Amino Acids at Positions 3 and 4 Determine the Membrane Specificity of Pseudomonas aeruginosa Lipoproteins

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Bacterial lipoproteins comprise a subset of membrane proteins that are covalently modified with lipids at the amino-terminal Cys (1). In Gram-negative bacteria, lipoproteins are anchored to either the inner or the outer membrane, and the final destinations of lipoproteins in Escherichia coli depend on the residue at position 2, which is immediately after the lipid-modified Cys (2–4). When the residue at position 2 is Asp, lipoproteins remain in the inner membrane. On the other hand, when the residue at position 2 is an amino acid other than Asp, lipoproteins are recognized by an ATP-binding cassette transporter, LolCDE, and are targeted to the outer membrane by a lipoprotein-specific targeting system comprising five Lol proteins (5). Lipoproteins possessing Asp at position 2 are not recognized by LolCDE and therefore remain in the inner membrane, indicating that the residue functions as the LolCDE avoidance signal. Genes for lipoproteins have been predicted based on the characteristic signal peptides and well conserved amino acid sequence immediately preceding the N-terminal Cys of the mature region. It is now established that bacterial genomes carry a considerable number of genes for lipoproteins (6, 7). More than 90 species of lipoproteins were biochemically found in E. coli (8). According to the “+2 rule” for lipoprotein sorting, most lipoproteins are predicted to be present in the outer membrane with some in the inner membrane.

Lipoprotein sorting signals have been comprehensively characterized in E. coli (2–4, 9). It is now clear that E. coli lipoproteins are targeted to the outer membrane by default. Only Asp at position 2 actively functions as an intrinsic inner membrane retention signal (4). Asn at position 2 also functions as an inner membrane retention signal when the residue at position 3 is Asp (4), as in the case of a native lipoprotein, AcrE (10, 11). Gly, Phe, Pro, Trp, and Tyr at position 2 also function as inner membrane retention signals when Asn is present at position 3, although these combinations of amino acids are not found in native lipoproteins (3, 4). Whether intrinsic or artificial, these residues function as inner membrane retention signals in the Enterobacteriaceae family of Gram-negative bacteria including E. coli, Shigella flexneri, Salmonella enterica serovar typhi-murium, Erwinia carotovora, and Klebsiella oxytoca (12). However, inner membrane lipoproteins that have residues other than Asp at position 2 are present in other branches of Gram-negative bacteria. For example, an inner membrane lipoprotein, MexA, involved in the multidrug efflux of Pseudomonas aeruginosa, has Gly at position 2 without Asn at position 3 (13). Because five Lol proteins are well conserved in this bacterium, it is mysterious why MexA is not targeted to the outer membrane.

Bacterial multidrug efflux pumps recognize and extrude multiple, structurally unrelated drugs from bacterial cells (14). Resistance-nodulation-division (RND)2-type multidrug efflux pumps play a major role in the drug resistance of Gram-negative bacteria. These pumps are composed of the RND protein in the inner membrane, the membrane fusion protein (MFP) in the periplasm with the N terminus anchored to the inner membrane through either a lipid moiety or an α-helical signal anchor sequence, and the outer membrane efflux protein (OEP) that forms an efflux channel
Sorting Signal for Pseudomonas aeruginosa Lipoproteins

The MexAB-OprM multidrug efflux pump is constitutively expressed and composed of MexA (MFP), MexB (RND), and OprM (OEP). This pump makes a large contribution to the natural resistance of P. aeruginosa against a broad spectrum of antibiotics (13, 16, 17).

Here, we show that Lys and Ser at positions 3 and 4, respectively, are critical for the inner membrane localization of MexA in P. aeruginosa. Moreover, we show that the residues at positions 3 and 4 generally function as sorting signals for P. aeruginosa lipoproteins.

EXPERIMENTAL PROCEDURES

Materials—n-Dodecyl-β-D-maltopyranoside was purchased from Dojindo Laboratories (Kumamoto, Japan). [9,10(n)-3H]Palmitic acid was from Amersham Biosciences.

