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Isolation of β-Lactamase Inhibitory Protein from *Streptomyces exfoliatus* SMF19 and Cloning of the Corresponding Gene

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The β-lactamase inhibitory protein (BLIP) produced by *Streptomyces exfoliatus* SMF19 was purified (33 kDa) and the N-terminal amino acid sequence was determined as NH2-ATSVVAWGGNND. Genomic DNA library of *S. exfoliatus* SMF19 was constructed in pWE15 and recombinants harbouring the corresponding gene were selected by colony hybridization to the mixture of 36-mer oligonucleotide designed from the N-terminal amino acid sequence. The corresponding gene (*bli*X) was isolated on a 4-kb *Apa*I fragment of *S. exfoliatus* SMF19 chromosomal DNA and then sequenced. The *bli*X consisting of 1,119 bp encoded a mature protein with a deduced amino acid sequence of 342 residues and also encoded a 40-amino-acid signal sequence. No significant sequence similarity to *bli*X was found by pairwise comparison using various protein and nucleotide sequences.

Resistance of pathogenic bacteria to the β-lactam antibiotics is usally due to the production of various β-lactamases, which cleave the β-lactam ring of β-lactam antibiotics (1). Resistant bacteria have frequently been isolated from the places where the antibiotics were widely used or disposed. Analysis of such results have suggested that the ability to produce β-lactamases has been aquired by horizontal gene transfer (15).

Chemical modifications of the β-lactam antibiotics have contributed to improvement of the therapeutic effects against pathogenic bacteria producing β-lactamases. In addition, useful β-lactamase inhibitors from microbial metabolites have been developed. β-lactamase inhibitors of low molecular weight, such as clavulanic acid, olivanic acid, thienamycin, and their derivatives have been clinically used. A proteinaceous β-lactamase inhibitor that inhibits β-lactamase from *Staphylococcus aureus* was first isolated from *Streptomyces gedanensis* (9). Later, a β-lactamase inhibitory protein (BLIP) produced by *S. clavuligerus* was characterized and the corresponding gene, *bli*, was sequenced (8). Transcriptional analysis and heterologous expression of the gene encoding BLIP in *S. lividans* were also reported (17). The 3-dimensional structure of BLIP was determined by X-ray crystallography (24).

We have purified and characterized β-lactamase inhibitory proteins produced extracellularly by *S. exfoliatus* SMF19 (11, 12). Here, we present on the cloning and sequencing of the gene encoding one of them.

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MATERIALS AND METHODS

Bacterial Strains, Cosmid, and Plasmids

Streptomyces exfoliatus SMF19 (11, 12) was used as a source of genomic DNA. *Escherichia coli* DH5α was used for the propagation of the recombinant cosmid and plasmids. *E. coli* Top10F' (Invitrogen, San Diego, CA) was used as the host for pZero-2 (Invitrogen). pWE15 (25), a broad-host-range cosmid vector, was used for the construction of the genomic librariy of *S. exfoliatus* SMF 19. Subcloning experiments were performed with the vectors pBluscript KS(+) (Stratagene Inc., La Jolla, CA, U.S.A.), pZero-2, and pUC18 (26).

Culture Conditions

S. exfoliatus SMF19 was maintained on a slope of R 2YE agar medium and spores formed on the medium were stored at -20°C in a 20% glycerol. For the preparation of vegetative mycelium, the strain was cultivated in tryptic soy broth (Difco Laboratories, Detroit, MI) at 28°C on a rotary shaker (150 rpm). All *E. coli* strains were grown in LB medium (20). Ampicillin (60 $\mu g \cdot ml^{-1}$) or kanamycin (50 $\mu g \cdot ml^{-1}$) was added to LB for the stable maintenance of plasmid or cosmid DNA.

Purification of BLIP from *S. exfoliatus* SMF19

The purification of BLIP was conducted as described previously (12). N-terminal amino acid sequence of the BLIP was determined by a modified phenylthiohydantoin method (14), using the Milligen/Biosearch 6600

Prosequencer (Millipore).

