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Molecular Cloning and Sequencing of Cell Wall Hydrolase Gene of an Alkalophilic *Bacillus subtilis* BL-29

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A DNA fragment containing the gene for cell wall hydrolase of alkalophilic *Bacillus subtilis* BL-29 was cloned into *E. coli* JM109 using pUC18 as a vector. A recombinant plasmid, designated pCWL45B, was contained in the fragment originating from the alkalophilic *B. subtilis* BL-29 chromosomal DNA by Southern hybridization analysis. The nucleotide sequence of a 1.6-kb *Hin*dIII fragment containing a cell wall hydrolase-encoding gene was determined. The nucleotide sequence revealed an open reading frame (ORF) of 900 bp with a concensus ribosome-binding site located 6 nucleotide upstream from the ATG start codon. The primary amino acid sequence deduced from the nucleotide sequence revealed a putative protein of 299 amino acid residues with an M.W. of 33,206. Based on comparison of the amino acid sequence of the ORF with amino acid sequences in the GenBank data, it showed significant homology to the sequence of cell wall amidase of the PBSX bacteriophage of *B. subtilis*.

Bacteria produce several types of cell wall hydrolases, enzymes capable of hydrolyzing the peptidoglycan of the cell envelope. On the basis of their cleavage specificities, the enzymes are classified as *N*-acetylmuramidases (lysozymes), *N*-acetylglucosaminidases, *N*-acetylmuramyl-L-alanine amidases, endopeptidases, and transglycosylases (23). Cell wall hydrolases are thought to be involved in cell wall turnover (10), cell separation (3), competence for genetic transformation, formation of flagella, sporulation and the lytic action of some general antibiotics. Recent evidence has also suggested a role for autolysins in bacterial pathogenicity. However, their precise roles are still speculative because of the difficulty in obtaining mutants for the genes determining these enzymes. Up to now, although many other alkalophilic enzymes of bacterial origin have been described, there have been few studies describing the cloning of the gene encoding bacteriolytic enzyme from alkalophilic bacteria.

We previously reported on the isolation and some properties of the extracellular lytic enzyme (8, 9). To study the molecular evolution of lytic enzyme, we report here on the cloning, sequencing of the cell wall hydrolase gene of *Bacillus subtilis* BL-29 (8) and on a comparison of the deduced polypeptide with that of other cell wall hydrolases.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids and Media

Alkalophilic *Bacillus subtilis* BL-29 (8) was used as the source of chromosomal DNA. *Escherichia coli* JM 109 (*recA*1, *endA*1, *gyrA*96, *thi*, *hsdR*17(r_k^-, m_k^+), *supE*44, Δ(*lac-proAB*), *relA*1, F'[*traD*36, *proAB*$^+$, *lacI*^{q}ZΔM15]) was used as a host bacterium for cloning purposes and M13 phage proliferation.

E. coli was grown in Luria-Bertani (LB) medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 10 g NaCl per liter) and *B. subtilis* BL-29 was grown in an alkaline medium AII (10 g soluble starch, 5 g yeast extract, 5 g polypeptone, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$ and 10 g Na_2CO_3 per liter, pH10.2). All cloning and subcloning work were conducted using the plasmid pUC18 and the bacteriophage vectors (M13mp18, M13mp19) were used for the determination of DNA sequence.

General DNA Techniques and Transformation

Molecular cloning techniques were performed essentially as described by Maniatis *et al.* (16). Restriction endonucleases, T4 DNA ligase, Calf intestinal alkaline phosphatase and Klenow polymerase were used according to the suppliers' instructions. DNA fragments for subcloning or preparation of probes were recovered from agarose gels with Gene Clean Kit (Bio101). Chromosomal DNA of *B. subtilis* BL-29 was obtained by phenol extraction from lysozyme and SDS-treated cells. Plasmids and M13 phage DNAs were isolated using the

method of SDS/alkaline lysis (2) from *E. coli*.

Preparation of *E. coli* Cell Wall and Assay of Cell Wall Lytic Activity

The cell walls of *E. coli* KCTC 1682 were prepared by treatment with SDS followed by Triton X-100, essentially as described by Potvin *et al.* (18). Lytic enzyme activities were measured basically as described previously (7).

