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Minireview

An ABC transporter mediating the membrane detachment of bacterial lipoproteins depending on their sorting signals

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Abstract **Bacterial lipoproteins are anchored to membranes through a lipid moiety attached to the N-terminal Cys. *Escherichia coli* possesses more than 90 species of lipoproteins, most of which are localized in the outer membrane and others in the inner membrane. Sorting of lipoproteins to the outer membrane requires the Lol system comprising five Lol proteins. An ATP-binding cassette transporter, LolCDE, initiates the lipoprotein sorting by mediating the detachment of outer membrane-specific lipoproteins from the inner membrane. LolCDE does not recognize lipoproteins possessing Asp at position 2, which therefore remain anchored to the inner membrane. We will discuss the mechanism of LolCDE based on data obtained through in vitro experiments.**

Keywords: Membrane anchor; Reconstitution; Membrane localization; Sorting signal; ATP hydrolysis

1. Overview of the lipoprotein-localization system in *Escherichia coli*

The outer membrane of Gram-negative bacteria is an asymmetric bilayer containing lipopolysaccharide (LPS) exclusively in the outer leaflet and phospholipids in the inner leaflet. The outer membrane contains two classes of proteins, which associate with the bilayer in different ways. A few major species of proteins span the bilayer by forming β-barrel structures, while lipoproteins are anchored to the outer membrane through the lipid moiety attached to the N-terminal Cys [1]. Modification of lipoproteins occurs after lipoprotein precursors with signal peptides have been translocated from the cytosol to the periplasmic side of the inner membrane through the action of Sec translocase. The modification reaction comprises three steps in Gram-negative bacteria: (1) formation of a thioether linkage between a Cys in the N-terminal region and diacylglycerol by phosphatidylglycerol:prolipoprotein diacylglyceryl transferase, which recognizes a consensus sequence, -L-(A/S)-(G/A)-C-, called a lipobox (or lipoprotein box), (2) cleavage of a signal peptide by signal peptidase II (prolipoprotein signal peptidase), which turns the S-lipidated Cys into the amino-terminal residue of the mature protein, and (3) amino-acylation of this Cys by phospholipid:apolipoprotein transacylase [2].

In Gram-negative bacteria, lipoproteins are localized in either the inner or outer membrane, depending on the sorting signals. In *E. coli*, the final destinations of lipoproteins are determined by the residue immediately after the lipid-modified Cys residue [3–5]. When Asp is present at this position (position 2), the lipoproteins remain on the inner membrane. On the other hand, when the residue at position 2 is other than Asp, the lipoproteins are sorted to the outer membrane. Thus, the residue at position 2 functions as a sorting signal for lipoproteins. Biochemical studies based on genomic analyses [6] revealed that the *E. coli* K-12 strain has at least 90 species of lipoproteins (Matsuyama, Tanaka-Masuda and Tokuda, unpublished observations), most of which are presumably located on the periplasmic surface of the outer membranes, while less than 10 lipoprotein species are expected to present on the inner membrane.

Lipoproteins are highly hydrophobic because of the lipid moiety attached to their N-terminus. Transport of lipoproteins to the outer membrane through the hydrophilic periplasm therefore requires a specialized mechanism. Lipoproteins are detached from the inner membrane by an ATP-binding cassette (ABC) transporter, LolCDE, in the presence of a periplasmic carrier protein, LolA. LolCDE recognizes lipoproteins possessing amino acid residues other than Asp at position 2 and releases them from the periplasmic leaflet of the inner membrane in an ATP-dependent manner, leading to the formation of a soluble complex between one molecule each of LolA and lipoprotein. The LolA-lipoprotein complex thus formed moves across the periplasm, probably through diffusion, and then interacts with a lipoprotein-specific receptor, LolB, which is itself a lipoprotein and anchored to the outer membrane. A lipoprotein is then transferred from LolA to LolB according to the affinity difference between LolA and LolB. LolB then incorporates lipoproteins into the inner leaflet of the outer membrane (Fig. 1) [7,8].