β-Lactamase Inhibition Assay

β-lactamase activity was estimated at pH 7.0 and 30°C by the modified iodometric assay method (22). β-lactamase inhibition activity was calculated as follows: β-lactamase inhibition activity (%)=[(A-B)/A]× 100, where A is the β-lactamase activity without the inhibitor and B is the β-lactamase activity with the inhibitor. One unit of β-lactamase inhibitory activity was defined as the amount of inhibitor needed for 50% inhibition of 0.96 unit of β-lactamase (Bacto-penase from Difco).

Preparation of Recombinant DNA

DNA manipulations for *Streptomyces* species were performed as described by Hopwood *et al.* (10). The large-scale purification of cosmid and plasmid was achieved by the alkaline lysis method (20). Small-scale preparation of plasmid DNA, transformation of *E. coli*, and construction of recombinant cosmid and plasmids were followed the established standard techniques (20). A library of *S. exfoliatus* SMF19 was constructed in the cosmid vector pWE15 as follows. One hundred micrograms of *S. exfoliatus* SMF19 chromosomal DNA was partially digested with *Sau*3AI (Poscochem, Sungnam, Korea) to produce an average fragment size of approximately 50 kb. The reaction was stopped by phenol extraction, and the mixture was applied to a 50 ml 10%-40% sucrose gradient and then centrifuged for 20 h at 26,000 rpm in a Beckman SW28 rotor. Fractions (1.0 ml) containing DNA fragments of 40-60 kb were pooled and concentrated by ethanol precipitation. 500 ng of the size-fractionated DNA was then ligated in a total volume of 20 μl with 1.4 μg of *Bam*HI-digested pWE15 that had been treated with calf intestinal alkaline phosphatase (Poscochem) to prevent recircularization. After 12 h of ligation at 16°C, the ligated DNA was packaged in bacteriophage lambda particles by using the Giga Pack II kit (Stratagene) and was introduced into *E. coli* DH5α by transduction.

A collection of *E. coli* colonies containing recombinant cosmids was screened by colony hybridization under highly stringent condition. The biased mixture of 36-mer oligonucleotide was synthesized in Genosys, Woodland, Texas. The hybridization probe was labelled by using ECL 3'-oligolabelling and detection sytems (Amersham, Buckinghamshire, England). Southern blot hybridization and colony hybridization were performed by the standard techniques (20). Physical mapping of the selected recombinant plasmid was conducted by an analysis of the products of single and double-restriction enzyme digestion and consideration of the results of corresponding hybridization experiments (20).

DNA Sequence Determination

DNA sequencing was conducted by the dideoxy method of Sanger *et al.* (21), using Sequenase™ 2 and utilizing 7-deaza-GTP in place of dGTP in oder to relieve compressions in the banding pattern on DNA sequencing gels. The DNA sequence of each fragment was determined repeatedly in oder to provide unequivocal results in the regions of high G+C content which are characteristic of *Streptomyces* DNA and difficult to sequence. For this purpose, DNA fragments of various sizes were generated by use of an exonuclease III kit. The entire sequence was assembled from overlapping clones analysis using either the DNASIS (Hitachi, Japan) or Macframe (13).

The nucleotide sequence data were analyzed for the presence of restriction sites, regions of dyad symmetry, RNA secondary structure, and codon usage by using the DNASIS and PC-Gene programs (Intelligenetics Corp., Mountan View, CA, U.S.A.). The corresponding protein sequence was examined by using the FASTP protein sequence analysis programs (18) and their accompanying protein sequence data banks.

RESULTS

Purification of BLIP and Determination of N-Terminal Amino Acid Sequencing of the Protein

BLIP was purified from culture filtrate of *S. exfoliatus* SMF19 as described previously (12). The molecular weight was determined as 33 kDa by SDS-PAGE (Fig. 1).

