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Cloning of Isopenicillin N Synthase Gene from *Lysobacter lactamgenus*

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The gene for isopenicillin N synthase (cyclase; IPNS) was cloned from *Lysobacter lactamgenus* using DNA probe amplified with primers based on the consensus sequences of isopenicillin N synthase genes of other β-lactam-producing microorganisms. The genomic library of *L. lactamgenus* using pUC18 plasmid cloned at the *SacI* site were screened with the PCR-generated DNA probe and three positive clones were isolated. Enzyme activities in *E. coli* clones were confirmed by bioassay and HPLC assay. Throughout the functional mapping, it was observed that the gene for isopenicillin N synthase is located at the 1.3-kb *XhoI-BamHI* fragment of insert of positive clones. Nucleotide sequencing at both ends of the *XhoI-BamHI* fragment revealed that IPNS of *L. lactamgenus* has the common amino acid sequences at amino- and carboxy-termini.

The β-lactam antibiotics, especially penam and cephem antibiotics, are produced by a variety of fungi and streptomycetes. It is well known that the initial biosynthetic pathways of these antibiotics have common steps, including the formation of L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) tripeptide followed by the first penam ring formation named isopenicillin N (IPN) (1, 4, 6, 12, 18).

Since 1982, certain bacteria including *Xanthomonas*, *Pseudomonas*, *Flavobacterium*, and *Lysobacter* have been reported to produce cephem antibiotics (13, 23). Among them, *Lysobacter lactamgenus* has known to be capable of synthesizing a new class of cephem compounds named cephabacins, which have oligopeptides at the 3-position and methoxy or formylimdino group at the 7α-postion of the cephem ring (5, 24). It was also confirmed by bioassay and HPLC assay that this strain has the enzyme activity of an isopenicillin N synthase (IPNS) (9, 16).

In this paper, we report the cloning and initial characterization of the IPNS gene from *L. lactamgenus*. The gene was probed by PCR product amplified using from the the consensus sequences of IPNS genes from other β-lactam producing microorganisms.

MATERIALS AND METHODS

Strains and Media

L. lactamgenus IFO 14288 was grown on a medium of 2% glucose, 1% casamino acid, 0.1% sodium sulfate, and 0.01% nickel chloride (pH 7.0) at 30°C with rotary shaking at 150 rpm (17). *Escherichia coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ [*lac-proAB*]) as the host cell was cultivated on LB (Luria-Bertani) media composed of 1% trypton, 0.5% yeast extract, and 1% sodium chloride (pH 7.2) at 37°C. When necessary, the LB media was supplemented with 50 μg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), or 0.4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). *Micrococcus luteus* ATCC 9341 for antibiotic susceptibility test was cultured on nutrient agar at 30°C for 24 h.

Vectors and DNAs

The pUC18 plasmid was employed as a cloning vector to construct a genomic library. For preparation of *L. lactamgenus* chromosomal DNA, harvested cells from a 100-ml culture in SET buffer (25% sucrose, 20 mM Tris, 5 mM EDTA) was treated with 1 mg proteinase K and 0.5% sodium dodecyl sulfate (SDS) at 37°C for 1 h. From the resulting solution, the chromosomal DNA was precipitated with 0.6 volumes of isopropanol after extracting with chloroform and isoamyl alcohol (24 : 1), and preserved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing RNase (0.25 μg/ml) at -20°C. The primer oligonucleotides were supplied from Bioneer Co. (Cheongwon, Korea).

Polymerase Chain Reaction (PCR)

The polymerase chain reaction was carried out using 50 ng of *L. lactamgenus* chromosomal DNA as template, 100 pmole of forward primer and 100 pmole of reverse

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primer in the presence of 15 mM magnesium chloride and 5 units of Taq polymerase (15). The reaction proceeded at 94°C for 1.5 min for denaturation, at 50°C for 2 min for annealing, and at 72°C for 2 min for extension for 30 cycles in a DNA thermal cycler (Model FPROG05D, Techne, U.K.).

