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Membrane Transporter Genes in Cephabacin Biosynthetic Gene Cluster of *Lysobacter lactamgenus*

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Abstract In order to clone the peptide synthetase gene from *Lysobacter lactamgenus* IFO 14,288, the gene fragments were amplified using primers for the adenylation domain and the thionylation domain of the peptide synthetase genes in other organisms by polymerase chain reaction (PCR). The resulting 0.5-kb fragment was cloned in a pGEM-T vector, and the nucleotide sequences were determined. Six different PCR products were obtained; three were identified to be a part of L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) synthetase and three to be other peptide synthetases. Using each of the two different classes of PCR products as mixed probes, a cosmid library of *L. lactamgenus* chromosomal DNA constructed in a pHC79 vector was screened by an *in situ* hybridization procedure, and one positive clone was selected which was bound by peptide synthetase gene fragments as well as ACV synthetase gene fragments. The partial sequence analysis from the obtained pPTS-5 cosmid showed the presence of more than two open reading frames. These were for two putative membrane transporters, which were homologous with several integral membrane proteins including the ABC transporter ATP-binding protein of *E. coli* (YbjZ) and the metal ion uptake protein of *Bacillus subtilis* (YvrN). A 45% homology was also found between the two transporter proteins at the carboxy terminus. Through a hydropathy analysis and transmembrane analysis, 4-5 transmembrane domains were found in these two proteins. When the genes were expressed in *Escherichia coli*, the gene products inhibited the host cell growth, probably due to the disturbance of the membrane transport system.

Key words: Cephabacin, *Lysobacter lactamgenus*, membrane transporter, cephem antibiotic, antibiotic biosynthetic gene

Cephabacins are a class of cephem antibiotics that are produced by the Gram-negative bacillus, *Lysobacter lactamgenus* or *Xanthomonas lactamgena* [11, 28]. As are cephalosporin C from *Acremonium chrysogenum* and cephamycin C from *Streptomyces clavuligerus*, cephabacin is produced in these bacilli through the same initial biosynthetic pathway, including L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) synthesis, ring formation to isopenicillin N (IPN), and ring expansion into deacetoxycephalosporin C (DAOC). This has been identified and confirmed by cloning of a biosynthetic gene cluster for cephabacin from *L. lactamgenus* [14-16, 25]. However, the initial biosynthetic enzyme, lysine ε-aminotransferase, for the production of L-α-aminoadipic acid in actinomycetes [22], has not been reported in cephabacin producers till now.

Recent progress in antibiotic research, based on the analysis of antibiotic biosynthetic gene clusters, has revealed that the major enzymes involved in antibiotic biosynthesis are peptide synthetase (PTS) for nonribosomal peptide synthesis, and polyketide synthase (PKS) in the repeated condensation of acetyl and malonyl units [4, 13]. These enzymes are composed of multiple modules, each with an active domain for a specific structural unit.

Based on the chemical structure of cephabacins with an oligopeptidylacetyl side chain on a 3-hydroxyl group of a cephem ring (Fig. 1) [11, 28], it can be deduced that the biosynthetic gene cluster may contain nonribosomal PTS and/or PKS. Accordingly, the cloning and characterization of the PTS gene involved in cephabacin biosynthesis was attempted from the genomic library of *L. lactamgenus*. During the analysis of the screened gene cluster, two membrane transporter genes were identified between the PTS gene and the ACV synthetase gene. This report presents the sequences and some analytical data on these transporter genes.

Amplification of Nonribosomal Peptide Synthetase of *L. lactamgenus*

The *L. lactamgenus* IFO 14,288 used in this study was grown on a medium of 2% glucose, 1% casamino acid,

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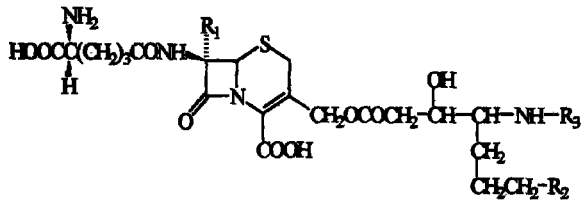

Cephabacin F R_1 = NH-CHO
R_2 = NH-C(=NH)-NH_2 or CH_2NH_2
R_3 = (L-Ala)$_{1\sim3}$, (L-Ser)$_{1\sim2}$, or (L-Ser)$_2$-L-Ala

Cephabacin H R_1 = H
R_2 = NH-C(=NH)-NH_2
R_3 = (L-Ala)$_{1\sim3}$, (L-Ser)$_{1\sim2}$, or (L-Ser)$_2$-L-Ala

Fig. 1. Chemical structures of cephabacins produced by *L. lactamgenus*.
At position R_2, cephabacin F_1~F_6 and cephabacin H_1~H_6 have -NH-C(=NH)-NH_2, yet cephabacin F_7~F_9 have -CH_2NH_2. Following to oligopeptides at position R_3, cephabacin F is classified as F_1~F_6 and cephabacin H as H_1~H_6.

