Characterization of the *Pseudomonas aeruginosa* Lol System as a Lipoprotein Sorting Mechanism*1

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*Escherichia coli* lipoproteins are localized to either the inner or the outer membrane depending on the residue that is present next to the N-terminal acylated Cys. Asp at position 2 causes the retention of lipoproteins in the inner membrane. In contrast, the accompanying study (9) revealed that the residues at positions 3 and 4 determine the membrane specificity of lipoproteins in *Pseudomonas aeruginosa*. Since the five Lol proteins involved in the sorting of *E. coli* lipoproteins are conserved in *P. aeruginosa*, we examined whether or not the Lol proteins of *P. aeruginosa* are also involved in lipoprotein sorting but utilize different signals. The genes encoding LolCDE, LolA, and LolB homologues were cloned and expressed. The LolCDE homologue thus purified was reconstituted into proteoliposomes with lipoproteins. When incubated in the presence of ATP and a LolA homologue, the reconstituted LolCDE homologue released lipoproteins, leading to the formation of a LolA-lipoprotein complex. Lipoproteins were then incorporated into the outer membrane depending on a LolB homologue. As revealed in vivo, lipoproteins with Lys and Ser at positions 3 and 4, respectively, remained in proteoliposomes. On the other hand, *E. coli* LolCDE released lipoproteins with this signal and transferred them to LolA of not only *E. coli* but also *P. aeruginosa*. These results indicate that Lol proteins are responsible for the sorting of lipoproteins to the outer membrane of *P. aeruginosa*, as in the case of *E. coli*, but respond differently to inner membrane retention signals.

Bacteria lipoproteins are anchored to either the inner or the outer membrane through acyl chains attached to the N-terminal Cys depending on the sorting signal (1). In *Escherichia coli*, the Asp residue at position 2 causes the retention of lipoproteins in the inner membrane, whereas other residues cause their outer membrane localization (2–4). Sorting of *E. coli* lipoproteins to the outer membrane is catalyzed by the Lol system (5) composed of five proteins, LolA through LolE. An ATP-binding cassette transporter LolCDE complex recognizes a newly synthesized outer membrane-directed lipoprotein on the perilamellar leaflet of the inner membrane and mediates the detachment of lipoproteins from the inner membrane in the presence of a periplasmic carrier protein, LolA. LolA forms a soluble complex with one molecule of lipoprotein and traverses the periplasmic space to reach a lipoprotein-specific outer membrane receptor, LolB. LolB, itself an outer membrane lipoprotein, receives a lipoprotein from LolA and then incorporates it into the outer membrane.

The role of Asp at position 2 in the lipoprotein sorting has been extensively studied, and it has been revealed that the Asp residue functions as a LolCDE avoidance signal because LolCDE does not recognize lipoproteins with Asp at position 2 (6). Subsequently, it was found that both the negative charge of the Asp residue and the positive charge of phosphatidylethanolamine are critical for the LolCDE avoidance (7). It was also found that LolCDE releases lipoproteins without recognizing the residue at position 2.

Lipoprotein sorting signals have been comprehensively characterized in *E. coli* (2–4, 8). It is now clear that *E. coli* lipoproteins are sorted to the outer membrane by default. Only Asp at position 2 actively functions as an intrinsic inner membrane retention signal (4). However, it was shown in the accompanying study (9) that lipoproteins of *Pseudomonas aeruginosa* are sorted according to the residues at positions 3 and 4. Here, we show that the five Lol proteins are responsible for the sorting of lipoproteins to the outer membrane of *P. aeruginosa*, as in the case of *E. coli* lipoproteins. Moreover, it is also shown that differences in lipoprotein sorting signals between *E. coli* and *P. aeruginosa* reflect differences in the properties of LolCDE.

**EXPERIMENTAL PROCEDURES**

*Materials—* n-Dodecyl-β-D-maltopyranoside (DDM)2 and sucrose monocaprate were purchased from Dojindo Laboratories (Kumamoto, Japan). *E. coli* phospholipids were obtained from Avanti Polar Lipids and washed with acetone as reported (10).

*Bacterial Strains, Plasmids, Primers, and Media—* The bacterial strains, plasmids, and primers used in this study are listed in supplemental Tables 1–3. *P. aeruginosa* strain PAO1 was a generous gift from Dr. Taiji Nakae (Tokai University). Bacteria were grown on LB medium (11). When required, carbenicillin, ampicillin, and chloramphenicol were added at concentrations of 100, 50, and 35 µg/ml, respectively.