2. Characterization of the LolCDE complex as a novel ABC transporter mediating the release of lipoproteins from membranes

Proteins destined to the periplasm or the outer membrane were released from the inner membrane of spheroplasts, in which the outer membrane was disrupted by EDTA and lyso-

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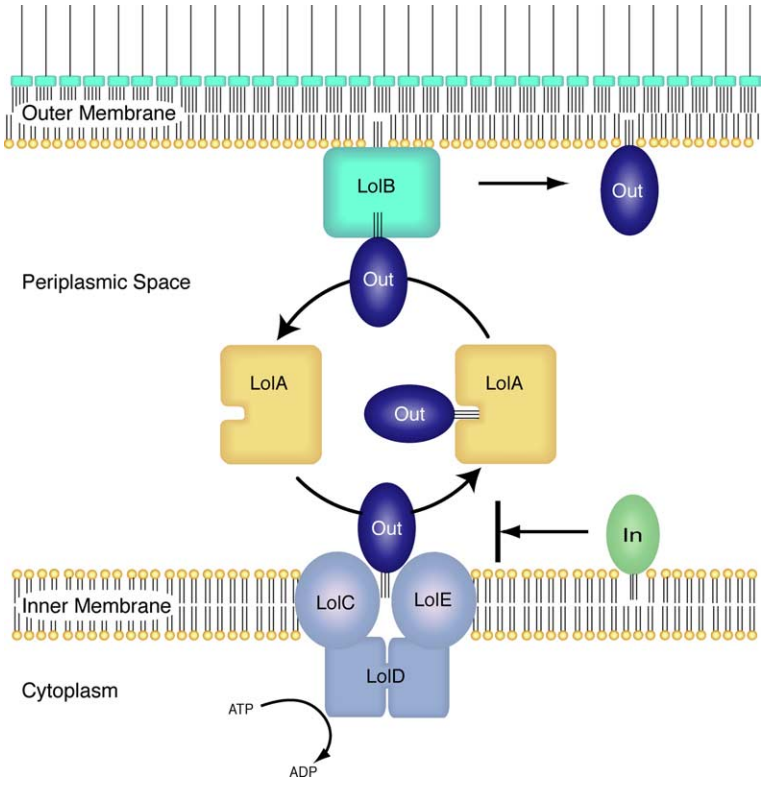

Fig. 1. Sorting and outer membrane localization of lipoproteins by the Lol system. "In" and "Out" represent inner membrane-specific and outer membrane-specific lipoproteins, respectively. The ABC transporter LolCDE recognizes outer membrane-directed lipoproteins and releases them from the inner membrane, causing the formation of a complex between one molecule each of lipoprotein and LolA, a periplasmic carrier protein. The LolA-lipoprotein complex traverses the periplasmic space and lipoproteins are transferred to the outer membrane receptor protein LolB, followed by incorporation into the outer membrane. LolB is itself an outer membrane lipoprotein. Inner membrane-specific lipoproteins with Asp at position 2 avoid recognition by LolCDE and thus remain anchored to the inner membrane.

zyme. On the other hand, outer membrane-specific lipoproteins remained anchored to the inner membrane unless the periplasmic fraction was added. A protein, named LolA, purified from the periplasm immediately induced the release of outer membrane lipoproteins into the spheroplast supernatant [9]. LolA did not release the inner membrane-specific lipoproteins, indicating that release depends on sorting signals. The release of outer membrane-specific lipoproteins from right-side out membrane vesicles occurred in the presence of LolA only when membrane vesicles were loaded with ATP, indicating that the release reaction requires ATP energy. GTP and UTP substituted for ATP, while ADP, AMP or AMP-PNP did not support the release of lipoproteins. Moreover, sodium vanadate completely inhibited the ATP-dependent release of lipoproteins [10]. These findings indicated that a certain membrane factor releases lipoproteins from the inner membrane by utilizing the energy of nucleotide triphosphate.

