The outer membrane, an essential organelle of Gram-negative bacteria, is composed of four major components: lipopolysaccharide, phospholipids, \( \beta \)-barrel proteins, and lipoproteins. The mechanisms underlying the transport of these components to outer membranes are currently under extensive examination. Among them, the sorting of lipoproteins to the outer membrane of *Escherichia coli* has been clarified in detail. The Lol system, composed of five proteins, catalyzes outer membrane sorting of lipoproteins. Various Lpt proteins have recently been identified as factors involved in the transport of lipopolysaccharide to the outer membrane, although the mechanism remains largely unknown. Proteins with \( \alpha \)-helical membrane spanning segments are found in the inner membrane, whereas amphipathic \( \beta \)-barrel proteins span the outer membrane. These \( \beta \)-barrel proteins are inserted into the outer membranes through a central core protein BamA (YaeT) with the help of four outer membrane lipoproteins. In contrast, little is known about how phospholipids are transported to the outer membrane.

**Key words:** outer membranes; bacterial lipoproteins; ABC transporters; Lol system; sorting signals

Gram-negative bacteria such as *Escherichia coli* have four compartments: the cytoplasm, the inner (cytoplasmic) membrane, the periplasm, and the outer membrane. Both the inner and outer leaflets of the inner membrane are composed of phospholipids. In contrast, the outer membrane is an asymmetrical bilayer possessing lipopolysaccharide (LPS) exclusively in the outer leaflet and phospholipids mostly in the inner leaflet (Fig. 1).

The outer membrane functions as a permeability barrier to hydrophobic substances and contains several major protein species, including OmpA, OmpC, OmpF, LamB, and PhoE. These proteins span the membrane by forming a \( \beta \)-barrel structure with amphipathic \( \beta \)-strands, which possess alternating hydrophobic residues, whereas proteins spanning the inner membrane contain hydrophobic \( \alpha \)-helical stretches. E. coli possesses at least 90 species of lipoproteins anchored to the periplasmic side of either the inner or the outer membrane through acyl chains attached to their N-terminal Cys residues. Most lipoproteins are anchored to the outer membrane, while less than 10 of them are localized on the inner membrane. Neither \( \beta \)-barrel proteins in the outer membrane nor lipoproteins anchored to the outer membrane have hydrophobic stretches that function as a stop transfer or as signal anchor sequences. As explained below (section III), acyl chains are attached to the N-terminal Cys of lipoproteins on the outer surface of the inner membrane after translocation across the inner membrane. Thus both \( \beta \)-structure and lipid attachment are important means of generating outer membrane-associated proteins.

The mechanisms underlying the transport of four components, LPS, phospholipids, \( \beta \)-barrel proteins, and lipoproteins, to the outer membrane are currently one of the most important subjects in the field of microbial cell biology (Fig. 1).

**I. Protein Secretion Systems in Gram-Negative Bacteria**

Periplasmic and outer membrane proteins are synthesized as precursors with a signal peptide at the N-termini in the cytoplasm, and then are translocated across the inner membrane through a Sec translocon. The Tat (twin-arginine translocation) pathway translocates a certain number of respiratory components across the inner membrane. The Sec translocon cannot translocate folded proteins, while a folded structure is essential for translocation by the Tat system. Specific signal peptides are essential for translocation by these systems, but do not determine the final destinations of the translocated proteins. They are localized through distinct mechanisms to their final destinations, viz., the inner membrane, the periplasm, the outer membrane, or the outside of cells. The Sec and Tat systems are widely conserved in bacteria, and are involved in fundamental functions and in the biogenesis of the structures of the cell envelope. In addition to these fundamental systems, various systems secrete proteins to the outside of the cells. Pathogenic Gram-negative bacteria frequently utilize these protein secretion systems to inject proteins into host cells (Fig. 2).
A lipoprotein precursor with a consensus lipobox sequence around the signal peptide cleavage site is synthesized in the cytosol and translocated across the inner membrane. The precursor is then sequentially processed to the mature form on the periplasmic side of the inner membrane. The mechanism by which phospholipids are transported to the inner leaflet of the outer membrane remains to be clarified.