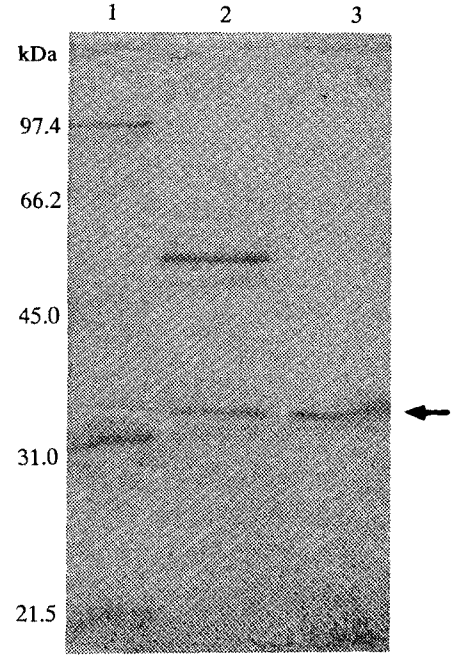

Fig. 1. Analysis of $BLIP_{SMF19}$ by SDS-PAGE.
Lane 1, molecular markers; lane 2, pooled fraction after DEAE-Sephadex A-50 chromatography; lane 3, pooled fraction of FPLC with Superdex (Pharmacia). The arrow indicates $BLIP_{SMF19}$.

The N-terminal amino acid sequence was determined as: NH_2-Ala-Thr-Ser-Val-Val-Ala-Trp-Gly-Gly-Asn-Asn-Asp. The protein was named as $BILP_{SMF19}$.

Cloning of Gene Encoding $BLIP_{SMF19}$

A 36-mer, mixed-oligonucleotide probe was designed from the N-terminal amino acid sequence as 5'-GC(G/C) AC(G/C)TC(G/C)GT(G/C)GTCGCGTGGGGCGGCA-ACAACGAC-3' considering the biased codon usage of *Streptomyces* sp. A library of *S. exfoliatus* SMF19 chromosomal DNA fragments was constructed in the cosmid vector pWE15 and forteen recombinants highly hybridizing with the probe were obtained in a population of about 8,000 colonies.

*Apa*I digests of the forteen hybridizing cosmids and the chromosomal DNA of *S. exfoliatus* SMF19 were separated by agarose gel electrophoresis and hybridized with the pool of 36-mer oligonucleotide. A 4-kb *Apa*I fragment was detected in chromosomal DNA of *S. exfoliatus* SMF19 and in cosmid DNA isolated from three recombinants. The fragment (4 kb) isolated from the cosmid DNA was subcloned into a positive selection vector (pZero-2) and the plasmid was designated as pSMF251. The position of the gene encoding $BLIP_{SMF19}$ determined by hybridization of the oligonucleotide probe to a Southern blot of *Sac*I, *Sac*II, *Sal*I, or *Bam*HI-digested pSMF251 DNA are shown in Fig. 2 and Fig. 3.

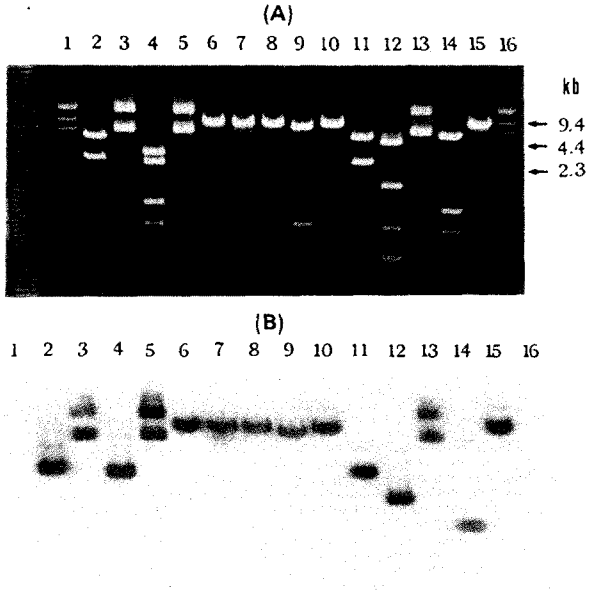

Fig. 2. Southern analysis of pSMF251.

The pSMF251 DNA was digested with the several restriction enzymes, separated by 1% agarose gel electrophoresis for southern analysis (A), and hybridized to the biased mixture of 36-mer oligonucleotide (B). Lane 1, 16, λ/*Hin*dIII size markers; lane 2, *Bam*HI; lane 3, *Bcl*I; lane 4, *Bgl*I; lane 5, *Cla*I; lane 6, *Eco*RI; lane 7, *Eco*RV; lane 8, *Kpn*I; lane 9, *Nco*I; lane 10, *Pst*I; lane 11, *Sac*I; lane 12, *Sac*II; lane 13, *Sal*I; lane 14, *Sma*I; lane 15, *Xho*I.