The activity of the lipoprotein-releasing factor(s) present in the inner membrane has been examined in reconstituted proteoliposomes. Inner membrane proteins solubilized with a detergent, purified outer-membrane lipoprotein Pal, and *E. coli* phospholipids were reconstituted into proteoliposomes in the presence of ATP (Fig. 2A). The proteoliposomes exhibited the ability to release Pal into the supernatant in a LolA-dependent manner. Pal was not released when AMP-PNP was added in place of ATP. The ATP-dependent release of Pal was inhibited by sodium vanadate. The inner membrane proteins responsible for the lipoprotein-releasing activity were then purified by successive chromatographies, and N-terminal sequencing revealed one protein was the product of *ycfV*. This protein possessed Walker A and B motifs, and a characteristic sequence called the ABC signature, which is conserved among ABC ATPases [11]. The *ycfV* gene, which is also referred to as o228b, is located between two genes encoding putative transmembrane proteins of an ABC transporter, together forming a possible transcriptional unit [12,13]. The products of these three genes were purified as a complex and then shown to be responsible for lipoprotein-releasing activity. Thus, the ATPase concerned was designated as LolD, while the integral membrane subunits were designated as LolC and LolE. The complex was composed of one copy each of LolC and LolE, and two copies of LolD (Fig. 2B). When Ser at position 2 of Pal was converted to Asp, the resulting derivative, Pal(S2D), was not released from proteoliposomes even in the presence of ATP, LolA and LolCDE (Fig. 2C) [13]. These results established that lipoprotein-sorting signals at position 2 function at the release step. The results also indicate that no other factor is required for recognition of the sorting signals.

Mutations in the Walker A, B, and ABC signature motifs of LolD severely inhibited the release of lipoproteins from spheroplasts, confirming that LolCDE is directly involved in the ATP-dependent release of lipoproteins. In contrast, these mutations had no effect on the processing of lipoprotein precursors to the mature forms, indicating that LolCDE is involved in neither the translocation of lipoprotein precursors from the cytosol to the periplasmic leaflet of the inner membrane nor