Gene Library of *B. subtilis* BL-29 Chromosomal DNA in *E. coli*

B. subtilis BL-29 chromosomal DNA, prepared as described by Harwood *et al.* and partially digested *Hin*dIII, was subjected to agarose gel electrophoresis. DNA fragments in the size range 2~10 kb were recovered from the agarose gel using Gene Clean (Bio101). The DNA fragments were ligated with pUC18 DNA, which had been digested with *Hin*dIII, followed by dephosphorylation with calf intestine alkaline phosphatase, and the ligated DNA was added to competent *E. coli* JM109 cells. The colonies containing insert (Ap^r, white) were selected on LB agar plates containing ampicillin and X-Gal, and used as gene library.

Selection of *E. coli* Harboring a Cell Wall Hydrolase Gene

Ampicillin-resistant (Ap^r) transformants were transferred with toothpicks to LB agar plates containing *E. coli* KCTC 1682 cell wall (0.05%) and ampicillin (50 μg/ml). The plates were incubated at 37°C for 2 to 3 h and then overlaid with soft agar containing D-cycloserine (3 mg/ml) which inhibits the cell wall synthesis. The bacteriolytic positive clone was selected by a clear zone around the colony in the agar medium after 16 to 18 h incubation.

Hybridization Procedures

For Southern hybridization (22), size fractionated DNA samples were transferred from agarose to nitrocellulose membranes by alkali blotting (19). Probes were prepared by the random priming labelling method using [α-^{32}P]dATP from Amersham, U.K.

DNA Sequencing

Nucleotide sequencing was performed by the dideoxy chain termination (21) method with a modified T7 polymerase of Sequenase™ kit. Phages M13mp18 and M13mp19 were used to generate templates for sequencing. Electrophoresis was performed on 6~8% (wt/vol) polyacrylamide-8M urea gels.

RESULTS AND DISCUSSION

Cloning of the Bacteriolytic Enzyme Gene

Chromosomal DNA of *Bacillus subtilis* BL-29 was partially digested with *Hin*dIII and ligated with *Hin*dIII-digested pUC18. *E. coli* JM109 was transformed with the ligation mixture and plated onto LB agar containing ampicillin. After overnight culture, transformants were picked onto LB agar plates containing *E. coli* KCTC1682 cell wall (0.05%) and ampicillin. Among 4500 Ap^r transformants tested, one clone was selected which produced a clear zone, indicating the presence of bacteriolytic activity (Fig. 1). Plasmid analysis showed that the recombinant clone contained a 6.7-kb insert (pCWL 45B, Fig. 2). Retransformation of *E. coli* JM109 with pCWL45B gave transformants that all expressed the lytic activity (Data not shown). This suggests that the insert encoded a protein with lytic activity.

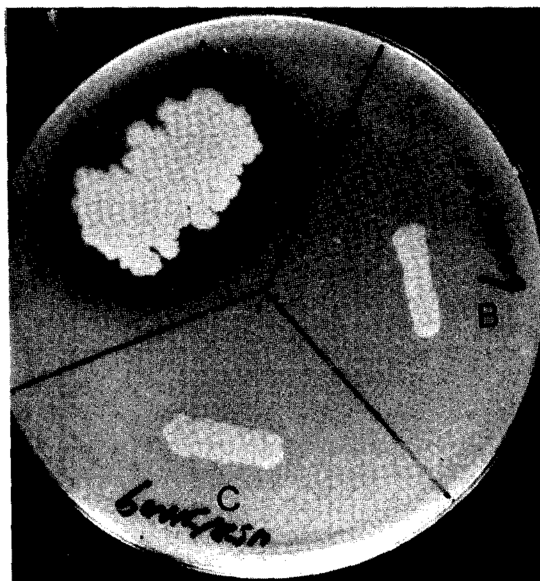

Fig. 1. Screening of recombinant *E. coli* JM109 containing cell wall hydrolase gene by D-cycloserine treatment.
A, *B. subtilis* BL-29; B, *E. coli* JM109 containing pUC18; C, Recombinant *E. coli* JM109 containing pCWL45B consisting 6.7-kb *Hin*dIII insert in pUC18.

