −, no lysis. ---, pBR322 vector sequence.

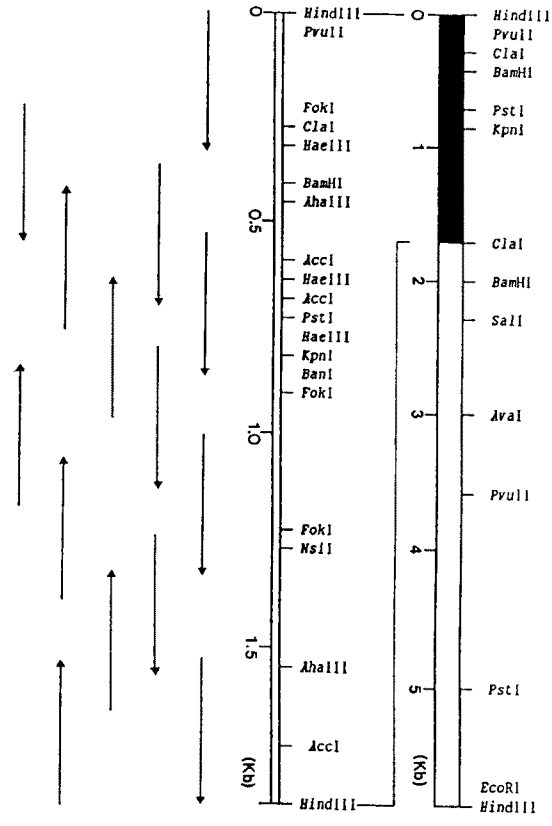

Fig. 2. Strategy for sequencing the 1.9 Kb *Hind*III/*Cla*I fragment of pYTR453 containing the bacteriolytic enzyme gene from *Bacillus* sp. YJ-451.

pYTR453 is hybrid plasmid containing of the 1.9 Kb fragment of *Bacillus* sp. YJ-451 DNA (filled bar) and pBR322 (open bar). The arrows show the direction of reading from inserts in M13mp19.

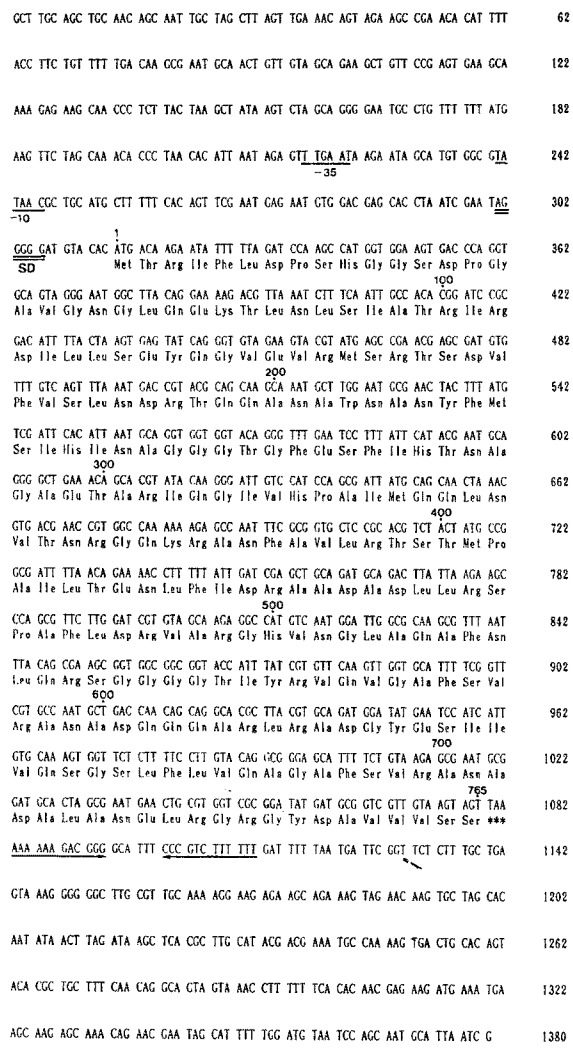

Fig. 3. Nucleotide sequence of the *Bacillus* sp. YJ-451 bacteriolytic enzyme gene and deduced amino acid sequence.

Numbering of both nucleotides and amino acids starts with the beginning of the coding sequence. Putative promoter sequences homologous to the −35 and −10 regions of the *B. subtilis* recognition sequences are underlined and labeled −35 and −10, respectively. The putative ribosome binding site is double-lined. An inverted repeat sequence capable of forming a stem-loop structure is marked by horizontal arrows.

27,420 daltons is proceeded by the inferred ribosome binding site AGGGGGG, which is separated from the initiation codon by 8 bp which complements to 3′ end of 16S ribosomal RNA of *Bacillus subtilis* (10). The regions at nucleotide 218~223 and 241~246 is presumed to be RNA polymerase binding site of the promoter with −35 (TTGAAT) and −10 (TATAAC) regions, with a space of 17 bp. Downstream from UAA stop codon was a potential stem-loop structure, followed by a stretch of U residues (Fig. 4), which may serve as a transcription terminator (19).

Amino Acid Analysis

The primary amino acid sequence deduced from the nucleotide sequence of *lyt*P gene revealed a putative protein of 255 amino acid residues with an apparent Mw. of 27,420. The putative bacteriolytic enzyme has an average pI of 11.0 and is thus a basic protein. When the deduced amino acid sequence was used to find homology, no significant homology could be found between the sequence of the *Bacillus* sp. bacteriolytic enzyme and that those of *Streptococcus pneumoniae* cell wall amidase (7), *Bacillus* bacteriolytic enzyme (20) or various *Bacillus* bacteriophage enzyme including *Bacillus* phage Φ29 gene 15 lysozyme (8), *B. subtlis* phage PZA gene 14 lysozyme (17), and *Bacillus* bacteriophage SF6 lysozyme (26).

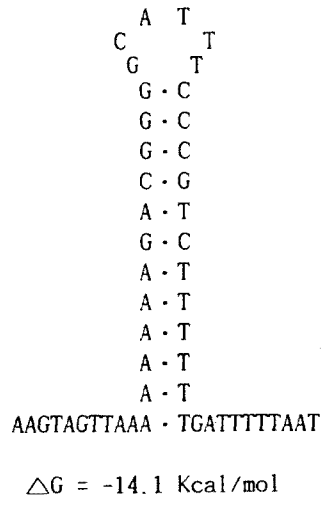

Fig. 4. Hairpin structure formed by nucleotides illustrating a putative terminator for transcription of bacteriolytic enzyme in *Bacillus* sp.

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