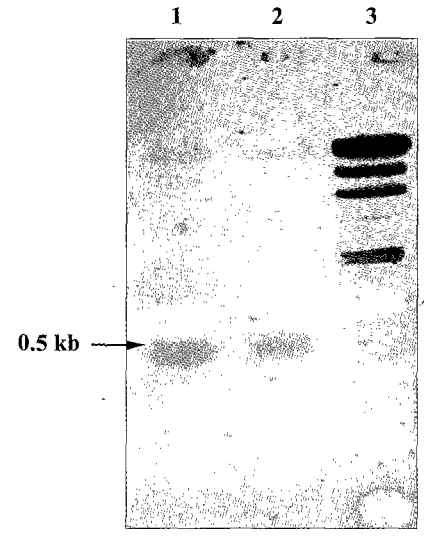

Fig. 2. PCR amplification of *L. lactamgenus* chromosomal DNA using the consensus core sequences 2 and 6 of nonribosomal peptide synthetase.
Core sequences 2 and 6 of nonribosomal peptide synthetase are described in the text in detail. Lanes 1 and 2: PCR product, lane 3: λDNA/*Hin*dIII (1.0% agarose gel electrophoresis).

0.1% sodium sulfate, and 0.01% nickel chloride (pH 7.0) at 30°C with rotary shaking at 150 rpm for 48 h [24]. The chromosomal DNA as a gene source was prepared following a previous report [25].

Generally, it is well known that there are 6 conserved sequences in bacterial nonribosomal PTSs; core sequence 1 (LKAGGAYVPID), 2 at the adenylation domain (YSGTTGxPKGV), 3 (GELCIGGxGxARGYL), 4 (YxTGD), 5 (VKIRGxRIELGEIE), and 6 at the thionylation domain (DNFYxLGGHSL) [27, 29].

In order to clone and characterize the gene for nonribosomal PTS from *L. lactamgenus* chromosomal DNA, the gene fragment was amplified by PCR using the conserved sequences of known PTSs: a forward primer (5'-TWYA-CHTCNGGMACBWCNGGBHDSCCMAARGSNRT-3') corresponding to the conserved sequence of the adenylation domain (core sequence 2) and a reverse primer (5'-AKRCTSTSNCCDCCBMDNBBRAASARRTYRBH-3') corresponding to that at the thionylation domain (core sequence 6). The PCR solution was composed of 50 ng of *L. lactamgenus* chromosomal DNA as the template, 100 pmole of each of two primers, 15 mM magnesium chloride, 5% dimethoxysulfoxide, and 5 units of *Taq* polymerase [21]. 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 with 30 cycles in a GeneAmp PCR System 2400 (Perkin-Elmer, CT, U.S.A.).

As a result, a 0.5-kb fragment of the PTS was obtained, as shown in Fig. 2. The amplified DNA fragment was then cloned into a pGEM-T vector (Promega Co., WI, U.S.A.), and transformed into *E. coli* JM109 (*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*)). Among the white colonies grown on the LB (Luria-Bertani) media supplemented with 50 μg/ml of ampicillin, 0.1 mM isopropyl-β-D-thiogalactoside (IPTG), and 0.4% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), 10 clones were selected and the nucleotide sequences of the fragments were partly determined by employing an ABI Automatic DNA Sequencer (Perkin-Elmer, CT, U.S.A.) using pUC/M13 universal primers in the DNA Sequencing Lab at the University of California, Davis, U.S.A. Through a BLAST search (http://www.ncbi.nlm.nih.gov/) of determined sequences, it was found that there were three *pcb*AB (ACV synthetase) gene fragments, previously reported by Kimura *et al.* [16]. It is already known that ACV synthetase in *L. lactamgenus* is composed of 3 nonribosomal PTS modules, as like other β-lactam producers [1, 3, 15-16, 18-20]. Therefore, by excluding the ACV synthetase gene fragments, the other fragments were then isolated and used as mixed probes in cloning the gene for the PTS of *L. lactamgenus*.