Restriction Analysis and Subcloning

The plasmid DNA, designated as pCWL45B, was digested with several restriction enzymes to map its restriction sites within the cloned fragment. The size of the cloned DNA fragment was about 6.7-kb. In order to localize the essential region of the gene coding for cell wall lytic enzyme in the 6.7-kb *Hin*dIII fragment, plasmid pCWL45B was partially digested with *Hin*dIII or *Eco*RI and religated with pUC18. The ligated DNA was introduced into competent cells of *E. coli* JM109 and several subclones were obtained. The lytic activity of the subclones was checked by patching colonies on LB agar containing cell wall and ampicillin as early described. As shown in Fig. 3, The plasmids pCWL580, 590 and 600 showed lytic activity. However, there was no activity observed in the plasmids pCWL570, 610 containing the H_1-H_3, E_1-E_2 fragment of the pCWL45B, respectively. These results suggest that the gene en-

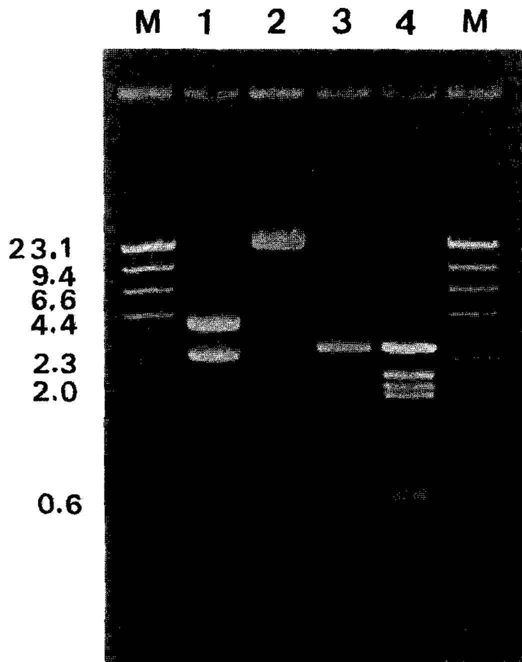

Fig. 2. Agarose gel electrophoresis of pCWL45B carrying cell wall hydrolase gene of *Bacillus subtilis* BL-29.
Lane M, Marker (λ-*Hin*dIII); Lane 1, pUC18; Lane 2, Recombinant DNA pCWL45B; Lane 3, pUC18 digested with *Hin*dIII; Lane 4, Recombinant DNA pCWL45B digested with *Hin*dIII.

coding the lytic enzyme is located within the H_3-H_4 region. In order to establish the fine restriction map of plasmid pCWL600, the plasmid DNA was digested with various restriction endonucleases and the resulting fragments were analyzed by agarose gel electrophoresis (data not shown). The physical map of pCWL600, constructed from the above results, is shown in Fig. 4. The recombinant plasmid pCWL600 had single site for *Pst*I, *Eco*RI, *Hinc*II, *Sca*I and *Hpa*I. No cutting site for *Sma*I,

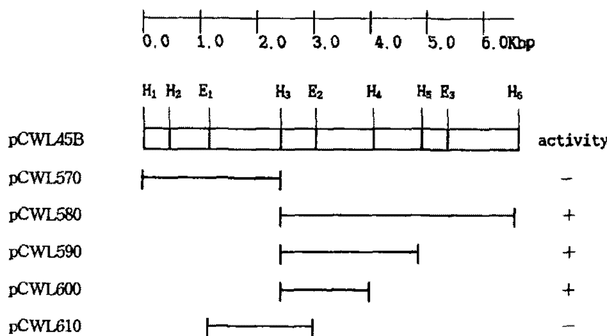

Fig. 3. Restriction map of the 6.7-kb pCWL45B containing cell wall hydrolase gene of *B. subtilis* BL-29 and localization of lytic enzyme gene by subcloning.
E, *Eco*RI; H, *Hin*dIII.