Nucleotide Sequence of the Gene Encoding $BLIP_{SMF19}$

To determine the nucleotide sequence of the gene encoding $BLIP_{SMF19}$, the two *Sal*I-*Sac*II fragments (about 0.7 kb and 0.9 kb) prepared from pSMF251 were subcloned into pBluescript KS(+) and one *Bam*HI-*Apa*I fragment (about 1.0 kb) obtained from pSMF251 was subcloned into pZero-2.

The nucleotide sequence of the gene encoding $BLIP_{SMF19}$ was determined from the fragments prepared and through the strategy given in Fig. 3.

The sequence are shown in Fig. 4 and the G+C content of the entire sequenced region as a function of codon position was analyzed by using the Macframe program. An open reading frame (ORF) was initially recognized by the presence of a DNA sequence which corresponded precisely to the N-terminal 12-amino-acid sequence of the $BLIP_{SMF19}$. Further analysis by DNASIS program revealed three unique ORFs starting at an ATG codon (nucleotide 476), a GTG codon (nucleotide 521), and an ATG (nucleotide 524). Among them, the ATG codon (nucleotide 476) was adopted because it is preceded by a potential ribosome-binding site (4) and a typical leader peptide sequence (underlined in Fig. 4) that has been observed in other *Streptomyces* sp. (8) was found after the codon.

The ORF terminated at a TAG translation stop codon adjacent to an inverted repeat sequence (underlined arrows in Fig. 4) which was thought to encode the stable RNA stem-loop structure ($\triangle G$=-9.70 kcal·mol^{-1} [27]). The overall mean G+C content of this ORF was 73%;

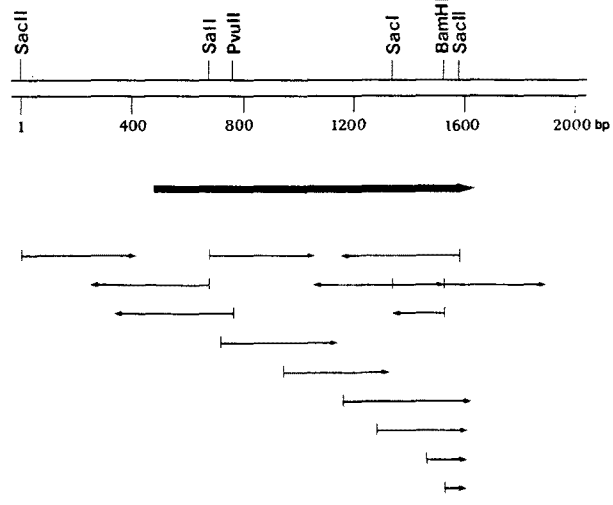

Fig. 3. Sequencing strategy for the $BLIP_{SMF19}$ of *S. exfoliatus* SMF19.

The sites used for cloning of DNA fragments obtained by digestion with restriction enzymes and deletion by S1 exonuclease are indicated by arrows whose length and direction indicate the extent of sequence determination from these sites. The thick arrow shows the coding region of the putative $BLIP_{SMF19}$ gene product.

Fig. 4. DNA sequence of region encompassing *S. exfoliatus* SMF19 $BLIP_{SMF19}$ gene.
The deduced amino acid sequence of the $BLIP_{SMF19}$ protein precursor is presented. The leader peptide is underlined and the presumed site of N-terminal processing of the precursor is indicated by a vertical arrowhead. The nucleotide constituting the putative $BLIP_{SMF19}$ ribosome-binding-site and the region of dyad symmetry presumable encode a transcriptional terminator are bold underlined and underlined with horizontal arrows.

the values for codon positions 1, 2, and 3 were 68%, 59%, and 89%, respectively.

Assuming that the ORF starts at the ATG codon, the gene encoding $BLIP_{SMF19}$ was determined to be 1,116 bp in length. The comparison of amino acid sequence deduced from the ORF with the N-terminal amino acid sequence of $BLIP_{SMF19}$ revealed that the sequence (1,116 bp) might be encoding a precursor protein which included a leader peptide consisting of 40 amino acid residues and the mature protein ($BLIP_{SMF19}$). Amino acids deduced from the ORF is 372, of which 8.2% are acidic, 8.2% are basic, and 62.8% are hydrophobic. The calculated molecular weight of a mature form of $BLIP_{SMF19}$ is 33,335.9 Da. The leader peptide contained three positively charged amino acid two arginines at positions 7 and 10 and one histidine at position 9, followed by a long hydrophobic stretch (residues 11-32). These data indicated that the sequence (1,116 bp) is a complete structural gene encoding $BLIP_{SMF19}$ and the gene was named as *bli*X.