Cloning of Nonribosomal Peptide Synthetase of *L. lactamgenus*

The chromosomal DNA of *L. lactamgenus* was digested with *Sau*3A, and DNA fragments larger than 15 kb were inserted into a pHC79 cosmid (American Type Culture Collection, U.S.A.) at the *Bam*HI site. The recombinant cosmid was *in vitro* packaged and transfected into *E. coli* HB101 (*F⁻ supE44 lacY1 ara-14 galK2 xyl-5 mtl1 leuB6 proA2 hsdS20 mcrB recA13 rpsL20 thi-1 λ⁻*).

For *in situ* colony hybridization, the amplified PCR probes were labeled with digoxigenin-11-dUTP by a DIG DNA

Labelling Kit (Boehringer Mannheim GmbH, Germany). The labelled DNA fragments were employed as probes after being precipitated with the same volume of 4 M lithium chloride and washed with 70% ethanol. A Southern transfer from agar plates was performed according to the procedure of Sambrook *et al.* [26], followed by fixation onto a positively-charged nylon membrane (Boehringer Mannheim GmbH, Germany) by a UV-crosslinker (Model CL-1000, UVP, CA, U.S.A.). The membrane was then hybridized overnight with the digoxigenin-labeled PCR probes at 60°C for 24 h after being prehybridized with a blocking solution at 60°C for 6 h, and washed twice with 0.5× SSC (75 mM sodium chloride, 7.5 mM sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 60°C. Finally, the membrane was colorized using an anti-digoxigenin-alkaline phosphatase conjugate in a DIG DNA Detection Kit (Boehringer Mannheim GmbH, Germany).

Among 2,000 clones selected on the LB-ampicillin agar plate, 11 clones were then chosen which were strongly bound by the above-mixed PTS gene fragments. When these clones were re-hybridized with the ACV synthetase gene fragments, based on the fact that antibiotic biosynthetic genes are clustered on the chromosome, one clone was selected and called pPTS-5, which was then bound with mixed probes for PTS as well as probes for ACV synthetase.

The restriction map of the pPTS-5 cosmid is shown in Fig. 3. When this cosmid was digested and analyzed with *Apa*I, *Eco*RI, *Nru*I, *Sal*I, *Sph*I, and *Xho*I restriction enzymes, it was found that the insert size was approximately 23.5 kb. When comparing the restriction map with that of the cephabacin biosynthetic gene cluster reported by Kimura *et al.* [16], it was found that the 8.0-kb fragment from the *Eco*RI site at the cloning junction adjacent to the first *Apa*I site was nearly the same as the ACV synthetase gene of *L. lactamgenus*. This was also confirmed by sequencing both terminal regions of the 2.0-kb *Xho*I fragment. Accordingly, it was decided to determine the nucleotide sequences next to the first *Apa*I site.

In order to do this, a more accurate restriction map of the 4.0-kb insert from the first *Apa*I site at 8.0 kb position

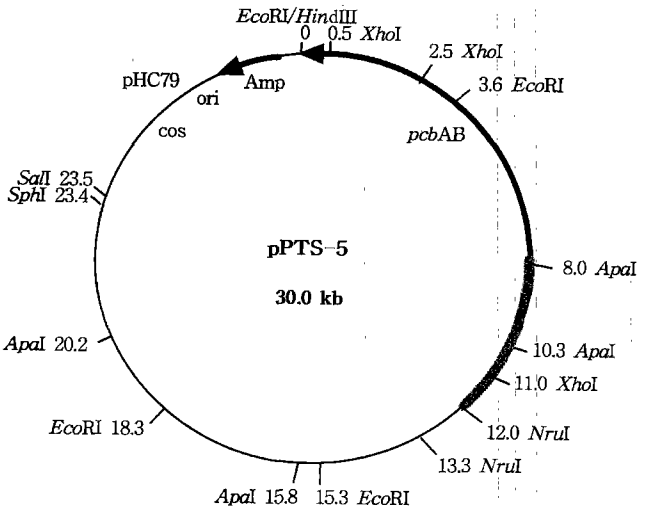

Fig. 3. Restriction map of the pPTS-5 cosmid clone.
The 23.5 kb fragment of *L. lactamgenus* chromosomal DNA was inserted in the pHC79 cosmid vector. From this insert, the 8.0-kb region from the cloning junction adjacent to the first *Apa*I site was found to be the *pcb*AB gene for ACV synthetase, as reported by Kimura *et al.* [16]. The gray part is the DNA region for which the sequences were determined in this work.

to the first *Nru*I site at the 12.0 kb position was made by digestion with several restriction enzymes, as shown in Fig. 4. Based on this map, several subclones were made in a pGEM-5Zf vector (Promega Corporation, WI, U.S.A.) for nucleotide sequencing.