**Cloning of lol Genes from P. aeruginosa—** The gene for His-tagged LolA was amplified from *P. aeruginosa* TPN072 (12) by

*□*

1 The abbreviations used are: DDM, n-dodecyl-β-D-maltopyranoside; CBB, Coomassie Brilliant Blue; IPTG, isopropyl-1-thio-β-D-galactopyranoside.

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Sorting Mechanism of Pseudomonas aeruginosa Lipoproteins

PC extracts were dialyzed against 25 mM Tris–HCl (pH 7.5). EcLolA was constructed using primers loLB-Pae-1 and loLB-Pae-2 and cloned into the EcoRI–HindIII sites of pUCP20. The loLB gene was amplified from TNP072 by PCR using primers loLB-Pae-1 and loLB-Pae-2 and cloned into the EcoRI–HindIII sites of pMAN885EH to construct pPLOLB. pPLOLB2 was constructed by subcloning the loLB gene from pPLOLB into the EcoRI–HindIII sites of pTTQ18 (14). The DNA fragment encoding LoLC, LoLD, and LoLE was amplified from TNP072 by PCR using primers loLE-Pae-1 and loLE-Pae-2 and then cloned into the EcoRI–HindIII sites of pTTQ18. A 1.3-kb fragment corresponding to the loLE gene was removed from pPLOLCDE. An oligonucleotide encoding hexahistidine was inserted into the EcoRI–HindIII sites of pTTQ18. A 1.3-kb fragment corresponding to the loLE gene was removed from pPLOLCDE.D. Since expression of LoLE from pPLOLCDE was inefficient, a plasmid expressing LoLE was constructed. A DNA fragment encoding LoLE was amplified from TNP072 by PCR using primers loLE-Pae-1 and loLE-Pae-2 and then cloned into the EcoRI–HindIII sites of pTTQ18. A 1.3-kb fragment corresponding to the loLE gene was removed from pPLOLCDE by digestion with StuI and HindIII, followed by self-ligation, to construct pPLOLCDE.

Construction of Plasmids Encoding Pal Derivatives—Plasmid pTAN21KS was constructed by site-directed mutagenesis of pTAN21 (15) with primers Pal(SLI)-1 and Pal(SLI)-2. pTAN21LI was constructed in the same way with primers Pal(SLI)-1 and Pal(SLI)-2.

Purification of LolA—P. aeruginosa PAO1 was transformed with pUCP–PLOLA–His encoding His-tagged PaLolA and then grown on LB at 37 °C. When the OD at 660 nm reached 0.2, expression of PaLolA in the periplasmic fraction was adsorbed to TALON resin (Clontech) and then dialyzed against 2,000 ml of the same buffer at 4 °C three times (16). The cell suspension was centrifuged at 10,000 g at 37 and 0 °C three times (16). The cell suspension was centrifuged at 10,000 g at 37 °C for 60 min at 4 °C. Lipoproteins in the supernatant were separated from the precipitate by centrifugation at 100,000 g for 30 min. The supernatant was dialyzed against 10 mM Tris–HCl (pH 7.5) containing 2% sucrose monolactate at 4 °C overnight. Reconstituted lipoproteins were collected by centrifugation at 100,000 × g for 2 h at 4 °C and then subjected to cold-shock treatment by repeated incubation of the cell suspension alternately at 37 and 0 °C three times (16). The cell suspension was centrifuged at 10,000 × g for 10 min at 4 °C, and the supernatant fraction was centrifuged at 100,000 × g for 60 min at 4 °C to obtain a soluble periplasmic fraction. After dialysis against 50 mM sodium phosphate (pH 7.2), His-tagged PaLolA in the periplasmic fraction was adsorbed to TALON resin (Clontech) and then eluted with the same buffer supplemented with 300 mM NaCl and 250 mM imidazole. The eluate containing PaLolA was dialyzed against 25 mM Tris–HCl (pH 7.5). EcLolA was purified from E. coli TT015 (17) transformed with pAM201 (18). A spheroplast supernatant was purified as described previously (24). His-tagged MexA was expressed in JM83 carrying pUCP–MEXA–His (9).