**Fig. 1.** Pathways for the Transport of Outer Membrane Components.

Sorting of the three major components to the outer membrane is depicted. A, LPS is transported from the inner to the outer leaflet of the inner membrane by an ABC transporter, MsbA, followed by transport to the outer membrane through unknown mechanisms involving an ABC transporter LptFGB complex, membrane protein LptC, periplasmic LptA, outer membrane protein LptD, and lipoprotein LptE. It is not yet clear whether LPS transport takes place through the periplasm, as indicated, or a postulated contact site connecting the inner and outer membranes (not shown). B, Lipoproteins are transported to the outer membrane by the Lol system, composed of an ABC transporter LolCDE complex, periplasmic chaperone LolA, and outer membrane receptor LolB, which is itself a lipoprotein. The structure and detailed functions of the Lol system have been extensively studied, as discussed in the text. C, β-barrel proteins are inserted into the outer membrane from the periplasm by an apparatus composed of BamA (YaeT) and the four indicated lipoproteins, BamB/C/D/E (formerly called YfgL, NlpB, YfiO, and SmpA respectively). Periplasmic chaperones, Skp, DegP, and SurA, are known to be involved in the formation of the folded structure of β-barrel proteins. Proteins with a membrane-spanning α-helix remain in the inner membrane. The mechanism by which phospholipids are transported to the inner leaflet of the outer membrane remains to be clarified.

**II. Roles of Lipoproteins in the Biogenesis of the Envelope Apparatus**

The complete genome sequence revealed many putative lipoproteins in various bacteria. One hundred and fourteen lipoproteins have been predicted for Gram-positive Bacillus subtilis,14) 105 for Lyme disease spirochete Borrelia burgdorferi,15) and more than 100 for E. coli.16,17) The presence of many lipoproteins in bacteria suggests that various membrane-associated activities are dependent on them but the majority of lipoproteins, including even those of E. coli, have no known function.

Most lipoproteins in E. coli appear to have their protein moieties exposed to the periplasm and to be involved in various biological activities in the cell envelope. Several lipoproteins have been reported to be involved in outer membrane sorting of β-barrel proteins, LPS, and lipoproteins (Fig. 1). Since some of these are essential for E. coli,3) they play critical roles in the biogenesis of outer membranes, which are essential for most bacteria. Lipoproteins involved in drug transport and in signal transduction are also known. Moreover, lipoproteins often comprise the protein secretion apparatus mentioned above. They are therefore an important class of envelope proteins.

More than 100 putative lipoprotein genes of E. coli have been cloned and examined as to whether they encode lipoproteins.3) These analyses revealed that E. coli possesses at least 90 species of lipoproteins. Disruption of each of the 90 lipoprotein genes revealed that two of them, BamA (YfiO)18) and LolB,19) are essential, as reported. BamA has been reported to be involved in β-barrel protein insertion,6) and LolB is an outer membrane component of the lipoprotein sorting Lol system.20) Disruption of many lipoprotein genes rendered the growth of cells temperature-sensitive or hypersensitive to various drugs.3)

**III. Biogenesis and Subcellular Localization of Lipoproteins**

A lipoprotein precursor with a consensus lipobox sequence around the signal peptide cleavage site is synthesized in the cytosol and translocated across the inner membrane.21) The precursor is then sequentially processed to the mature form on the periplasmic side of the inner membrane (Fig. 3). Step 1, Lgt forms a
fourth residue at position 2 is the inner membrane retention signal, and the residue at position 3 considerably affects outer membrane localization of lipoproteins. The five residues that are used throughout the discussion are Asp, five residues, Phe, Trp, Tyr, Gly, and Pro, at position 2.26) On the other hand, secretion of cellular proteins by the outer membrane vesicles is also called a two-partner system, in which the passenger domain and pore domain are present in separate polypeptides. Substrate proteins for a type-V system are translocated to the periplasm by the Sec or Tat translocon. Although not shown here, a type-VI system has been reported as a new secretion system.27) On the other hand, secretion of cellular proteins by the outer membrane vesicles is also called a two-partner system. The specificity of the cargo proteins is questionable in this outer membrane vesicle system.28) The definition of a type-VI secretion system also requires further study.