Bacterial Strains, Plasmids, and Media—P. aeruginosa strains PAO1 (prototroph), PAO4290 (leu-10 argF10 aph-9004; FP -) (18), and TNP070 (∆mexA; derivative of PAO4290) (18) were generous gifts from Dr. Taiji Nakae (Tokai University). E. coli K12 DH5α (endA hsdR17 supE44 thi-1 recA1 gyrA96 relA1 Δ(lacZYA-argF)U169 deoR (880 lacZΔM15)) (19) was also used. The plasmids and primers used in this study are listed in supplemental Tables 1 and 2, respectively. Bacteria were grown on LB medium (20). When required, carbenicillin, ampicillin, and chloramphenicol were added at concentrations of 100, 50, and 35 μg/ml, respectively.

Cloning of the Gene for His-tagged MexA—To construct pUCP-MEXA-His, the gene for His-tagged MexA was amplified by PCR from pMEXA1 (21) using primers A-U-Bam and A-D-His and then cloned into the BamHI-HindIII sites of pUCP20 (22). Site-directed mutagenesis of pUCP-MEXA-His was performed with primers mexA(D)-1 and mexA(D)-2 to construct pUCP-MEXA(G2D)-His and with primers mexA(S)-1 and mexA(S)-2 to construct pUCP-MEXA(G2S)-His.

Construction of Plasmids Encoding OprM-MexA Fusion Proteins—To construct pMA20 encoding OM-1, a 111-bp fragment encoding the signal peptide plus 20 amino acid residues of OprM was amplified by PCR using pOprM (23) as a template and the specified primers shown in supplemental Table 1. The PCR product was then used as a primer to amplify the whole plasmid sequence using pLMALE1-His as a template. Plasmids encoding other OprM-MexA fusion proteins were constructed by site-directed mutagenesis using pLMALE2-His or one of its derivatives as a template and the specified primers shown in supplemental Table 2. The lineage of plasmids encoding lipopMalE derivatives is shown in supplemental Fig. 1B.

Membrane Localization of Proteins—P. aeruginosa PAO1 or E. coli DH5α harboring the specified plasmid was grown on LB at 37 °C to the mid-exponential phase of growth. Cells were harvested, suspended in 50 mM Tris-HCl (pH 7.5), and then disrupted by a single passage through a French pressure cell at 10,000 p.s.i. A total membrane fraction was recovered by centrifugation at 100,000 × g for 1 h at 4 °C, suspended in 50 mM Tris-HCl (pH 7.5) containing 1 mM EDTA, and then layered on a 20–60% (w/w) linear sucrose gradient. After centrifugation at 60,000 × g for 12 h at 4 °C, fractions were collected from the gradient with a piston gradient fractionator (BioComp Instruments), and then analyzed by SDS-PAGE and immunoblotting.

Lipoprotein Labeling—P. aeruginosa PAO1 cells expressing lipopMalE derivatives were labeled with 10 μCi/ml [9,10(n)-3H]palmitic acid for 24 h in LB medium at 37 °C. Cells were harvested, extracted with BugBuster reagent (Novagen) in the presence of 200 μg/ml lysozyme and 100 μg/ml DNase I, and then centrifuged at 10,000 × g for 2 min. The supernatant was incubated for 1 h with TALON resin (Clontech), which had been equilibrated with buffer A comprising 20 mM sodium phosphate (pH 7.2) containing 150 mM NaCl and 0.01% n-dodecyl-β-D-maltopyranoside. The resin was packed into a column, washed with buffer A supplemented with 20 mM imidazole, and then eluted with buffer A containing 250 mM imidazole. The eluate was concentrated by triacrylxylic acid precipitation and then subjected to SDS-PAGE followed by immunoblotting with anti-MalE antiserum. The same membrane was exposed to an imaging plate, and radiolabeled proteins were detected with a PhosphorImager STORM 820 (Amersham Biosciences).

Other Techniques—The minimum inhibitory concentrations (MICs) of antibiotics were determined by the agar dilution method using Mueller-Hinton agar (25). SDS-PAGE was carried out as described (26). Proteins in SDS-polyacrylamide gels were transferred to polyvinylidene fluoride membranes, and the blots were developed with enhanced chemiluminescence substrate (ECL-Plus; Amersham Biosciences) followed by detection with a lumino-image analyzer (LAS-1000plus; Fujifilm).
Anti-hexahistidine tag antiserum was raised in rabbits against the purified His-tagged RpmJ protein (27). Anti-OmpA (28), -Pal (24), and -SecG (29) antisera were raised in rabbits against the purified proteins. Anti-MexA and -MexB antisera were generous gifts from Dr. Taiji Nakae. Anti-MalE antiserum was purchased from New England Biolabs.