Release of Lipoproteins from Proteoliposomes—EcLolCDE (1 µg) or PaLolCDE (5 µg) was incubated for 1 min on ice with 0.8 mg of E. coli phospholipids and 2 µg of lipoproteins in 100 µl of 50 mM Tris–HCl (pH 7.5) containing 5 mM MgSO₄ and 12% sucrose monolactate. The mixture was diluted with 900 µl of 50 mM Tris–HCl (pH 7.5) containing 5 mM MgSO₄, 100 mM NaCl and then dialyzed against 2,000 ml of the same buffer at 4 °C overnight. Reconstituted lipoproteins were collected by centrifugation at 100,000 × g for 2 h at 4 °C and then subjected to the lipoprotein release assay at 30 °C for 20 min in the presence of 4 µg of LoLA and 2 mM ATP as reported previously (25). Where specified, sodium orthovanadate was added to the reaction mixture at 5 mM. The reaction mixtures were fractionated into proteoliposomes and supernatants by centrifugation at 100,000 × g for 2 h at 4 °C. Lipoproteins in the precipitates and supernatants were analyzed by SDS-PAGE and immunoblotting. ¾ of the precipitated material, and ½ of the supernatant material was applied to the gel.

LolB-Dependent Incorporation of Lipoproteins into Membranes—LolB-depleted outer membranes were prepared from E. coli KT60 (ΔlolB) harboring pYKT123, which encodes a soluble periplasmic variant of LolB. PaLolB-containing outer membranes were prepared from KT60 harboring pYKT123 and pPLOLB2 encoding PaLolB. The outer membrane fractions containing PaLolB were diluted 18-fold with the LolB-depleted outer membrane fraction to give the same amount of PaLolB as that of EcLolB in the outer membrane fraction prepared from E. coli JC7752. Pal-LolA complexes were prepared by means of

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release assays performed with proteoliposomes and mixed with specified outer membrane fractions followed by incubation for 30 min at 30 °C. The reaction mixtures were centrifuged at 100,000 × g for 60 min at 4 °C. The precipitate and supernatant fractions were analyzed by SDS-PAGE and immunoblotting with anti-His antiserum.

**ATPase Activity Assay**—ATP hydrolysis by proteoliposomes reconstituted with EcLolCDE or PaLolCDE was determined as described previously (6). The amounts of inorganic phosphate were determined according to the reported method (26).

**Other Techniques**—SDS-PAGE was carried out as described (27). Western blotting was performed as described (19). Anti-His tag antiserum was raised in rabbits against purified hexahistidine-tagged RpmJ protein (28). Anti-Pal (29) antiserum was raised in rabbits against the purified protein.

**RESULTS**

**Cloning and Purification of Lol Proteins from P. aeruginosa**—To examine the sorting mechanism for *P. aeruginosa* lipoproteins *in vitro*, we cloned the genes for the Lol proteins of this bacterium. The PA2614 locus encodes a *P. aeruginosa* LolA homologue (PaLolA) comprising 208 amino acids, which contains the N-terminal signal peptide and the mature region. PaLolA exhibits 34% sequence identity with *E. coli* LolA. The gene encoding PaLolA was engineered to have a His tag at the C terminus and then cloned into *P. aeruginosa* PAO1 cells. PaLolA was then purified from the periplasm by metal affinity chromatography for His-tagged proteins (Fig. 1A). The N-terminal sequence of the purified protein was DDSAVQRLT, indicating that the signal peptide was cleaved after the residue at position 21 of the PaLolA precursor. PaLolA exhibits 34% sequence identity with *E. coli* LolA, the PaLolCDE complex was overproduced in the presence of 0.2% arabinose and 20 μM IPTG (Fig. 1B). Since both PaLolC and PaLolE were required for the reconstituted activity (see below), the PaLolCDE complex was expressed in *E. coli* JM83 in the presence of various concentrations of IPTG and 0.2% arabinose. Total membrane fractions were solubilized and His-tagged LolD was adsorbed to a metal affinity resin followed by elution with imidazole as described under “Experimental Procedures.” The eluates were analyzed by SDS-PAGE followed by staining with CBB.

**Reconstitution of the Lipoprotein-releasing Apparatus of P. aeruginosa**—To examine the lipoprotein-releasing activity, PaLolCDE or EcLolCDE was reconstituted into proteoliposomes together with *E. coli* outer membrane lipoprotein Pal.
followed by incubation with either PaLolA or EcLolA. The efficiency of lipoprotein release from proteoliposomes is generally low because the orientation of the reconstructed lipoproteins is random, which leaves a major fraction of lipoproteins incompetent as to release (6, 25). Nevertheless, reconstitution of LolCDE revealed important aspects of the lipoprotein release reaction (6, 7, 21). As shown in Fig. 3A, PaLolCDE released Pal from proteoliposomes in the presence of PaLolA in orthovanadate-sensitive and ATP-dependent manners, indicating that PaLolCDE and PaLolA are functional homologues of the respective E. coli Lol proteins. The amount of PaLolCDE required to detect the Pal release was 5-fold higher than that of EcLolCDE. EcLolCDE released Pal in the presence of not only EcLolA but also PaLolA, whereas PaLolCDE only released Pal in the presence of PaLolA. It is not clear at present whether this difference is caused by more specific subunit interaction of PaLol proteins or simply by lower activity of PaLolCDE.

