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Biochemical Characterization of an ABC Transporter Gene Involved in Cephabacin Biosynthesis in *Lysobacter lactamgenus*

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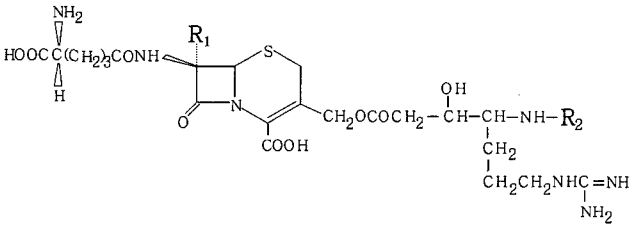
Abstract An ATP-binding-cassette (ABC) transporter gene in the cephabacin biosynthetic gene cluster of *Lysobacter lactamgenus* was characterized. The amplified orf10 (*cpbJ*) gene was subcloned into pET-28a(+) vector and expressed in *E. coli* BL21(DE3) strain by 0.5 mM IPTG at 30°C. The membrane fraction of recombinant *E. coli* cells was separated by ultracentrifugation, and solubilized using 2.5% octyl-β-D-glucoside. Using the solubilized membrane fraction, the artificial proteoliposomes were reconstituted and analyzed for the biological activity of CpbJ protein. Upon measuring ATPase activity, the proteoliposome made from recombinant *E. coli* membrane proteins showed slightly higher activity than that from host *E. coli* membrane proteins. In the measurement of membrane transport activity, the reconstituted proteoliposome of recombinant *E. coli* membrane proteins exhibited higher activity when both substrates of cephalosporin C and L-Ala-L-Ser were applied, compared to the case of cephalosporin C or L-Ala-L-Ser only. It implies that the CpbJ protein is an ABC transporter secreting cephabacin antibiotics synthesized in cytoplasm.

Key words: ABC transporter, cephabacin, cephem antibiotic, *Lysobacter lactamgenus*, proteoliposome, antibiotic biosynthesis

Cephabacins are a class of cephem antibiotics produced by Gram-negative bacillus, *Lysobacter lactamgenus* [8, 13] or *Xanthomonas lactamgena* [7, 14]. Different from cephalosporin C or cephamycin C, cephabacins have oligopeptides at the C-3 position of the cephem ring. In addition, cephabacins are classified into F and H groups depending on the presence (F) or absence (H) of formylamino residue at the C-7 position (Fig. 1).

A gene cluster responsible for the initial biosynthetic steps of cephabacins in *L. lactamgenus* was cloned by a Japanese research group [2–4]. They have already characterized the biochemical properties of L-α-aminoadipyl-L-cysteinyl-D-valine (ACV) synthetase, isopenicillin N (IPN) synthase, deacetoxycephalosporin C (DAOC) synthase, and other enzymes engaged in the early biosynthetic steps of cephabacin. However, neither the genes nor the enzymes responsible for the biosynthesis of oligopeptide moiety or formylamino residue have been identified or isolated.

Based on the chemical structure of cephabacins, it can be hypothesized that another nonribosomal peptide synthetase (NRPS) system in addition to ACV synthetase is required for the introduction of oligopeptide moiety at position C-3. By chromosome walking, the upstream region of the known cephabacin biosynthetic gene cluster has been cloned using NRPS probes [10, 12]. In the 24-kb upstream region, the genes for 4 NRPS domains, 1 polyketide synthase (PKS) domain, and 2 ATP-binding cassette (ABC) transporters were deduced by comparison of sequence homology (Fig. 2).



Cephabacin	R1	R2
F_1	-NHC(=O)H	- L-Ala
F^2	-NHC(=O)H	- L-Ala- L-Ala
F_3	-NHC(=O)H	-L-Ala-L-Ala-L-Ala
H_1	-H	- L-Ala
H_2	-H	- L-Ala- L-Ala
H_3	-H	-L-Ala-L-Ala-L-Ala

Fig. 1. Chemical structure of cephabacins.
Among them, the cephabacin F group is the major antibiotics produced by *Lysobacter lactamgenus* IFO 14,288.