RESULTS

Membrane Localization of MexA in E. coli and P. aeruginosa— Genome-wide computational analysis of the proteome of P. aeruginosa PAO1 has revealed 2123 signal peptides, which correspond to 38.1% of the total proteins encoded in the genome (30). Among them, 185 signal peptides (3.3% of the total proteome) contain a consensus motif for lipoproteins. The membrane localization of some P. aeruginosa lipoproteins can be predicted from their functions (Fig. 1). However, none of the inner membrane lipoproteins in Fig. 1 have Asp at position 2. Instead, the residues at position 2 of these lipoproteins are known to cause outer membrane localization in E. coli (4). Nevertheless, MexA has been reported to be localized in the inner membrane of P. aeruginosa (31). To determine the mechanism underlying the inner membrane localization of MexA, we expressed hexahistidine (His)-tagged MexA in E. coli and fractionated membranes by means of sucrose density gradient centrifugation followed by SDS-PAGE and immunoblotting with antibodies against the His tag (Fig. 2A). Outer membrane protein OmpA and inner membrane protein SecG were also examined as controls. Inner membrane-specific lipoprotein MexA of P. aeruginosa was localized in the outer membrane in E. coli.

However, when Gly at position 2 of MexA was replaced by Asp, the MexA derivative MexA(G2D) was detected in the inner membrane fraction (Fig. 2A). It was therefore suggested that the lipoprotein sorting signals differ between P. aeruginosa and E. coli.

We next examined the membrane localization of His-tagged MexA and its derivatives in P. aeruginosa (Fig. 2B). Outer membrane protein OprL, which is homologous to E. coli outer membrane lipoprotein Pal (32), and inner membrane protein MexB were examined as controls. In contrast to the results obtained for E. coli (Fig. 2A), MexA was localized in the inner membrane of P. aeruginosa (Fig. 2B). To determine whether or not Gly at position 2 functions as an inner membrane retention signal in P. aeruginosa, we replaced the residue by Asp and Ser. Asp is the general inner membrane signal in E. coli, and Ser is frequently found at position 2 of outer membrane lipoproteins in not only E. coli but also P. aeruginosa (Fig. 1). However, both MexA(G2D) and MexA(G2S) remained in the inner membrane (Fig. 2B), suggesting that Gly2 does not cause the inner membrane localization of MexA. The level of MexA(G2S) expressed from a plasmid was about 5-fold higher than that of chromosomally encoded wild-type MexA (Fig. 3). It is therefore unlikely that the interaction with chromosomally encoded MexB caused the inner membrane localization of MexA(G2S).

Taken together, these results suggested that the inner membrane retention signal of MexA is localized at a position other than 2.
OprM is an outer membrane lipoprotein that functions as the OEP of the MexAB-OprM multidrug efflux pump. It has been reported that a non-lipidated derivative of \textit{P. aeruginosa} OprM was functional and localized to the outer membrane (23, 33). Moreover, a Ser to Asp mutation at position 2 of OprM affected neither its localization nor its function (33). These observations suggested that the outer membrane localization of OprM is determined by information in its protein moiety. However, since OprM must be released from the inner membrane for outer membrane targeting, it should not contain any inner membrane retention signal. To identify the residue or region of MexA that functions as an inner membrane retention signal, we constructed an OprM-MexA chimeric lipoprotein, OM-1, comprising the signal peptide and 20 N-terminal residues of the mature region of OprM, the rest being derived from MexA. This construct was located in the outer membrane of \textit{P. aeruginosa} PAO1 (Fig. 2C). On the other hand, OM-8, in which only the signal peptide of MexA was replaced by that of OprM, was located in the inner membrane (Fig. 2C). These results indicated that the inner membrane retention signal is present in the 20 N-terminal residues of MexA. Moreover, it is clear that the signal peptide does not affect the final destinations of lipoproteins. To identify the sorting signal, we constructed a series of OprM-MexA chimeras (Fig. 2C). OM-2 through OM-5 contained the 4–10 N-terminal residues of OprM and were located in the outer membrane. On the other hand, OM-6 containing the 3 N-terminal residues of OprM was detected in both the inner and the outer membrane fractions. The sequence of the mature region of OM-7 was identical to that of MexA(G2S) (Fig. 2B) and largely located in the inner membrane. Both OM-5 and OM-9 were located in the outer membrane, confirming that Gly$^2$ of MexA does not cause the inner membrane localization. In contrast, the outer membrane localization of these two chimeras seemed to be caused by the introduction of Leu and/or Ile at positions 3 and 4, respectively. We therefore constructed two derivatives by substituting Leu$^3$ and Ile$^4$ of outer membrane-specific OM-9 with Lys and Ser, respectively. Significant portions of both derivatives, OM-10 and OM-11, were localized in the inner membrane, indicating that both Lys$^3$ and Ser$^4$ function to retain lipoproteins in the inner membrane. We also constructed an OprM derivative having Lys and Ser at positions 3 and 4, respectively. However, this construct could not be expressed in \textit{P. aeruginosa} for an unknown reason. Although