Southern Hybridization

The amplified DNA was labelled with 20 μCi of [α-P^{32}]dCTP (10 mCi/mL, Amersham, U.K.) at 15°C for 1 h using a Nick translation kit (Gibco BRL, MD, U.S.A.). The labelled DNA was employed as probe after purifying through an Elutip-d column (Schleicher & Schuell, Germany). Southern transfer was performed following the procedure of Sambrook *et al.* (19) after 0.7% agarose gel electrophoresis and fixation onto nitrocellulose membrane by UV-crosslinker (Model CL-1000, UVP, CA, U.S.A.). The membrane was then hybridized overnight with the labelled PCR probe at 42°C after prehybridization with 100 μg/ml denatured salmon sperm DNA for 6 h, and washed twice with 2×SSC (0.3 M sodium chloride, 0.03 M sodium citrate) and 0.1% SDS at 50°C. Finally the membrane was exposed on X-ray film at -70°C.

DNA Manipulation

The recombinant plasmid library was made by ligating *Sac*I-digested *L. lactamgenus* chromosomal DNA with *Sac*I-digested and calf intestinal phosphatase (CIP)-treated pUC18 plasmid. After transformation into *E. coli* JM109, the white colonies on LB media containing ampicillin, IPTG and X-Gal were screened by *in situ* hybridization with the labelled probe (19). Based on a restriction map of the positive clone, subclones were made using several restriction enzymes, and hybridized with the probes.

DNA Sequencing

The nucleotide sequence of IPNS gene was determined using universal primers by Custom service of Bioneer Co. (Cheongwon, Korea). The determined nucleotide sequence was further analyzed using PC-DOS HIBIO DNASIS Program (version 7.0; Hitachi Software Engineering Co., Japan).

Analysis of Enzyme Activity

Cell-free extracts of clones were prepared by sonication of the cultured cells for 30 seconds 5 times with ultrasonic desmembrator (Model XL2010, Heat Systems-Ultrasonics, Inc., NY, U.S.A.). The enzyme reaction mixture consisted of 100 μg of *bis*-ACV, 40 mM dithiothreitol, 0.1 mM ferrous sulfate and 3 mM ascorbic acid in 0.5 M Tris buffer (pH 7.2). After reaction at 27°C with vigorous shaking at 250 rpm for 2 h, the same volume of methanol was added to precipitate proteins. The amount of isopenicillin N produced in the supernatant was then analyzed by bioassay on *M. luteus* ATCC 9341 or by high performance liquid chromatographic (HPLC) assay (7, 8).

RESULTS AND DISCUSSION

Southern Hybridization

In order to confirm the presence of the IPNS gene (*pcbC*) in *L. lactamgenus* chromosome, the PCR primers were designed according to the consensus sequences of *pcbC* genes of *Penicillium* (2, 3), *Cephalosporium* (20), *Aspergillus* (14) and *Streptomyces* (10, 11, 21, 22, 25) reported previously. The forward primer was synthesized as 5'-(A/G)AAGGCCGTCGA(A/G)TC(G/C)T-3' (primer #1) for amino acids [98-102] of IPNS, and the reverse primers were 5'-ACGGTGATCA(G/T)(G/C)GA(A/G/C/T)ACGTC-3' (primer #2) for amino acids [216-212] and 5'-(G/C)(G/C)GT(C/T)TC(G/C)ACCTGGAGGTT (primer #3) for amino acids [229-235]. Using these primers, the IPNS gene (*pcbC*) of *L. lactamgenus* was amplified by PCR. As seen in Fig. 1, a 368-bp fragment of the IPNS gene was found between primer #1 and primer #2, and a 412 bp between primer #1 and primer #3. This implies that *L. lactamgenus* carries an IPNS gene having the consensus sequences of other β-lactam producers.