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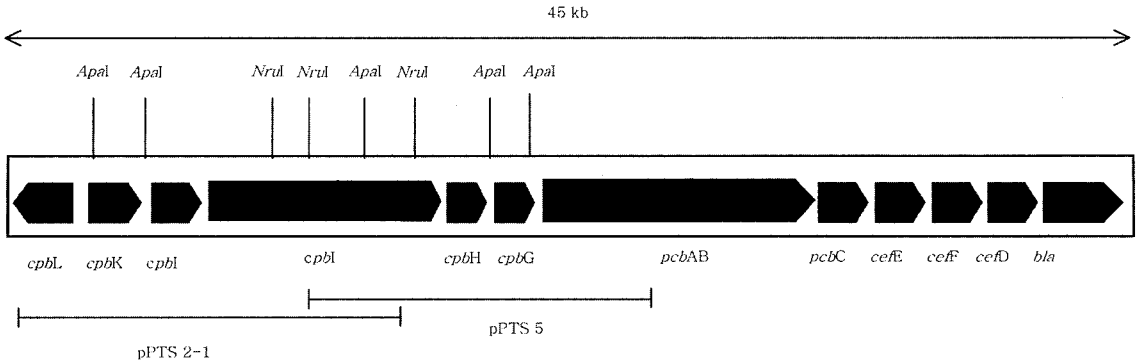

Gene	Proposed functions	Reference
*pcb*AB	ACV synthetase	[3]
*pcb*C	ACV cyclase	[4, 10]
*cef*E	Penicillin N expandase	[2]
*cef*F	DAOC hydroxylase	[2]
*cef*D	IPN epimerase	[3]
bla	β-lactamase	[3]
*cpb*G	ABC transporter homolog	[5]
*cpb*H	ABC transporter homolog	[5]
*cpb*I	Hybrid NRPS3/NRPS2/NRPS1/PKS	[12]
cpbJ	**ABC transporter**	[12]
*cpb*K	NRPS4	[12]
*cpb*L	Methyltransferase homolog	[12]

Fig. 2. The gene organization for the biosynthesis of cephabacins.
The key genes responsible for the early steps of cephem biosynthesis from orf1 to orf6 was reported by Kimura *et al.* [2-4], and the upstream region for the modification of the cephem ring from orf7 (*cpb*H) to orf12 (*cpb*M) was described by Sohn *et al.* [12]. Among them, the biological function of the gene product of orf10 (*cpb*J) was identified in this work.

Here, we tried to characterize the biochemical properties of ORF10 (CpbJ) protein, a presumable ABC transporter among the biosynthetic genes located at the upstream region [12]. To do that, orf10 (*cpbJ*) gene was amplified from pPTS2-1 cosmid using forward primer (5'-GGG ACT GTC GAC ATA TGG TGT TGG-3') and reverse primer (5'-GAT CAG CCC GGA TCC GAA CTT CAG-3'). The amplified gene product of 1.6 kb was firstly cloned into pGEM-T easy vector (Promega Co., WI, U.S.A.), and transformed into *E. coli* XL1-Blue [*F' proAB*$^+$ *lacI*^{q} *lacZ ΔM15 Tn10 tet*^{r}]. The orf10 (*cpbJ*) gene cut by *Nde*I and *Bam*HI enzymes was then re-cloned into pET-28a(+) vector (Novagen Inc., WI, U.S.A.), and transformed into *E. coli* BL21(DE3) [*F' ompT hsdSb* ($r_B^-m_B^-$) *gal dcm* (*DE3*)] cells. The recombinant *E. coli* cells were cultivated on Luria-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 1% sodium chloride, pH 7.5) at 30°C, and the gene induction was attempted by 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). When the gene induction was performed at 30°C for 8 h, a lot of the recombinant CpbJ protein was produced as soluble form in the cytoplasm fraction as well as a membrane protein in the membrane fraction, as like other ABC transporters (Fig. 3).

The recombinant *E. coli* cells were recovered from culture broth by centrifugation at 5,000 ×*g* for 10 min, suspended in 1/50 volume of buffer A (50 mM Tris-HCl, 5 mM magnesium chloride, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 8.0), and subjected 2 times to French press (SLM Instruments, Rochester, NY, U.S.A.) under 12,000 psi at 4°C. The supernatant separated by centrifugation at 10,000 ×g for 20 min was further separated by ultracentrifugation at 100,000 ×*g* for 1 h at 4°C to isolate the membrane fraction. The precipitate from ultracentrifugation was suspended in buffer B (20 mM Tris-HCl, 15 mM sodium chloride, 1 mM dithiothreitol, 1 mM EDTA, pH 8.0), and membrane proteins were solubilized by stirring with solubilization buffer (20 mM Tris-HCl, 5 mM imidazole, 150 mM sodium chloride, 20% (v/v) glycerol, 2.5% octyl-β-D-glucoside, pH 8.0) for 2 h at 4°C, and recovered by centrifugation at 10,000 ×*g* for 10 min [6, 9, 11].