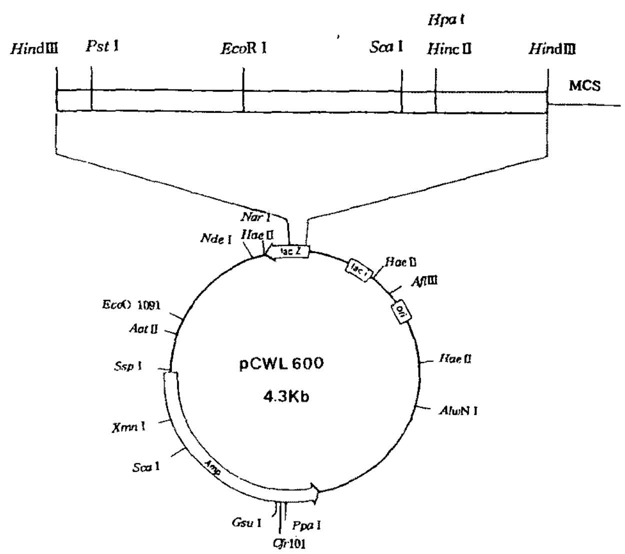

Fig. 4. Restriction endonuclease map of recombinant plasmid pCWL600.

*Xba*I and *Bam*HI was observed.

Southern Hybridization

To confirm the inserted fragment of pCWL600 was derived from the chromosomal DNA of *B. subtilis* BL-29, a hybridization experiment was performed. The chromosomal DNA and plasmid pCWL600 were digested with *Hin*dIII, fractionated by 0.8% agarose gel electrophoresis, and then DNA were blotted with the 0.6-kb *Eco*RI-*Hinc*II probe fragment prepared from pCWL600. Fig. 5 shows that the 23-kb, 6.7-kb, 3.9-kb, 2.3-kb and 1.6-kb *Hin*dIII-digested chromosomal DNA correspond to the size of the *Hin*dIII digested fragment of the plasmid pCWL600 were hybridized with the 0.6-kb *Eco*RI-*Hinc*II probe fragment. This result demonstrates that the cloned DNA fragment was originated from the *B. subtilis* BL-29 chromosomal DNA. Southern hybridi-

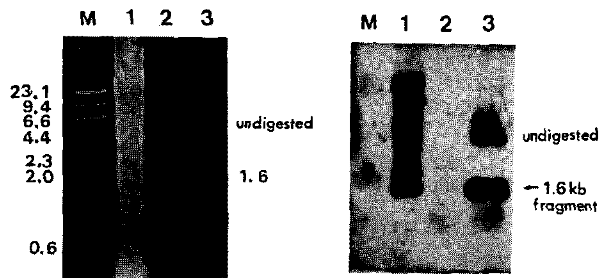

Fig. 5. Southern hybridization analysis of the *Hin*dIII digested *B. subtilis* BL-29 chromosomal DNA. The 0.6 kb *Eco*RI-*Hinc*II DNA fragment of pCWL600 was used as a probe.
A, Photograph of EtBr-stained gel; B, Southern hybridization pattern. Lane M, λ-*Hin*dIII; 1, *B. subtilis* BL-29 DNA digested with *Hin*dIII; 2, pUC18 digested with *Hin*dIII; 3, pCWL600 digested with *Hin*dIII.

zation experiments revealed that a 0.6-kb *Eco*RI-*Hinc*II probe hybridized with five, different loci, suggesting the presence of one or more copies of this gene. Presence of multiple copies of lytic genes has been reported in *B. licheniformis* (17) as well as in *Streptococcus pneumoniae*, where the close relationship between host and phage enzymes (20) has provided strong support for the concept of the modular organization of proteins.