After labelling these PCR products with [α-P^{32}]dCTP, the chromosomal DNA of *L. lactamgenus* digested with several restriction enzymes was hybridized by Southern blotting. As shown in Fig. 2, the chromosomal DNA cleaved by *Sac*I or *Xho*I gave single hybridization bands at 4.7 kb or 4.5 kb, regardless of using any of two labelled probes. However, two DNA blots at 4.0 kb and 5.0

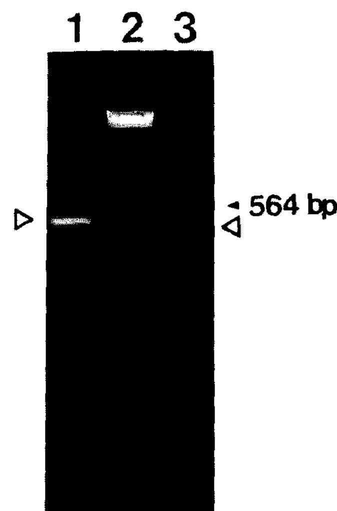

Fig. 1. DNA amplification of *L. lactamgenus* chromosomal DNA using the consensus sequences of IPNS from other β-lactam producing organisms.
Lane 1, PCR product from primer #1 and #3; lane 2, molecular weight marker (λ DNA/*Hin*dIII); lane 3, PCR product from primer #1 and #2 on 1.1% agarose gel electrophoresis.

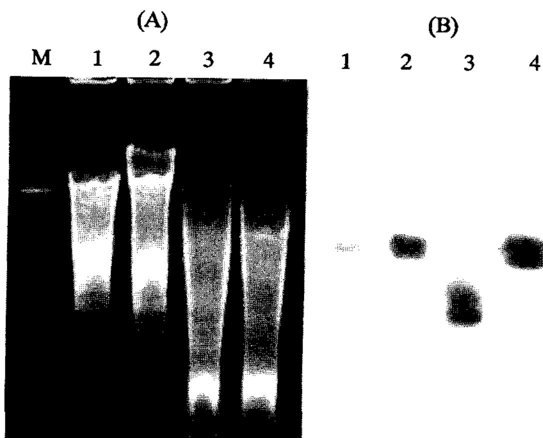

Fig. 2. Southern blotting of *L. lactamgenus* chromosomal DNA with amplified PCR products.

(A), 0.7% agarose gel electrophoresis of *L. lactamgenus* chromosomal DNA digested with restriction enzyme; (B), Southern blot of (A) with PCR products. Lane M, λ DNA/*Hin*dIII; lane 1, *L. lactamgenus* chromosomal DNA/*Sac*I; lane 2, *L. lactamgenus* chromosomal DNA/*Xho*I; lane 3, *L. lactamgenus* chromosomal DNA/*Sal*I; lane 4, *L. lactamgenus* chromosomal DNA/*Pvu*II.

kb appeared on the gel of *Pvu*II-cleaved chromosomal DNA, and 2.1 kb and 1.8 kb on *Sal*I-cleaved chromosomal DNA. From these results, it was deduced that the IPNS gene of *L. lactamgenus* might be located in the *Sac*I- or *Xho*I-digested fragments of chromosomal DNA.

Selection of Positive Clones

Based on the above result, the genomic library of *L. lactamgnus* was constructed in pUC18 plasmid after digesting the chromosomal DNA with *Sac*I. The transformed *E. coli* JM109 were screened by colony hybridization with the 412-bp PCR product of the IPNS gene. Three clones giving strong signals were chosen as positive clones.

The enzyme activities of IPNS in the positive *E. coli* clones were also examined using the cell-free extracts. In bioassay, the reaction mixture showed antibacterial activity against *M. luteus* ATTC 9341 due to the production of isopenicillin N from *bis*-ACV by IPNS (Fig. 3). This was also confirmed by the appearance of a new isopenicillin N peak in an HPLC chromatogram of reaction mixture of cell-free extracts.

Restriction and Functional Mapping

Restriction analysis of selected plasmids showed that all inserts of the clones have identical restriction patterns. 2 *Xho*I sites, 2 *Nco*I sites and 1 *Bam*HI site were recognized as drawn in Fig. 4.