Lipoproteins in Gram-negative bacteria are localized to the mature region and diacylglycerol. Step 2, LspA cleaves the signal peptide. Step 3, Lnt catalyzes aminoaoylation of the N-terminal Cys residue. The mature lipoprotein thus formed has three acyl chains attached to its N-terminal Cys.4) Globomycin22) is a specific inhibitor of LspA (prolipoprotein signal peptidase), and Lnt (phospholipid:apolipoprotein transacylase). X represents a derivative of maltose binding protein (MalE) having an aminoacylated Cys residue at the N-terminus. In addition to these factors, Lol proteins have been systematically examined.29) Only Asp at position 2 is critical for the membrane specificity of lipoproteins in E. coli. This was first suggested by Yamaguchi et al.,20) who showed that replacement of Ser at position 2 of an outer membrane-specific lipoprotein by Asp caused mislocalization of the lipoprotein in the inner membrane. Moreover, an inner membrane-specific lipoprotein was localized to the outer membrane when Asp at position 2 was replaced by another residue. These results indicate that Asp at position 2 is the inner membrane retention signal, although the residue at position 3 considerably affects inner membrane retention by Asp at position 2.26) Lipoprotein sorting signals have been systematically examined as to an artificial lipoprotein, lipomMalE, a derivative of maltose binding protein (MalE) having an acylated Cys residue at the N-terminus. In addition to Asp, five residues, Phe, Trp, Tyr, Gly, and Pro, at position 2 were found to cause inner membrane localization of lipomMalE, which had Asn at position 3,23) but it should be noted that these five residues are not found at position 2 of native E. coli lipoproteins.

Although the details are discussed in the following section, LolA was found to be a key factor in the release of lipoproteins from spheroplasts.28) It was found that LolA is essential for the release of outer membrane-specific lipoproteins from spheroplasts, whereas inner membrane-specific ones remain in spheroplasts even in the presence of LolA. Taking advantage of this LolA-dependent release, the residues at positions 2 and 3 were systematically examined.29) Only Asp at position 2 caused retention of lipoproteins in the inner membrane when the residue at position 3 was Ser. The five residues

**Fig. 2.** Protein Secretion Systems.

Gram-negative bacteria, especially pathogenic ones, have developed various protein secretion systems, which transport effector (toxic) proteins into the extracellular milieu or directly into host cells. The type-I, type-III, and type-IV systems secrete proteins from the cytosol in one step. In contrast, the type-II and type-V systems secrete proteins in two steps. Secretion of α-haemolysin is performed by a type-I system in an ATP-dependent manner. A type-II system is driven by ATP and secretes proteins from the periplasm, to which substrate proteins are translocated by the Sec or Tat translocon. The biogenesis of a type-II system resembles that of type-IV pili.6) A type-III system has a needle-like structure and injects effector proteins into host cells. A type-IV system injects proteins and nucleic acids into host cells in an ATP-dependent manner. A type-V system comprises an N-terminal passenger domain and a C-terminal β-barrel domain, which serves as a pore for the passenger domain. The passenger domain is then auto-catalytically cleaved and secreted into the extracellular milieu. This system is therefore called an autotransporter. A variant of a type-V system is called a two-partner system, in which the passenger domain and pore domain are present in separate polypeptides. Substrate proteins for a type-V system are translocated to the periplasm by the Sec or Tat translocon. Although not shown here, a type-VI system has been reported as a new secretion system.27) On the other hand, secretion of cellular proteins by the outer membrane vesicles is also called a two-partner system. The specificity of the cargo proteins is questionable in this outer membrane vesicle system.28) The definition of a type-VI secretion system also requires further study.