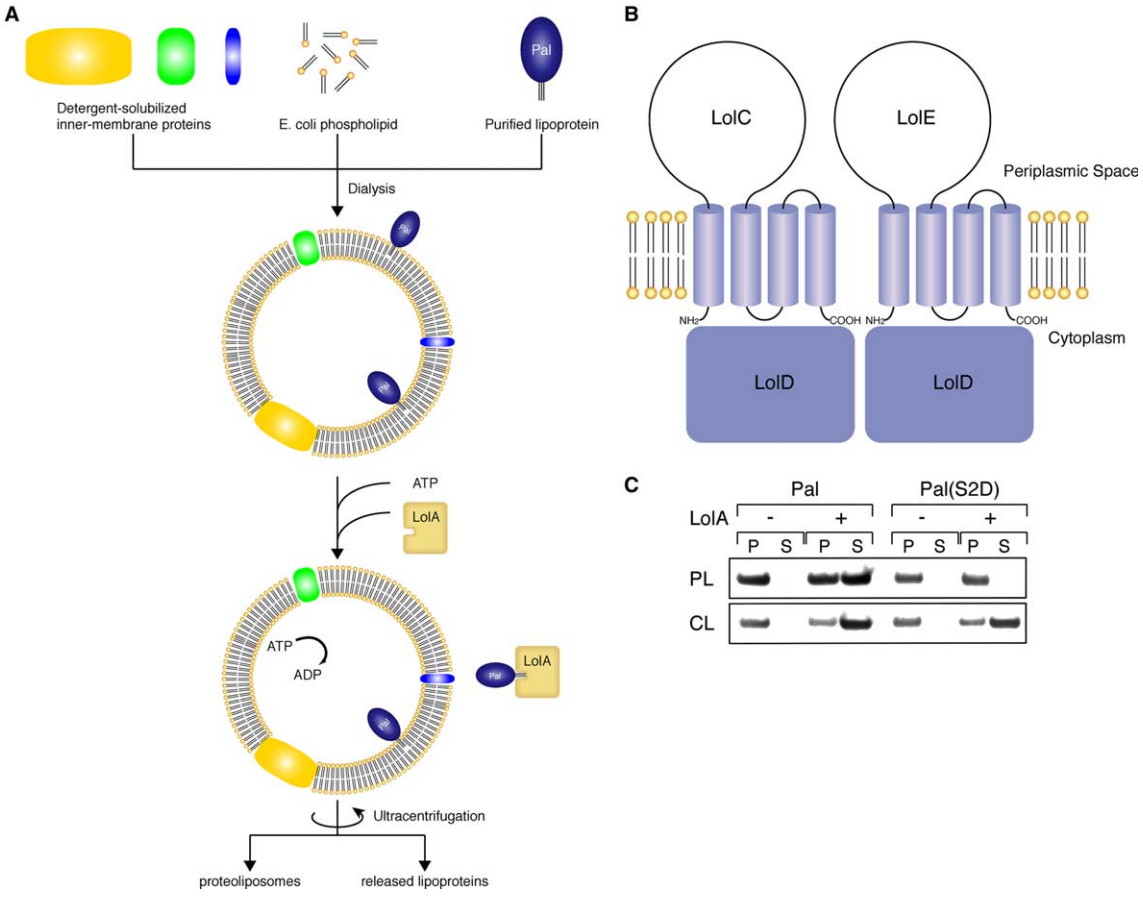

Fig. 2. The lipoprotein-releasing apparatus. (A) Reconstitution of lipoprotein-releasing activity into proteoliposomes. Inner membrane proteins are solubilized with a detergent and then reconstituted into proteoliposomes together with purified lipoproteins and *E. coli* phospholipids. Proteoliposomes are frozen, thawed and sonicated to load ATP, followed by incubation with LolA. Lipoprotein-releasing activity is measured as the amount of lipoproteins recovered in the supernatant after ultracentrifugation. (B) The LolCDE complex conferring the lipoprotein-releasing activity. The membrane topology of LolC and LolE is based on topology prediction and biochemical analyses (Yasuda, Iguchi, Matsuyama, Narita and Tokuda, unpublished observations). (C) The LolCDE avoidance function of Asp depends on phospholipids. Proteoliposomes are reconstituted with purified LolCDE, *E. coli* phospholipids (PL) or cardiolipin (CL), and Pal or Pal(S2D). Inner membrane-specific Pal(S2D) possessing Asp in place of Ser at position 2 is not released from proteoliposomes reconstituted with PL but is released from ones reconstituted with CL in a LolA-dependent manner. P and S indicate the pellet and supernatant fractions, respectively.

the subsequent formation of mature lipoproteins [13]. In addition to the conserved sequences in ABC transporters, LolD contains a characteristic sequence that is conserved among LolD homologues in proteobacteria, and located between the Walker A and ABC signature motifs (Fig. 3A). It has been speculated that the LolD motif is involved in the unique function of LolCDE. LolC and LolE are homologous to each other, the amino acid identity being 26%. Moreover, the two proteins span the membrane four times, and have a large domain exposed to the periplasm between the first and second transmembrane helices (Fig. 2B; Yasuda, Iguchi, Matsuyama, Narita and Tokuda, unpublished observations). Therefore, the number of transmembrane helices in the LolCDE complex is eight, which is smaller than that in most ABC transporters [11]. This may be related to the unique function of LolCDE, because unlike other ABC transporters, LolCDE does not catalyze the transmembrane transport of substrates.

Removal of a plasmid carrying the *lolCDE* genes in a chromosomal *lolC–lolD–lolE* null mutant caused mislocalization of the outer membrane lipoproteins in the inner membrane and inhibited the growth of *E. coli* [14]. These results established that the LolCDE complex is the sole apparatus mediating the release of *E. coli* lipoproteins. So far as reported, only two ABC transporters, LolCDE and MsbA, are essential for the growth of *E. coli*. MsbA has been proposed to be involved in the biogenesis of LPS and/or outer membrane phospholipid [15,16]. Five Lol proteins, LolA–LolE, are well conserved in various Gram-negative bacteria, and the genes encoding these proteins are all essential for *E. coli* growth [14,17,18]. Two outer membrane lipoproteins were found to be essential for the growth of *E. coli* [18,19]. Depletion of Lol proteins causes the depletion of these essential lipoproteins from the outer membrane, cell death thereby being caused. Since lipoproteins and their localization system are unique in bacteria, Lol proteins are good targets for drug development as to infectious diseases caused by pathogenic Gram-negative bacteria.