Two Putative Transporter Genes in the Cephabacin Biosynthetic Gene Cluster

The DNA sequencing results of the subclones implied that there were more than two open reading frames (ORF) in front of the ACV synthetase gene. The two deduced complete ORFs had their own Shine-Dalgarno sequence, however no promoter regions were found, as shown in Fig. 5.

The deduced amino acid sequences from the two ORFs indicated that ORF-1 had 437 amino acid residues while ORF-2 had 410 residues. Through a BLAST search of amino acid sequences, it was found that ORF-1 was homologous with the ABC transporter ATP-binding protein of *E. coli*

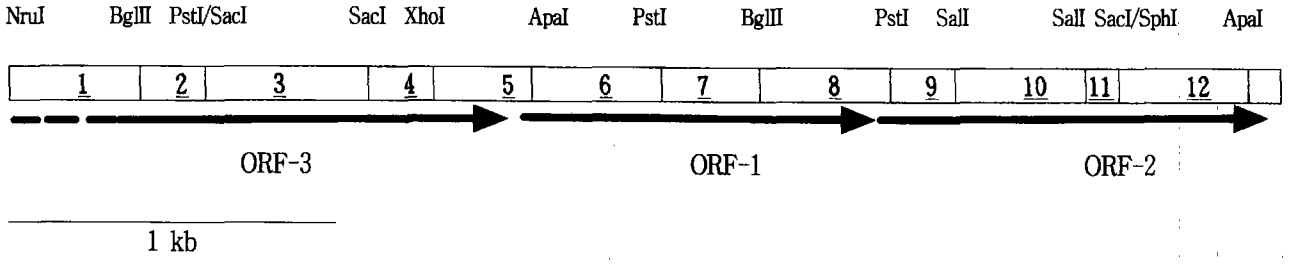

Fig. 4. Detail restriction map of the 4.0-kb fragment from the *Apa*I site at the 8.0 kb position to the *Nru*I site at the 12.0 kb position on the pPTS-5 clone.
Based on this restriction map, several subclones were made in the pGEM-5Zf plasmid for the nucleotide sequencing. The sequenced results show that there are two complete ORFs (ORF-1 and ORF-2) and one incomplete ORF (ORF-3).

Fig. 5. DNA nucleotide sequences and deduced amino acid sequences of ORF-1 and ORF-2.
The nucleotide sequence is numbered from the 5'-end of the sequenced region (GenBank accession number; LLMTRP AF315510). The numbering of the amino acids starts at the initiation codon of the ORFs. The initiation codon and termination codon of the putative ORFs are denoted in bold letters, and the potential Shine-Dalgarno sequences in italic letters. Some restriction sites are described on the nucleotide sequence. The nucleotide sequence of the downstream of the *Apa*I site at the 3'-end denoted in a parenthesis is the same one as the *pcb*AB gene for ACV synthetase, previously reported by Kimura *et al.* [16].

(YbjZ; 37% among 372 amino acids) [2] and the metal ion uptake protein of *Bacillus subtilis* (YvrN; 37% among 372 amino acids) [31]. The other ORF-2 also exhibited a good homology, in a BLAST search, with the metal ion uptake protein of *B. subtilis* (YvrN; 43% among 300 amino acids) [31], the integral membrane protein of *Treponema pallidum* (43% among 267 amino acids) [9], the ABC transporter ATP-binding protein of *E. coli* (YbjZ; 40% among 283 amino acids) [2], and the ATP-binding cassette transporter-like protein of *Streptococcus crista* (40% among