Following the constructed restriction map of the positive clones, the location of the putative IPNS gene in the inserts was examined by functional mapping. As shown in Fig. 4, enzyme activity was present in the 1.3 kb *Xho*I-*Sac*I fragment, which was one of DNA fragments generated by *Xho*I-digestion of insert. When 0.1 kb of *Bam*HI-*Sac*I fragment was deleted in this 1.3 kb, the subclone still showed the enzyme activity. Based on previous reports that the size of the IPNS genes is nearly 1.0 kb in all the β-lactam producers (2, 3, 10, 11, 14, 20, 22, 25), it can be deduced that the full sequence of IPNS of *L. lactamgenus* might be located in this 1.2-kb *Xho*I-*Bam*HI fragment.

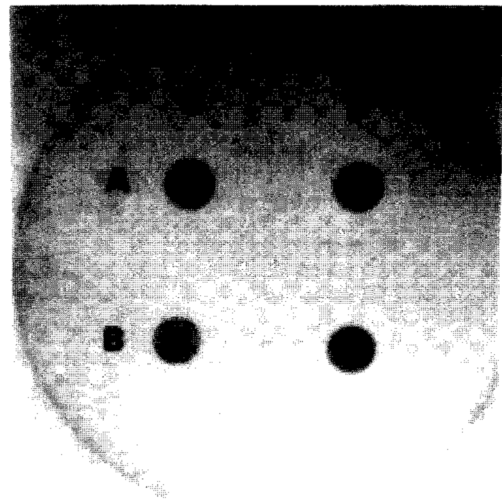

Fig. 3. The determination of IPNS activity in positive clones by the growth inhibition of *M. luteus*.

A, cell-free extract of *L. lactamgenus*; B, cell-free extract of pLIPS clone; C, control (0-h reaction); R, reaction for 2 h at 27°C.

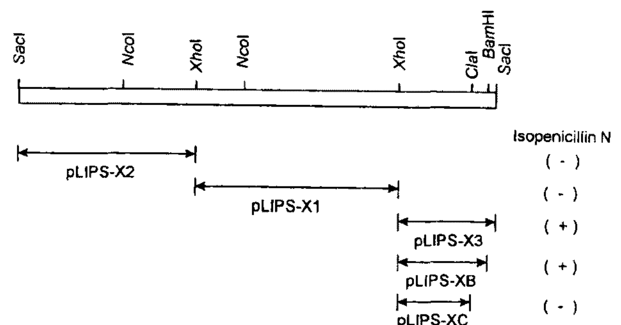

Fig. 4. Restriction map of insert and its functional mapping for the identification of IPNS locus.

Isopenicillin N: synthesis of isopenicillin N from ACV by IPNS.

DNA Sequencing

The IPNSs are known to have several consensus amino acid sequences. One of these is V-P-(K/R/T/V)-I-D-(V/I)-S-(P/G)-L-(F/S)-G at the amino terminus and L-(I/Y)-(N/V/A/R)-(K/A)-N-(G/V)-Q-T at the carboxy terminus (2, 3, 10, 11, 14, 20, 22, 25) (Fig. 6).

Based on these reports, the sequence of *L. lactamgenus* IPNS in the *Xho*I-*Bam*HI fragment was deter-