3. How does the LolCDE complex recognize the residue at position 2 as a sorting signal?

The mode of lipid modification at the amino-terminal Cys is the same between the inner membrane-specific and outer membrane-specific lipoproteins [20], indicating that the lipoprotein

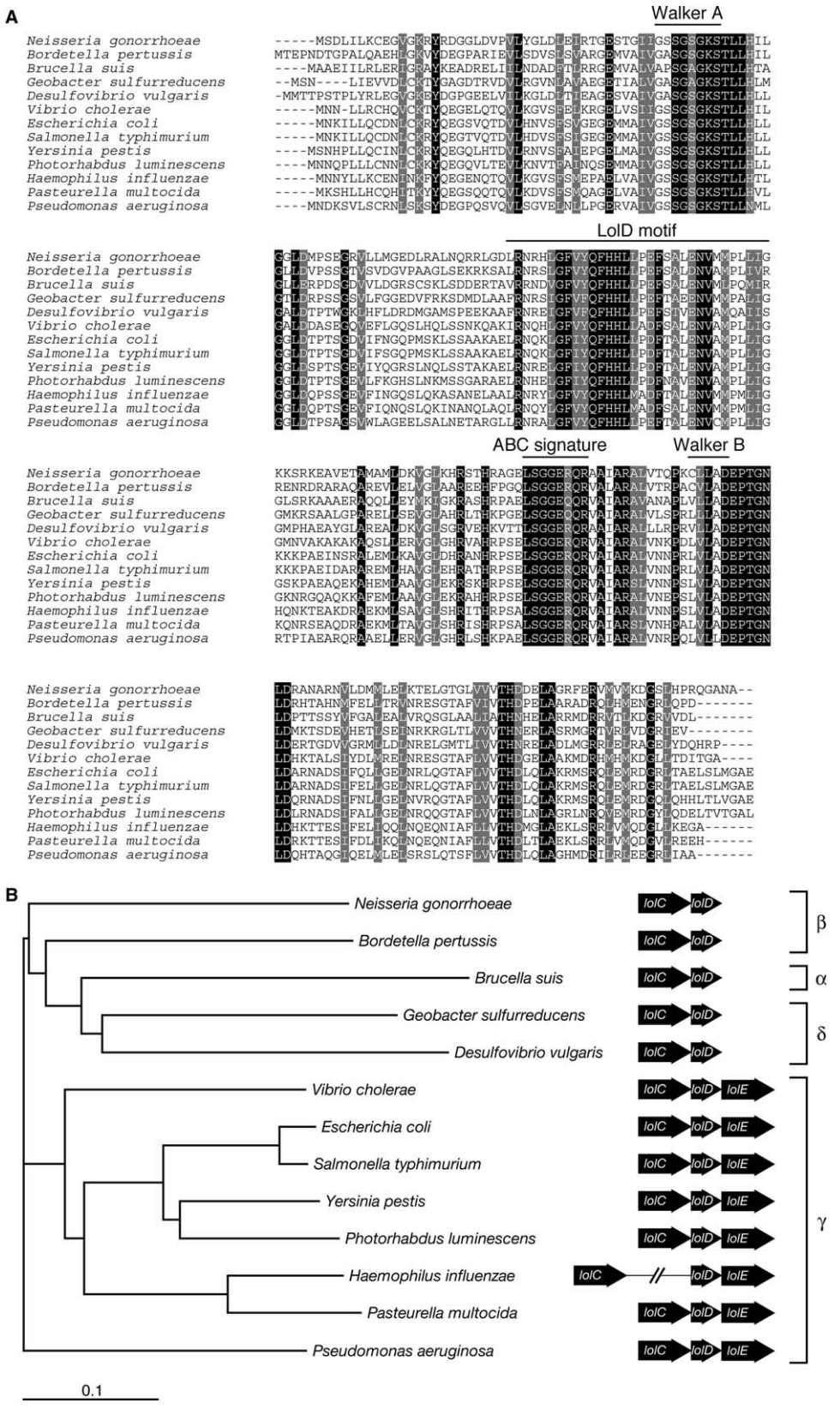

Fig. 3. (A) Sequence alignment of LolD homologues. The amino acid sequences of LolD and its homologues were aligned using ClustalW [37]. Residues that are identical in all sequences are highlighted in black. Highly conserved residues are highlighted in grey. Motifs characteristic of ABC proteins are indicated above the sequences. The LolD motif region is also indicated. (B) A phylogenetic tree of LolD homologues was drawn using TreeView ver.1.6.6. Subgroups of proteobacteria are shown at the right.

sorting signal does not affect lipid modification. Moreover, apolipoproteins lacking the amino-linked acyl chain are not the substrate for the LolCDE complex [20,21]. Therefore, the amino-acid residue at position 2 of lipoproteins determines the membrane localization specificity after the completion of lipid modification, which has been called the "+2 rule" [4]. Only Asp at this position functions as the inner membrane retention signal of native lipoproteins, although the residue at position 3 influences the strength of the Asp residue [5,22]. Therefore, the Asp residue at position 2 is the native inner membrane retention signal. In contrast, inner membrane retention signals other than Asp at position 2 were found by systematic mutagenesis of the residue at position 2 [4]. These signals were found to be combinations of Trp, Phe or Pro at positions 2 and Asn at position 3 [4,5]. Since native *E. coli* lipoproteins do not have these residues at positions 2 and 3, these are artificial inner membrane retentions signals. A LolCDE mutant causing the mislocalization of inner membrane lipoproteins to the