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ORF-1: 302 KVVPPESKISALLGLGFLLICLVNTIGLL-LAKFM--RRASEIGVRRALGASRGAIYRQFLVEAATIGLAGGL 371
ORF-2: 277 QVYKSDRGMATLMGAISLVLLAITAAGIFGLTSFWVGQRRKQIGVRRALGATRGDIMGYFLTENLLIGVGGVA 349
YvrN   281 ELHSFNQSFALLLGGIASISLLVGGIGVMNIMLVSVTERTREIGIKKALGAKRRVILFQFLTEAVVLTSIGGI 354
YbjZ   513 TVEKTTRTLQLFLTLVAVISLVVGGICVMNIMLVSVTERTREIGIRMAVGARASDVLQQFLIEAVLVCLVGGA 585
           +V  +  ++ L+G  L++  +  G+  L  F  +R  +IGVRRALGA+RG I  FL E + IG++G++

ORF-1: 372 LGLPLTGLGMLCTDLVFEPEIARLATLDTSLAALTLVVA-----TLATVIAALGPALRASRVSPVEAIRSV 437
ORF-2: 350 LGTAM-AIG-LNTWLMTHEELTRLSP--------TYVVAGAAALLVLGQIAVLYRIWRAAQVQPAWQLKTN 410
YvrN   355 LGVLA-GFG-IAKLLTVIEPMPFIVSI--------PAVVGALIFSMAVGIIFGLLPSIKASKLQPVDALRYE 416
YbjZ   586 LGITLSLLIAFTLQLFLPGWEIGFSP--------LALLLAFLCSTVTG-IFGWLEARNAARLDPVDALARE 648
           LG  + ++G L T L+  E+ RL+   +   T VVA          IA+L P  RA++V P   ++
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Fig. 6. Alignment of two putative transporters with corresponding regions of two selected homologous bacterial transporters.
ORF-1 and ORF-2 are the putative transporters identified in this work. YvrN is the metal ion uptake protein of *Bacillus subtilis* [31] and YbjZ is the ABC transporter ATP-binding protein of *E. coli* [2]. The sequence homology was analyzed by the CLUSTAL W Multiple Sequence Alignment Program [12]. The sequences appearing simultaneously in ORF-1 and ORF-2 are shown in the gray boxes and denoted in the lower column. The similar amino acid residues in the 4 transporters are described as '+'.

201 amino acids) [5]. These search results strongly suggest that these two putative proteins are membrane-bound transporters, possibly involved in cephabacin secretion after its biosynthesis. The homology between these two transporter proteins was 45% among 138 amino acid residues at the carboxy terminus (Fig. 6), when employing the CLUSTAL W multiple sequence alignment program [12] supplied by The Department of Medical Biochemistry, Goeteborg University, Sweden (http://www.medkem.gu.se/edu/msf.html).

A hydrophobicity analysis of the two ORFs, according to the Kyte and Doolittle method [17] (http://fasta.bioch.virginia.edu/fasta/cgi/pgrease.cgi), showed that there were at least 9 hydrophobic regions in the sequences. The mean hydrophobicity indices of ORF-1 and ORF-2 were estimated as 0.1 and 0.17. However, the mean index values for the hydrophobicity of 138 amino acids at the carboxy terminus (H7, H8, and H9 regions) were calculated as 0.85 for ORF-1 and 0.73 for ORF-2, which were both higher than 0.42 which is the minimum threshold for hydrophobicity [8]. Similarly, an analysis of the transmembrane domain using the DAS program [7] (http://www.biokemi.su.se/~server/DAS/) showed that ORF-1 had 5 transmembrane regions whereas ORF-2 had 4, as shown in Fig. 7. Among the hydrophobic domains, the three transmembrane regions at the carboxy terminus of each ORF comprised of more than 20 consecutive hydrophobic or uncharged amino acids, which were enough to span the membrane. Accordingly, it can be assumed that the cephabacin secretion system is composed of two different subunits, as in other transporting systems; one for substrate binding and the other for substrate translocation through the membrane, which are encoded by two transporter genes.

However, no canonical A and B sequences of the Walker motif for ATP binding [30] were observed in these two ORFs, which means that this transporting system does not require ATP for secretion.

Expression of the Two Transporter Genes in *E. coli*

In order to investigate the biological function of the two transporter genes, ORF-1 and ORF-2 were amplified by PCR using primers designed under the consideration of reading frame, and subcloned into a pET-28a expression vector (Novagen, Inc., WI, U.S.A.). When ORF-1 was expressed in *E. coli* BL21 [*F⁻ ompT hsdS*$_B$($r_B^- m_B^-$) *gal dcm*] (DE3) by 1 mM IPTG, the growth of the host cells immediately ceased. Furthermore, the simultaneous expression of ORF-1 and ORF-2 also inhibited the host cell growth, even though the expression of ORF-2 itself did not exhibit any effect on the host cell growth (Fig. 8). This implies that the gene product of ORF-1 exerts some cytotoxicity on *E. coli*, probably due to the disturbance of the membrane transport system. Accordingly, it can be deduced that ORF-1 may be involved in antibiotic translocation across the membrane, whereas ORF-2 may play a role in antibiotic binding.