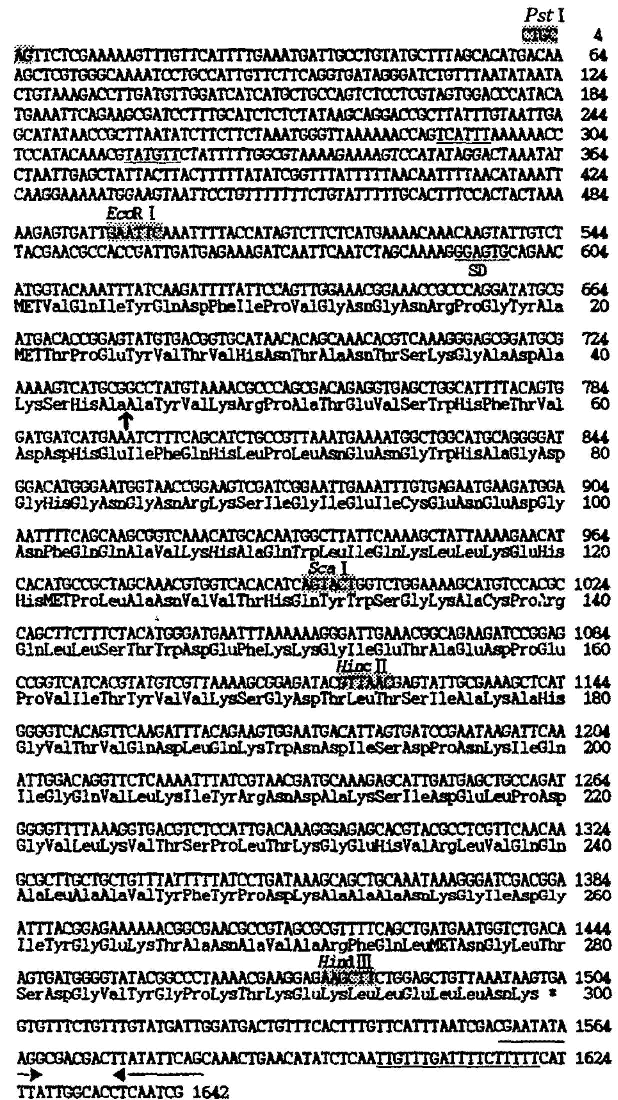

Fig. 6. Nucleotide sequence of the cloned *B. subtilis* BL-29 cell wall hydrolase gene.

Only the sequence of the nontranscribed DNA strand is shown from position +1 (*Pst*I site). The deduced amino acid sequence is given below the nucleotide sequence. Putative promoter sequences (-35, TCATTT and -10, TATGTT) and terminator (inverted repeat sequences, 1558-1582), as well as a putative ribosome-binding sequence (SD) are indicated. ↑ indicates putative signal sequence cleavage site. * indicates a stop codon.

DNA Sequences Determination

The 1.6-kb *Hin*dIII region of pCWL600 was sequenced by means of the dideoxy chain termination reaction (Fig. 6). The nucleotide sequence showed one long ORF, starting at nucleotide 605 and ending at nucleotide 1501, which encodes a polypeptide of 299 amino acid residues with a molecular mass of 33,206 Da. Six nucleotide upstream from the start codon (ATG) was a putative ribosome binding site, 5'-GGAGTG-3', which exhibits complementarity to the 3' end of the 16S ribosomal RNA of *Bacillus subtilis* (6). A putative leader sequence was found in the first 44 amino acids and the cleavage site was located after the sequence Ser-His-Ala using the strategy described by von Heijne (24). Downstream from the stop codon of the gene (TGA at bp 1502) a perfect inverted 8-bp repeat with a loop of 7 nucleotides is located, that is followed by a stretch of T residues. This region could form a stable stem-loop structure (if transcribed, with a free energy ΔG=-10 kcal/mol) and may function as a rho-independent terminator of transcription (5). The total G+C content of the ORF was 45.5% and the G+C content of the second position of codons has the lowest mol% (36%).

Deduced Amino Acid Sequences and Homology Comparisons

The deduced amino acid sequences of the ORF presumed in the 1682 bp *Hin*dIII fragment were compared with the sequence of other cell wall lytic enzymes in the National Biomedical Research Foundation (NBRF), SWISS-PROT, Genbank, and EMBL databases. A similarity search of protein databases showed that the ORF product has extensive similarity to the cell wall lytic amidase of *B. subtilis* (*cwlA*, 13; *cwlC*, 16), *B. licheniformis* (*cwlM*, 14; *cwlL*, 19), *Bacillus* sp. (18) and a prophage PBSX amidase of *B. subtilis* (*xlyA*, 15). In particular, the highest homology was found between a *xlyA*