outer membrane has been isolated [23]. This mutant carries an Ala to Pro mutation at position 40 of LolC. Once the inner membrane-specific lipoproteins are released through the action of this LolC mutant, LolA and LolB both interact with the lipoproteins, and finally sort them to the outer membrane, indicating that lipoprotein sorting signals only function at the release step. The ATPase activity of LolCDE reconstituted into proteoliposomes was stimulated by outer membrane-specific, but not inner membrane-specific lipoproteins. The release of Lpp, the major outer membrane lipoprotein, from proteoliposomes was inhibited when another outer membrane lipoprotein, Pal, was also used for reconstitution, whereas reconstitution with inner membrane-specific derivative Pal(S2D) had no effect [24]. These results strongly suggested that LolCDE does not recognize lipoproteins with Asp at position 2 because Asp functions as a LolCDE avoidance signal. We then asked what is required for the Lol avoidance signal.

A derivative of Pal, Pal(S2C), with Cys in place of Ser at position 2 was released from the reconstituted proteoliposomes in LolA- and LolCDE-dependent manners. When the Cys residue was oxidized with performic acid to cysteic acid, oxidized Pal(S2C) was no longer released from membranes, indicating that cysteic acid functioned as a LolCDE avoidance signal. Furthermore, when an inner membrane-specific lipoprotein with Asp at position 2 was modified with a carboxylate-specific reagent, the inner membrane retention of this lipoprotein was abolished [25]. The distance between Cα and the negative charge of cysteic acid is very similar to that in the case of Asp. Taken together, these results indicated that the Lol avoidance signal should have a negative charge that is within a certain distance from Cα of the second residue. This also explains why Glu at position 2 is not an inner membrane retention signal [5,22].