It has been shown in E. coli that a water-soluble complex formed between LolA and a lipoprotein released by LolCDE represents the physiological intermediate of the lipoprotein release reaction (5). LolA efficiently transports hydrophobic lipoproteins through the hydrophilic periplasm to LolB on the outer membrane, and then LolB incorporates lipoproteins into the outer membrane. We next examined the outer membrane incorporation of Pal released by PaLolCDE in the presence of PaLolA. P. aeruginosa chromosomal locus PA4668 encodes a putative outer membrane lipoprotein exhibiting 27% amino acid identity to E. coli LolB (EcLolB). This LolB homologue of P. aeruginosa (PaLolB) was overexpressed in the E. coli Δloll strain, and an outer membrane fraction was prepared. When the supernatant obtained after the release reaction performed with PaLolCDE and PaLolA was mixed with the outer membrane fraction containing PaLolB, Pal was recovered in the membrane fraction, whereas PaLolA remained in the supernatant (Fig. 3B), indicating that Pal was transferred from the PaLolCDE complex to the outer membrane containing PaLolB. In contrast, Pal remained in the supernatant together with PaLolA when the outer membrane containing EcLolB was used. In contrast to the heterologous release reaction with EcLolCDE and PaLolA (Fig. 3A), EcLolA did not transfer Pal to PaLolB, suggesting that the specificity of the interaction between LolA and LolB is strict. Taken together, these results establish that the PaLol system mediates the transfer of lipoproteins from the inner to the outer membrane. Neither the PaLolC/D complex nor the PaLolD/E complex purified in the absence and presence of IPTG, respectively (Fig. 4A), supported PaLolA-dependent release of Pal from proteoliposomes (Fig. 4B). Thus, both PaLolC and PaLolE are required for the lipoprotein-releasing activity.

Sorting of Lipoproteins by the PaLolCDE Complex—To determine in vitro whether or not the residues at positions 3 and 4 function as sorting signals for P. aeruginosa lipoproteins, PaLolCDE was reconstituted into proteoliposomes together with Pal derivatives having various combinations of residues at positions 2, 3, and 4 (Fig. 5A) followed by release assays with PaLolA. When EcLolCDE and EcLolA were used as controls, not only Pal(SLI) but also Pal(SKS) was released (Fig. 5B), confirming that Ser² does not retain lipoproteins in the inner membrane of E. coli. In marked contrast, when PaLolCDE and PaLolA were used, Pal(SLI) was released, whereas Pal(SKS) was not (Fig. 5B). Furthermore, we also examined the release of MexA and found that it was released by the EcLol system but not the PaLol system. These results agree well with the in vivo localization of MexA in E. coli and P. aeruginosa (10). It is therefore clear that the residues at positions 3 and 4 function as lipoprotein sorting signals in P. aeruginosa but not in E. coli. It is concluded that the PaLol system functions to localize lipoproteins to the outer membrane as the EcLol system does but that their sorting mechanisms are not identical.
Although the residues at positions 3 and 4 determine the membrane specificity of lipoproteins in \textit{P. aeruginosa} both \textit{in vivo} and \textit{in vitro}, Asp at position 2 also functions as an inner membrane retention signal \textit{in vivo} (9). Consistent with the \textit{in vivo} results, Pal(DSN) having Asp at position 2 remained in proteoliposomes reconstituted with not only EcLolCDE but also PaLolCDE (Fig. 5B), indicating that Asp at position 2 is the common inner membrane retention signal. The release of Pal(SLI) by PaLolCDE appeared to be more efficient than that of Pal. However, Pal(DLI) still remained in proteoliposomes reconstituted with PaLolCDE (data not shown), suggesting that Leu$^3$-Ile$^4$ does not abolish the inner membrane retention function of Asp at position 2.