![Figure 2. Membrane localization of MexA derivatives in \textit{E. coli} and \textit{P. aeruginosa}](image)

- **A**: His-tagged MexA or its derivative, MexA(G2D), was expressed in \textit{E. coli} K12. Membranes were separated by sucrose density gradient centrifugation. Proteins were analyzed by SDS-PAGE and immunoblotting. MexA was detected with anti-His tag antiserum. Inner membrane protein SecG and outer membrane protein OmpA were also detected with the respective antisera.
- **B**: His-tagged MexA and its derivatives, MexA(G2D) and MexA(G2S), were expressed in \textit{P. aeruginosa} PAO1. Membranes were separated, and proteins were analyzed as in A. Inner membrane protein MexB and outer membrane protein OprL were detected with antisera against MexB and \textit{E. coli} Pal, respectively.
- **C**: OprM-MexA fusion proteins were expressed in \textit{P. aeruginosa} PAO1 cells, and their membrane localization was examined as in B. The N-terminal sequences of the respective derivatives are shown. Residues derived from MexA and OprM are shown as black and white letters, respectively.
Gly2 of wild-type MexA was not the inner membrane localization signal, it was not clear whether or not Asp at this position functions as an inner membrane localization signal in P. aeruginosa. To examine this, Ser2 of the outer membrane-specific derivative OM-5 was replaced by Asp. The resultant derivative, OM-12, was located in the inner membrane (Fig. 2C), indicating that Asp at position 2 functions as an inner membrane retention signal in P. aeruginosa as well as in E. coli.

MFPs such as MexA are essential for the function of RND-type multidrug efflux pumps (14, 16, 18). Although the precise functions of MFPs remain to be clarified, crystallographic and mutational studies suggest that MexA stabilizes the interaction between MexB and OprM by interacting with the former at the C-terminal domain and the latter at the α-helical hairpin (34–37). Therefore, the inner membrane localization of MexA is likely to be essential for proper interaction between MexB and OprM and thus for drug resistance. To examine the functions of MexA derivatives, P. aeruginosa ΔmexA strain TNP070 was transformed with a plasmid expressing each MexA derivative, and then the MICs of antibiotics were determined (Fig. 3). Western blotting of whole cell lysates revealed that the levels of MexA derivatives expressed from plasmids varied but were higher than that of chromosomally encoded wild-type MexA except in the case of OM-1. Wild-type MexA and its derivatives with Asp or Ser at position 2 conferred resistance to aztreonam and chloramphenicol, the MIC values being 1.56 and 25 μg/ml, respectively. The drug resistance conferred by these MexA derivatives was comparable with that exhibited by wild-type strain PAO4290. Expression of OM-1 through OM-5 and of OM-9 did not confer resistance to the two drugs. These derivatives were localized to the outer membrane (Fig. 2C). On the other hand, OM-6 through OM-8 and OM-10 through OM-12 conferred the drug resistance, although the resistance conferred by OM-12 was only half of that conferred by the others. These derivatives were partially or entirely localized in the inner membrane. Taken together, these results indicate that the antibiotic resistance requires the inner membrane localization of MexA derivatives.