Taking advantage of Cys-specific modification, a large nonprotein molecule such as iodo-biotin was introduced at Cys at position 2 of Pal(S2C). However, this modification did not inhibit the release of lipoproteins from proteoliposomes [25], indicating that LolCDE does not recognize the structure of the residue at position 2. On the other hand, LolCDE does not release apolipoprotein, which lacks an amino-linked acyl chain [20,21]. These results suggest that LolCDE only recognizes the N-terminal Cys when it is modified with an amino-linked acyl chain and thioether-linked diacyl glycerol. If LolCDE does not recognize the second residue of lipoproteins, how can Asp at position 2 cause avoidance of LolCDE? Reconstitution experiments revealed that phospholipids are directly involved in the LolCDE avoidance mechanism. *E. coli* contains phosphatidylethanolamine (PE), phosphatidylglycerol (PG), and cardiolipin (CL) as major phospholipids. When proteoliposomes contained a mixture of these phospholipids, LolCDE released lipoproteins in a sorting signal-dependent manner. On the other hand, when the phospholipids were treated with an amine-specific reagent prior to reconstitution, not only Pal but also Pal(S2D) was released. Furthermore, Pal(S2D) was released from proteoliposomes reconstituted with CL alone (Fig. 2C) in a manner sensitive to sodium vanadate, a specific inhibitor of LolCDE [25]. From these observations, we proposed that the electrostatic and steric interactions between Asp at position 2 of lipoproteins and PE make the N-terminal conformation of lipoproteins distinct [25], thereby inhibiting the recognition of LolCDE. The A40P mutation of LolC mentioned above may alter the conformation of the LolCDE complex so that it can accept the unique conformation of the N-terminal region of inner membrane lipoproteins. However, it has been reported that PE affects the membrane topology of some transporters [26–28]. The ability of LolCDE to discriminate lipoproteins might be disturbed by modification of the amino group of PE or reconstitution with CL alone. The effects of phospholipids on the LolCDE function remain to be clarified.

4. Role of ATP energy obtained by LolCDE in the transfer of lipoproteins to outer membranes

ATP hydrolysis by ABC transporters is generally stimulated by their substrates, although the extent of stimulation depends on the transporter. LolCDE in proteoliposomes exhibited ATPase activity of 0.02 μmol ATP min^{-1} mg^{-1} in the absence of lipoproteins. Reconstitution of Pal increased the activity by 2.8-fold in the absence of LolA [24]. The addition of LolA inhibited the lipoprotein-dependent increase in the ATPase activity, because LolA releases Pal from proteoliposomes and thereby reduces the amount of Pal in proteoliposomes. Since it is very difficult to maintain the amount of lipoproteins in proteoliposomes sufficiently high enough for a continuous release reaction, the ATPase activity of LolCDE, which is coupled to the release reaction, has not been measured.

The stimulation of the ATPase activity of LolCDE by outer membrane-specific lipoprotein Pal is comparable to that of bacterial ABC multidrug transporter LmrA by verapamil [29], but is significantly lower than that of bacterial ABC importers such as histidine permease $HisQMP_2$ [30] and maltose permease $MalFGK_2$ [31].

The only step that requires exogenous energy input is the release of lipoproteins from the periplasmic leaflet of inner membranes. Once the LolA-lipoprotein complex is formed in the periplasm, lipoproteins are efficiently localized to the outer membrane in the absence of energy input. The transfer of lipoproteins from LolA to LolB and then from LolB to the outer membranes takes place toward the direction of the higher affinity for lipoproteins [32,33]. Lipoproteins are most stably anchored to the outer membrane. Crystallo-

graphic analysis revealed that LolA has an unclosed β-barrel structure with a 'lid' composed of three α-helices, together constituting a hydrophobic cavity, which is considered to accept the lipid moiety of lipoproteins [34]. The lid of LolA is expected to open to accommodate lipoproteins. Energy obtained through ATP hydrolysis on the cytoplasmic side of the inner membrane is transferred to LolC and/or LolE, and then utilized for both the membrane-detachment of lipoproteins from the periplasmic leaflet of the inner membrane and opening of the LolA lid.