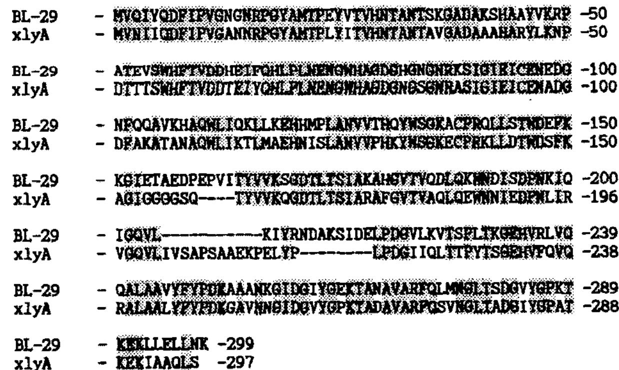

Fig. 7. Comparison of the deduced amino acid sequence of the gene (BL-29) to that of the *N*-acetylmuramyl-L-alanine amidase of *Bacillus subtilis* prophage PBSX (*xlyA*). Identical amino acids are indicated by shadow.

gene and the lytic enzyme of the BL-29 (66% identity over 299 residues) (Fig. 7). Computer analysis of the deduced amino acid sequences of the lytic enzyme revealed three domains with homologies to other enzymes. The *N*-terminal region, encompassing amino acids 1-99, is highly similar to the *N*-terminal region of XlyA (15), a prophage PBSX amidase of *B. subtilis* and to that of the *B. subtilis* amidase (Fig. 7). Table 1 shows the amino acids composition of other cell wall hydrolase. Cell wall-lytic amidases in the genus *Bacillus* are classified into two groups; class I contains CwlA (4, 11), CwlL (17), and a lytic enzyme from PBSX (XlyA) (15), and class II contains CwlB (13) and CwlM (12). Furthermore, amino acid sequence homology among other autolytic enzymes suggests that a lytic enzyme of a *Bacillus* species (18) and a sporulation phase-specific lytic enzyme, CwlC (14), belong to class I and class II, respectively. Because of the predicted amino acid sequence of the lytic enzyme from *B. subtilis* BL-29 showed significant similarity with the sequences of proteins in the class I family but low similarity with those in class II, it may be lytic enzyme belong to class I family. Recent reports suggest that the class I family may be lytic enzymes derived originally from phages, because the genes neighboring the lytic enzyme gene are functionally similar to phage genes (4, 15, 17). It is, however, imposible to unambiguously attribute such entities to PBSX-like phage or to their degenerate copies.

Table 1. Comparison of the amino acid composition of cell wall hydrolase determined from the nucleotide sequence.

Amino acid	*B. subtilis* BL-29	*B. subtilis* (XlyA)	*B. subtilis* (CwlA)
Non-polar	Num (%)	Num (%)	Num (%)
Ala	25 (8.36)	36(12.1)	25 (9.19)
Val	23 (7.69)	20 (6.73)	14 (5.15)
Leu	22 (7.36)	20 (6.73)	15 (5.51)
Ile	17 (5.68)	20 (6.73)	18 (6.62)
Pro	14 (4.68)	16 (5.38)	9 (3.31)
Met	4 (1.34)	3 (1.01)	6 (2.20)
Phe	7 (2.34)	8 (2.69)	6 (2.20)
Trp	6 (2.00)	6 (2.02)	4 (1.47)
Polar			
Gly	24 (8.03)	28 (9.43)	24 (8.82)
Ser	12 (4.01)	13 (4.37)	21 (7.72)
Thr	18 (6.02)	22 (7.41)	17 (6.25)
Cys	2 (0.67)	2 (0.67)	3 (1.10)
Tyr	11 (3.67)	12 (4.04)	12 (4.41)
Asn	18 (6.02)	20 (6.73)	16 (5.88)
Gln	16 (5.35)	13 (4.37)	9 (3.31)
Acidic			
Asp	18 (6.02)	17 (5.72)	10 (3.67)
Glu	17 (5.68)	12 (4.04)	10 (3.67)
Basic			
Lys	25 (8.36)	13 (4.37)	37(13.6)
Arg	7 (2.34)	8 (2.69)	11 (4.04)
His	13 (4.35)	8 (2.69)	5 (1.84)
Total A.A	299(100)	297(100)	272(100)
MW	33,206	31,913	29,957

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