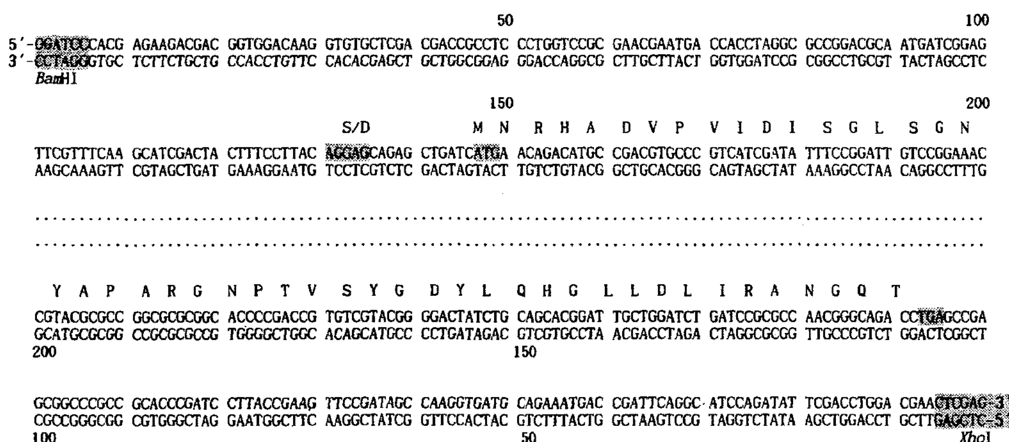

Fig. 5. Nucleotide sequences of the putative IPNS gene at 5'-end and 3'-end of *Xho*I-*Bam*HI fragment.

(Amino Terminus)

		(reference)
Penicillium chrysogenum	MAST**PKANVPKIDVSPLFGDN	(18)
Aspergillus nidulans	MGSV**SKANVPKIDVSPLFGDD	(20)
Cephalosporium acremonium	MGSVPVPVANVPRIDVSPLFGDD	(19)
Streptomyces clavuligerus	MPVL*MPSAHVPTIDISPLFGTD	(21)
Streptomyces lipmanii	MPVL*MPSADVPTIDISPLFGTD	(22)
Streptomyces jumonjinensis	MPIL*MPSAEVPTIDISPLSGDD	(23)
Streptomyces griseus	MPIP*MLPAHVPTIDISPLSGDD	(24)
Flavobacterium sp.	MN****RHADVPVIDISGLSGND	(25)
Lysobacter lactamgenus	MN****RHADVPVIDISGLSGLS	

(Carboxy Terminus)

		(amino acid residue)
Penicillium chrysogenum	SYGDYLQNGLVSLIDSKNGQT	331
Aspergillus nidulans	SYGDYLQNGLVSLINKNGQT	331
Cephalosporium acremonium	SYGEYLQGGLRGLINKNGQT	338
Streptomyces clavuligerus	SYGDYLQHGLRALYVKNGQT	329
Streptomyces lipmanii	TYGEYLQEGFHALIAKNVQT	333
Streptomyces jumonjinensis	SYGEYLQHGLRALYVKNGQT	329
Streptomyces griseus	RYGDYLQQASNALIAKNGQT	329
Flavobacterium sp.	SYGDYLQHGLLDLIRANGQT	326
Lysobacter lactamgenus	SYGDYLQHGLLDLIRANGQT	

Fig. 6. Comparison of amino acid sequences at amino terminus and carboxy terminus of *L. lactamgenus* IPNS with other IPNSs of β-lactam-producing microorganisms.
The conserved amino acids in all IPNSs of β-lactam producers are expressed in double letters, and the conserved area used in the identification of amino acid residues are indicated in black boxes. The number means the bases from *Bam*HI or *Xho*I ends sequenced. Total number of amino acid residues of IPNSs appeared in each references are noted at the end of carboxy termini.

mined from both ends using universal primers and compared with IPNS gene sequences of other β-lactam producing organisms (Fig. 5). It was found that the V-P-V-I-D-I-S-G-L-S-G peptide sequence was located 165~197 bp away from the *Bam*HI recognition site. The putative translation initiation codon was also recognized at 18 bp (6 amino acids) upstream from the above sequence, and the presumable Shine-Dalgarno (S/D) sequence, AG-GAG, was seen at 16 bp upstream of the translation initiation codon.

The L-I-N-A-N-G-Q-T peptide sequence followed by the translation termination codon, TGA, was also found 130~94 bp away from the *Xho*I restriction site.

These results suggest that the 1.2 kb *Xho*I-*Bam*HI fragment carries the full gene for *L. lactamgenus* IPNS.

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