5. Evolutionary conservation of the LolCDE apparatus

Five Lol factors, LolA through LolE, are highly conserved in the gamma subdivision of Gram-negative proteobacteria. Moreover, other subdivisions of proteobacteria also possess Lol factors, although the gene for LolE is missing (Fig. 3B). The lipoprotein-releasing ABC transporters in these organisms are most likely composed of a homodimer of the LolC-LolD heterodimer. Bacteria such as *Brucella suis* and *Desulfovibrio vulgaris* apparently lack a gene for LolB, while they possess genes for LolCD and LolA. The outer membrane lipoproteins of these bacteria may be released from the inner membrane through a mechanism homologous to that in *E. coli*, but may be incorporated into the outer membrane through a different mechanism. It may be possible that a certain outer membrane protein or lipoprotein has a dissimilar sequence to LolB but retains the LolB function. LolD homologues are also present in Archaea, for example the MJ0796 protein of *Methanococcus jannaschii* has been crystallized [35]. The structure of this protein provided valuable information, although it is not clear whether or not this protein is a functional homologue of LolD.

Despite the fact that Lol factors are highly conserved in gamma proteobacteria, sorting signals for lipoproteins may not be conserved throughout species. When the sequences of homologues of AcrA, a conserved inner membrane lipoprotein involved in antibiotic resistance, are compared, the residue at position 2 is not always Asp in many cases. For example, MexA, the AcrA homologue of *Pseudomonas aeruginosa*, has Gly at position 2. Detailed analysis revealed that Lys at position 3 and Ser at position 4, but not Gly at position 2, are critical for the inner membrane-localization of MexA, (Narita and Tokuda, unpublished observation), although these two residues are not conserved among MexA homologues. The release of lipoproteins from proteoliposomes reconstituted with *P. aeruginosa* LolCDE was examined in the presence of *P. aeruginosa* LolA. Lipoproteins with Lys and Ser at positions 3 and 4, respectively, remained in proteoliposomes. Moreover, lipoproteins with Asp at position 2 also remained in these proteoliposomes (Tanaka, Narita and Tokuda, unpublished observation). Taken together, these observations indicate that Asp at position 2 is the general avoidance signal, for which phospholipid compositions are important [25], while the Lys-Ser residues at positions 3 and 4 of *P. aeruginosa* lipoproteins and certain combinations of residues at positions 2 and 3 of *E. coli* lipoproteins [4,5] cause the inner membrane retention by unknown mechanisms. Therefore, the mechanisms for the sorting of lipoproteins seem to be more diverse and complex than the previous proposal [25], even though Lol factors are conserved.

6. Conclusions and perspectives

LolCDE is regarded as an interesting ABC efflux pump variant in that it moves hydrophobic substrates from membranes into the aqueous milieu [36]. While efflux pumps are considered to bind substrates in the inner leaflet of membranes, LolCDE binds lipoproteins in the outer leaflet of the inner membrane after they have been translocated across the membrane. This unique mode of transport may be reflected in the number of transmembrane helices; the total number of transmembrane helices in LolCDE is only eight, which is smaller than that in most ABC efflux pumps or importers.

The mode of recognition of lipoproteins by LolCDE has been extensively studied in the last few years. LolCDE most likely recognizes lipoproteins at their N-terminal Cys, which is modified with an amino-linked acyl chain and thioether-linked diacylglycerol. Asp at position 2 of lipoproteins interacts with phospholipids and disturbs the recognition of the N-terminal Cys by LolCDE, thereby functioning as a LolCDE-avoidance signal. On the other hand, we have no biochemical or biophysical data that directly confirm an interaction between the Asp residue and phospholipids. It is also open to question as to how phospholipids interact with LolCDE and influence its activity. Moreover, LolCDE of other bacteria, in which lipoprotein-sorting signals appear to be different from those in *E. coli*, will help us to understand the general mechanism of recognition of bacterial lipoproteins by this class of ABC transporters.

We identified all factors involved in the lipoprotein-targeting pathway by establishing in vitro assay systems, and examined their functions both genetically and biochemically. However, a number of questions remain to be answered. How does LolCDE change its conformation during the catalytic cycle? How is ATP energy transferred from the cytosol to the periplasm through LolCDE? Are the functions of LolC and LolE the same? Is a lipoprotein the only substrate for LolCDE? To answer these and other questions, further improvement of the in vitro systems is necessary.

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