Taking advantage of the heterologous release reaction involving EcLolCDE and PaLolA, we examined whether or not PaLolA distinguishes lipoprotein sorting signals. For this, proteoliposomes were reconstituted with PaLolCDE and PaLolA, followed by the release assay in the presence of PaLolCDE. As shown in Fig. 5C, only Pal(DSN) remained in proteoliposomes, the other Pal derivatives including Pal(SKS) being released from proteoliposomes in a PaLolA-dependent manner, indicating that the lipoprotein sorting signals of the 3rd and 4th residues of \textit{P. aeruginosa} lipoproteins only function against PaLolCDE, i.e. not PaLolA.

**DISCUSSION**

The lipoprotein sorting signals examined \textit{in vitro} using purified Lol proteins revealed that both Asp$^2$ and Lys$^3$-Ser$^4$ function as inner membrane retention signals for the PaLol system. In contrast, Lys$^3$-Ser$^4$ did not function as an inner membrane retention signal for the EcLol system. These lipoprotein sorting signals were found to only function against LolCDE, i.e. not LolA (Fig. 5C). Because \textit{E. coli} phospholipids were used to reconstitute both PaLolCDE and EcLolCDE, differences in the properties of membrane phospholipids between the two bacteria, if any, cannot account for the differences in the sorting signals. Instead, the different properties of the two LolCDE complexes most likely caused the differences in the sorting signals.

It has been found in \textit{E. coli} that phosphatidylethanolamine is critically important for the LolCDE avoidance function of Asp$^2$ (7). Lipoproteins having Asp$^2$ were therefore released from proteoliposomes reconstituted with EcLolCDE and cardiolipin alone. However, our preliminary experiments revealed that PaLolCDE did not release lipoproteins with Asp$^2$ or Lys$^3$-Ser$^4$ even when proteoliposomes were reconstituted with cardio-
lipin alone. Therefore, it remains to be determined how Asp\(^2\) functions as an inner membrane retention signal for PaLolCDE. The release of one outer membrane-specific lipoprotein by EcLolCDE was competitively inhibited by an excess amount of another outer membrane-specific, but not inner membrane-specific, lipoprotein. The ATPase activity of EcLolCDE was competitively inhibited by an excess amount of lipoproteins, we do not know whether or not inner membrane signals Asp\(^2\) and Lys\(^3\)-Ser\(^4\) function as PaLolCDE avoidance signals.

The membrane-impermeable sulphydryl reagent was inaccessible to Cys introduced to position 2 of lipoproteins, whereas it was accessible to Cys at positions 3 and 4 (7). It seems therefore likely that Asp\(^2\) and Lys\(^3\)-Ser\(^4\) cause the retention of lipoproteins through different mechanisms. The periplasmic loops of PaLolC and/or PaLolE may be involved in the recognition of the retention signals at positions 3 and 4 of lipoproteins, whereas phospholipids may play an important role in the retention of lipoproteins with Asp\(^2\).

Asp\(^2\) is generally found in the inner membrane lipoproteins of \(E.\) \(coli\), whereas the residues at positions 3 and 4 of \(P.\) \(aeruginosa\) inner membrane lipoproteins vary (9). For example, Glu\(^4\)-Ala\(^4\) of MexX, a parologue of MexA, also function as an inner membrane retention signal (9). We speculate that the secondary structure formed by these residues functions as a lipoprotein sorting signal.

We convincingly showed here that the PaLol system is involved in the lipoprotein sorting to the outer membrane of \(P.\) \(aeruginosa\), as the EcLol system is. Five Lol proteins are highly conserved in the \(\gamma\)-proteobacteria including \(E.\) \(coli\) and \(P.\) \(aeruginosa\). Moreover, other classes of proteobacteria also possess Lol proteins, although the gene for LolE is missing (5). In these cases, the lipoprotein-releasing ATP-binding cassette transporters are probably composed of a homodimer of the LolC-LolD heterodimer. \(\alpha\)-Proteobacteria apparently lack a gene for LolB, although they possess ones for LolCD and LolA. The outer membrane lipoproteins of these bacteria may be released from the inner membrane through a mechanism homologous to those in \(E.\) \(coli\) and \(P.\) \(aeruginosa\) but may be incorporated into the outer membrane through a different mechanism. It is not known whether or not lipoprotein sorting to the outer membrane in other Gram-negative bacteria is mediated by the Lol system. A recent report suggested that the sorting of \(Borrelia\) spirochaete lipoproteins to the outer surface of the outer membrane is mediated by a system other than the Lol system (30). Therefore, the lipoprotein sorting signals used in this bacterium are likely to be different from those used in \(E.\) \(coli\) and \(P.\) \(aeruginosa\).

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\(^4\) S. Tanaka, S. Narita, and H. Tokuda, unpublished observation.