From the analysis of amino acid sequence, very highly conserved repeats of amino acid sequences were observed (Fig. 5). One (region L) consisting of 13 amino acids (SLALKDGKVIAWG) was observed at 116 - 127, 154 - 166, 232 - 244, and 271 - 283. The other (region P) consisting of 19 amino acids (GQTTVPAEAQSGVDAIAGG) was observed at 132 - 150, 171 - 189, 210 - 228, and 249 - 267.

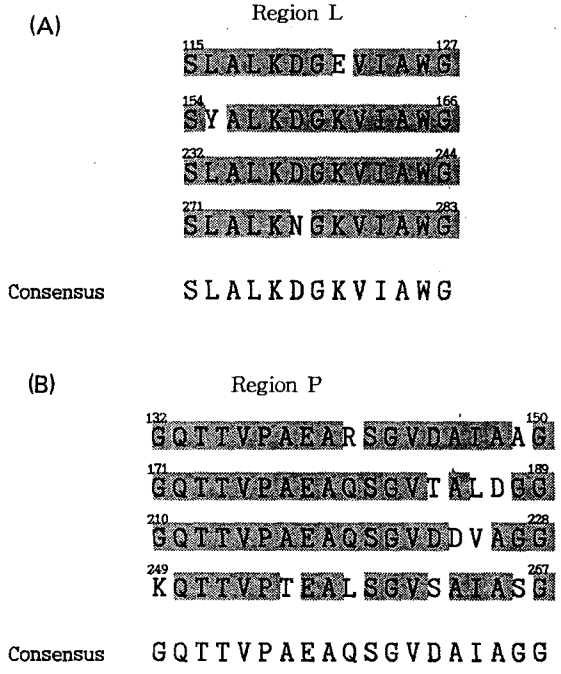

Fig. 5. Alignment of the repeated amino acid sequences of *bli*X.

Two kinds of the repeated amino acid sequence (region L and region P) was found in the deduced amino acid sequence. Strands are numbered sequentially from the N terminus of the deduced amino acid sequence from *bli*X. Identical residues are shaded. The repeated amino acid sequences of region L of 13 amino acid residues (A) and region P of 19 amino acid residues (B) are aligned.

DISCUSSION

The N-terminal amino acid sequence of $BLIP_{SMF19}$ was different from that of the βLIP-II, although the molecular weight was same (12). The sequence and size were also different from that of BLIP obtained from *S. clavuligerus* (17.5 kDa). Sequence similarity analysis of *bli*X and $BLIP_{SMF19}$ with the data currently available in the GenBank and EMBL revealed no significant similarity to any known amino acid sequence. Furthermore the deduced products of *bli*X of *S. exfoliatus* and *bli* of *S. clavuligerus* did not show any similarity (8).

*bli*X of *S. exfoliatus* encoded a 40 amino acid leader peptide, similar in length and position of the positively charged and hydrophobic residues to the leader peptides of other secreted proteins from *Streptomyces* sp. including *bli* of *S. clavuligerus* (8). In addition, *S. clavuligerus* produced a variety of β-lactam antibiotics including penicillin N and cephamycin C, BLIP and a small molecule β-lactamase inhibitor, clavulanic acid, containing a β-lactam ring (8). However, no antibiotic activity in the culture filtrate of *S. exfoliatus* SMF19 was detected (data not shown). These imply that the biological function of $BLIP_{SMF19}$ may not resemble to that of *S. clavuligerus*.

The 3-dimensional structure of BLIP produced in *S. clavuligerus* revealed that BLIP had a tandem repeat of a 76-amino acid domain (24). Analysis of amino acid sequence of $BLIP_{SMF19}$ showed several repetition of two highly conserved sequences (region L and region P). To investigate the realtionship between the conserved sequence, the protein structure, and the active site, site-specific mutagenesis, heterologous expression, and 3-dimensional structure are required.

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