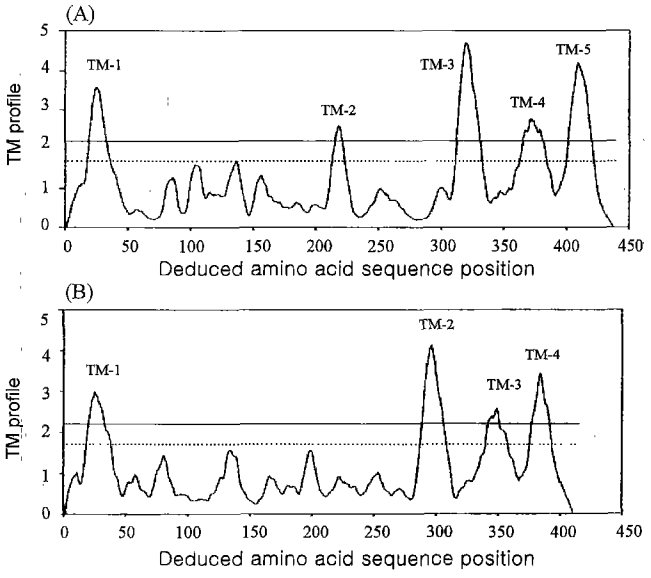

Fig. 7. Prediction of the transmembrane domain of two putative transporters by DAS program.

ORF-1 (A) and ORF-2 (B) are the putative transporters identified in this work. A prediction of the transmembrane domain was performed using the method reported by Cserzo *et al.* [7]. The lower broken line at the center is the loose cutoff and the upper straight line is the strict cutoff. Regions TM1-TM5 indicate the potential transmembrane α-helices in the two putative transporters.

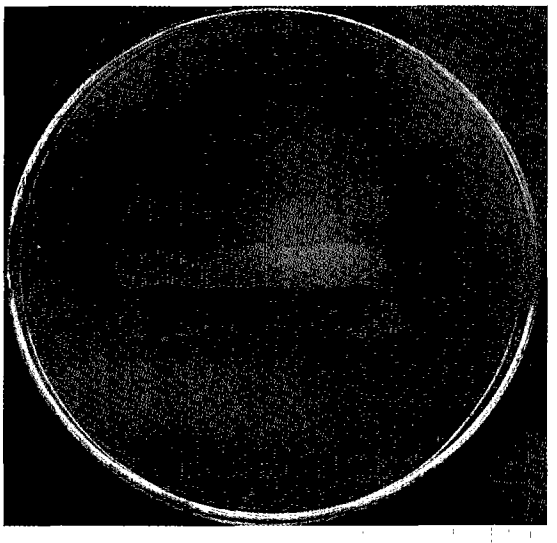

Fig. 8. Effect of transporter gene expression on cell growth of *E. coli*.

The genes of ORF-1 and ORF-2 were subcloned into the pET-28a vector and transformed into *E. coli* BL21(DE3). The gene expression was tested by streaking the solution of 1 mM IPTG on the middle of a culture plate. Thus the middle part of the agar plate shows the state of the gene expression, and the left and right sides give the results without the expression of the cloned genes.

Alignment of the Cephabacin Biosynthetic Gene Cluster

Even though the complete DNA sequences of the upstream region to the two putative transporter genes were not determined yet, the partially determined sequences of ORF-3 in Fig. 4 showed that there were an acyl carrier protein (ACP) domain (FEAGGDSLL) and thioesterase domain (LLGWSFGAIV) (in preparation), for which consensus sequences were previously reported by Robinson [23]. In addition, the clone obtained appeared to include PTS module(s), because the clone was selected by hybridization with the amplified PTS fragments. This strongly supports that mixed PKS/PTS modules for the biosynthesis of cephabacin may be clustered with cephem biosynthetic genes.

Recently, two mixed PKS/PTS modules were reported in the biosynthesis of pristinamycin [6] and yersiniabactin [10]. Based on its chemical structure with a 3-oligopeptidylacetyl group, the gene cluster for cephabacin biosynthesis may also have mixed PKS/PTS modules.

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