The clarified membrane protein was gently mixed with NTA-chelating Sepharose resin (Peptron Inc., Korea) equilibrated with binding buffer (5 mM imidazole, 150 mM sodium chloride, 20% glycerol, 0.05% octyl-β-D-glucoside, 20 mM Tris-HCl, pH 8.0) for 2-3 h at 4°C. The resin was then

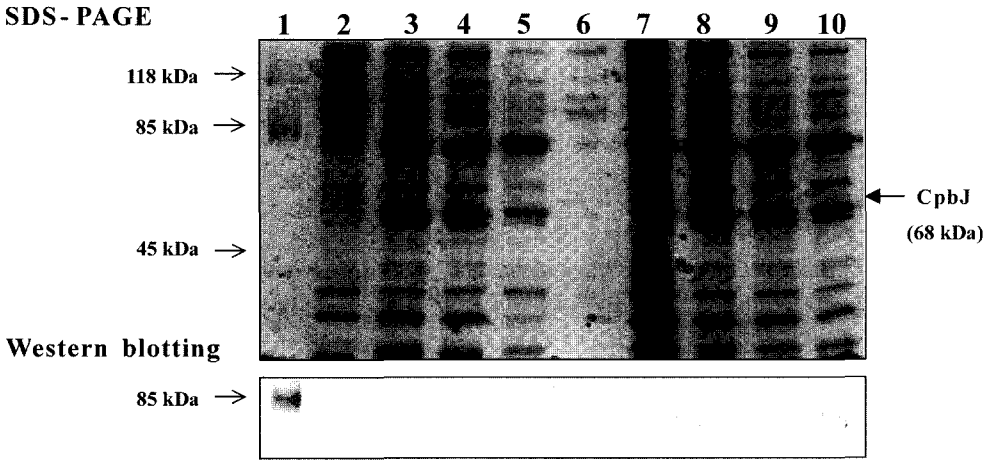

Fig. 3. Identification of CpbJ protein from the recombinant *E. coli* BL21(DE3).
The *cpbJ* gene in pET-28a(+) plasmid transformed into *E. coli* BL21(DE3) cells was induced by 0.5 mM IPTG at 30°C for 8 h, and the cells were ruptured by a French press (12,000 Lb/in^2) at 4°C. From the precipitates obtained by centrifugation at 10,000 ×*g*, the cell membrane fractions were isolated by ultracentrifugation at 100,000 ×*g* for 1 h. The same amount of cell membrane protein (20 μg) was analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. The CpbJ protein was also identified by Western blotting with Penta-His antibody on electro-transferred nitrocellulose membrane. Lane 1, high molecular weight marker; lane 2, total cell protein of recombinant *E. coli* cells, not induced; lane 3, total cell protein of the recombinant *E. coli* cells, induced by IPTG; lane 4, soluble fraction after rupture by a French press; lane 5, insoluble fraction after rupture by a French press; lane 6, cytosol fraction after ultracentrifugation; lane 7, membrane fraction in pellet after ultracentrifugation; lanes 8- 10, membrane fraction solubilized by dilution with octyl-β-D-glucoside (1/4, 1/10, and 1/20 fold).

packed in chromatography column (1×3.5 cm), and washed with 10 volumes of binding buffer and 6 volumes of washing buffer (20 mM imidazole, 150 mM sodium chloride, 20% glycerol, 0.05% octyl-β-D-glucoside, 20 mM Tris-HCl, pH 8.0) [1]. Finally, His-tagged CpbJ protein was eluted with elution buffer (500 mM imidazole, 150 mM sodium chloride, 10% glycerol, 0.05% octyl-β-D-glucoside, 20 mM Tris-HCl, pH 7.9), and confirmed by 10% SDS-PAGE. The purified protein was also identified by Western blotting, where Penta-His antibody (Qiagen GmbH, Hilden, Germany) was employed as primary antibody and alkaline phosphatase-conjugated anti-mouse IgG (Sigma-Aldrich Co., St. Louis MO, U.S.A.) as secondary antibody. However, enough amount of protein as a pure form was not obtained (data not shown).

The comparative analysis of biological activity was tried using membrane proteins of recombinant *E. coli* cells and host cells. For this, the artificial proteoliposomes were reconstituted using the solubilized membrane proteins from recombinant cells or host cells according to the procedure of the octyl-β-D-glucoside dilution method [6, 9, 11]. The proteoliposome was made from 500 mg of membrane protein and 5 mg of *E. coli* phospholipid (Avanti Polar-lipids, Alabaster, AL, U.S.A.) in the presence of 2.5% octyl-β-D-glucoside, and collected by ultracentrifugation at 200,000 ×*g* at 4°C. The generated liposomes (about 2 μm) were observed by transmission electron microscopy under the magnification of 18,000× (Fig. 4).