Membrane Localization of lipoMalE—To determine whether or not the sorting signals revealed with MexA in P. aeruginosa are applicable to another lipoprotein, the periplasmic maltose-binding protein (MalE) of E. coli was converted into a lipoprotein (lipoMalE) by attaching the signal peptide and 4 N-terminal residues of OprM to the N terminus of mature MalE. The indicated lipoMalE derivatives were expressed in P. aeruginosa, and then their membrane localization was determined as in Fig. 2. Residues derived from inner and outer membrane lipoproteins are shown as black and white letters, respectively.
membrane localization (lipoMalE-6 and -7). If the derivatives had both Lys at position 3 and Ser at position 4, lipoMalE molecules were completely localized in the inner membrane (lipoMalE-8 and -9). These results were consistent with those obtained with MexA derivatives (Fig. 2). Moreover, lipoMalE-10 having Glu and Ala at positions 3 and 4, respectively, was localized in the inner membrane. The inner membrane lipoprotein MexX has the same signal (Fig. 1). In contrast, the Val-Gly signal present in outer membrane lipoproteins OpmD and OprN (Fig. 1) caused the outer membrane localization of lipoMalE-11. The introduction of Gly3 and Leu4, which are present in outer membrane lipoprotein LolB, into lipoMalE-1 did not affect the outer membrane localization (lipoMalE-12). These results indicate that residues at positions 3 and 4 determine the membrane localization of P. aeruginosa lipoproteins.

DISCUSSION

The lipoprotein sorting signals examined in vivo using OprM-MexA chimeric lipoproteins and model lipoproteins, lipoMalEs, clearly revealed that the residues at positions 3 and 4 determine the membrane specificity of lipoproteins in P. aeruginosa but not in E. coli. In contrast, it was found that Asp2 functions as an inner membrane retention signal in P. aeruginosa as well as in E. coli. Among 185 putative lipoprotein genes in P. aeruginosa PAO1 (30), five chromosomal loci (PA1222, PA1592, PA2137, PA3262, and PA3396) are predicted to encode lipoproteins with Asp2. However, none of these putative lipoproteins has been characterized as to lipid modification and membrane localization. The inner membrane retention of lipoMalE-3 was less efficient than that of OM-12, both of them having Asp2-Leu3-Ile4 (Figs. 2C and 4B). MexA forms oligomers during crystallization (34, 35). It is therefore possible that the inner membrane retention of OM-12 is facilitated by its oligomerization in the inner membrane. In contrast, because MalE is unlikely to oligomerize itself, the localization of lipoMalE-3 in both membranes may indicate that Asp2-Leu3-Ile4 is a less efficient inner membrane retention signal. This may be the reason why very few inner membrane lipoproteins use Asp2 for inner membrane retention.

In E. coli, lipoproteins are released from the inner membrane and then transported to the outer membrane by default. Therefore, only the Lol avoidance signal functions to retain lipoproteins in the inner membrane, the “outer membrane-specific signal” being neutral and having no effect on the release reaction. When outer membrane-specific lipoproteins of P. aeruginosa were mutated to have Lys3 or Ser4, a significant portion of the lipoproteins became localized in the inner membrane (Figs. 2 and 4). If the lipoprotein sorting to the outer membrane of P. aeruginosa also takes place by default, these results indicate that the introduction of any one of the three inner membrane-specific signals is sufficient to alter the membrane localization of outer membrane-specific lipoproteins. However, it cannot be excluded that the outer membrane localization of lipoproteins is also determined by specific signals, not by default. If this is the case, the outer membrane localization of lipoproteins should be determined by the combined action of the residues at positions 3 and 4, for example, Leu3 and Ile4. Moreover, Asp2 should abolish the ability of the Leu3-Ile4 signal to localize lipoproteins to the outer membrane.

We have analyzed the amino-terminal sequences of MexA homologues in proteobacteria (Fig. 5). Conservation of Asp at positions 2 or 3 is found only in the Enterobacteriales. The Vibrionales, Pseudomonadales, and Xanthomonadales are γ-proteobacteria with a highly conserved Lol system, but their MexA homologues do not have conserved residues at positions 3 and 4, although Gly2 is considerably conserved in the Vibrionales and Pseudomonadales. The very low conservation of the 3rd and 4th residues in the Pseudomonadales suggests that the inner membrane-specific signals are diverse. Indeed, the Glu3-Ala4 signal, which is present in MexX, was found to be inner membrane-specific in P. aeruginosa (Fig. 4B). It is therefore likely that other combinations of the 3rd and 4th residues, or one of them, also function to retain lipoproteins in the inner membrane. To completely reveal the inner membrane retention signals of P. aeruginosa, an outer membrane-specific MexA derivative was subjected to random mutagenesis followed by expression in ΔmexA strain TNP070. The isolation of drug-resistant mutants and identification of mutations are currently in progress.

Five Lol proteins are conserved in P. aeruginosa. In the accompanying study, it is shown that the Pseudomonas Lol system is also involved in the sorting of lipoproteins to the outer membrane, albeit with different sorting signals (38).

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