The ATPase activity of ABC transporter in proteoliposomes was measured according to the procedure of Van Veldhoven and Mannaerts [15]. Upon measuring ATPase activity of the reconstituted liposomes, the proteoliposome made from recombinant *E. coli* membrane proteins showed slightly higher activity than that from host *E. coli* membrane proteins (Fig. 4). Relative high ATPase activity in host cells may be attributed to the pre-existence of a lot of other ABC transporters.

On the other hand, the membrane transport activity was determined by exposing the proteoliposome on substrates. Because of difficulty to get cephabacin compounds, cephalosporin C having the similar structure of cephem ring, L-Ala-L-Ser dipeptide as an oligopeptide side chain, or both was employed as substrate. The proteoliposomes

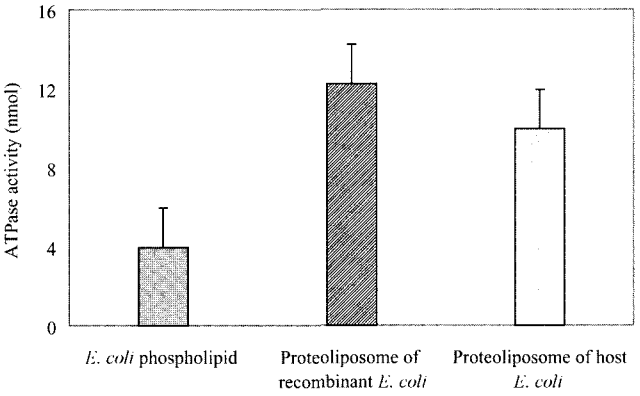

Fig. 4. ATPase activity of CpbJ protein expressed in *E. coli* BL21(DE3) cells.
ATPase activity was measured using artificial proteoliposomes reconstituted with *E. coli* phospholipid.

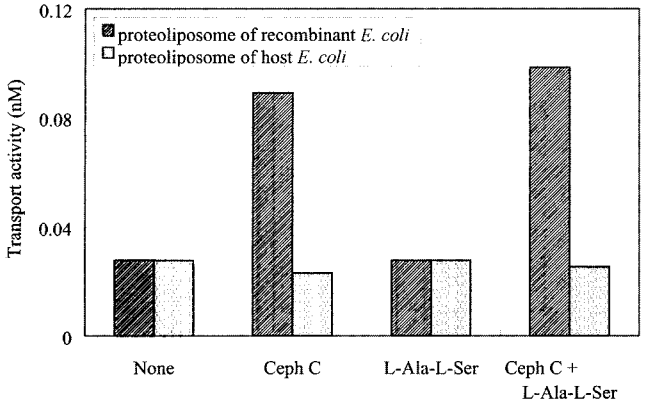

Fig. 5. Transport activity of CpbJ protein expressed in *E. coli* BL21(DE3) cells.

The artificial proteoliposomes reconstituted with *E. coli* phospholipid were exposed to no substrate (none), cephalosporin C (Ceph C), L-Ala-L-Ser dipeptide (L-Ala-L-Ser), or cephalosporin C and L-Ala-L-Ser dipeptide (Ceph C+L-Ala-L-Ser), in the presence of ATP and magnesium chloride. The amount of substrates transported into proteoliposomes was measured by ninhydrin assay.

(2 mg) were incubated overnight in 1 ml of reaction buffer (20 mM Tris-HCl, 10 mM magnesium chloride, 4 mM ATP, 1 mM substrate, pH 7.5) at 37°C [6, 11]. The membrane transport of substrate was stopped by diluting with 10 ml of transport buffer (20 mM Tris-HCl, 10 mM magnesium chloride, pH 7.4), and the proteoliposomes were recovered by ultracentrifugation at 200,000 ×*g* for 1 h, followed by washing 3 times with the same buffer. The finally obtained proteoliposomes were suspended in 1 ml of transport buffer, and sonicated to disrupt liposomes. After ultracentrifugatin at 200,000 ×*g* for 1 h, the substrates released from liposome were assayed by ninhydrin reaction (0.35 g ninhydrin in 100 ml ethanol) at 80-100°C for 20 min. The absorbance was measured at 410 nm.

In measurement of membrane transport activity, the reconstituted proteoliposome of recombinant *E. coli* membrane proteins exhibited a significantly high activity on cephalosporin C, a part of the cephem nucleus of cephabacin, but not on L-Ala-L-Ser, a mimic of the oligopeptide side chain of cephabacin (Fig. 5). The tranport activity was further increased when this liposome was treated with cephalosporin C and L-Ala-L-Ser simultaneously. It implied that CpbJ protein may be specific to both the cephem ring and oligopeptide side chain.

In conclusion, it was confirmed that the CpbJ protein is an ABC transporter having a function that transports cephabacin by the energy of hydrolyzing ATP in the process of its biosynthesis.

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