**Fig. 3.** Processing of Lipoproteins.

Lipoproteins are synthesized as precursors with an N-terminal signal peptide (dotted box) that contain a consensus sequence called a lipobox, Leu-(Ala/Ser)-(Gly/Ala)-Cys, and are then translocated across the inner membrane by the Sec translocon. Processing of lipoprotein precursors to their mature forms is catalyzed by three well-conserved enzymes, Lgt (phosphatidylglycerol:prolipoprotein diacylglycerol transferase), LspA (prolipoprotein signal peptidase), and Lnt (phospholipid:apolipoprotein transacylase). X represents a residue other than Asp at position 2 of lipoproteins.

**IV. Sorting of E. coli Lipoproteins**

The species of the N-terminal second residue is critically important to the membrane specificity of lipoproteins in E. coli. This was first suggested by Yamaguchi et al.,20) who showed that replacement of Ser at position 2 of an outer membrane-specific lipoprotein by Asp caused mislocalization of the lipoprotein in the inner membrane. Moreover, an inner membrane-specific lipoprotein was localized to the outer membrane when Asp at position 2 was replaced by another residue. These results indicate that Asp at position 2 is the inner membrane retention signal, although the residue at position 3 considerably affects inner membrane retention by Asp at position 2.26) Lipoprotein sorting signals have been systematically examined as to an artificial lipoprotein, lipoMalE, a derivative of maltose binding protein (MalE) having an acylated Cys residue at the N-terminus. In addition to Asp, five residues, Phe, Trp, Tyr, Gly, and Pro, at position 2 were found to cause inner membrane localization of lipoMalE, which had Asn at position 3,23) but it should be noted that these five residues are not found at position 2 of native E. coli lipoproteins.

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localizing lipoMalE to the inner membrane were not inner membrane retention signals. The effects of different residues at position 3 on inner membrane retention of lipoproteins with Asp at position 2 were then examined. It was found that residues such as Asp, Glu, Asn, and Gln at position 3 substantially inhibited inner membrane retention by Asp at position 2. The native inner membrane-specific lipoproteins in *E. coli* have Asp at position 2 together with residues that enhance Asp-dependent retention at position 3. The role of Asp at position 2 is discussed in more detail in section VI below.

V. The Lol System

1. *LolA*

Various periplasmic and outer membrane proteins expressed in spheroplasts are secreted into the spheroplast medium via the Sec-dependent translocon after signal peptide cleavage. In marked contrast, outer membrane-specific lipoproteins, such as the major outer membrane lipoprotein Lpp are not secreted, remaining in the inner membrane of spheroplasts even though Lpp precursors have been completely processed to mature forms. However, when Lpp is expressed in the presence of concentrated periplasmic fraction, it is secreted into the medium, strongly indicating the presence of a periplasmic factor required for the release of mature lipoproteins from the inner membrane. The periplasmic factor LolA was thus identified after fractionation and purification of periplasmic materials. LolA also releases outer membrane lipoproteins such as Pal, BamC (formally NlpB), Sip, and RlpA. On the other hand, inner membrane-specific lipoproteins, such as AcrA and NlpA, remain in the spheroplasts even in the presence of LolA, indicating that the release is a critical step in lipoprotein sorting. One molecule each of LolA and a secreted lipoprotein have been found to form a water-soluble complex in the medium. Due to the N-terminal acyl chains, lipoproteins are highly hydrophobic, but become hydrophilic in complexes with LolA. In order to reach the outer membrane via the hydrophilic periplasm, the formation of a water-soluble LolA-lipoprotein complex is critical.

2. *LolB*

When the LolA-lipoprotein complex is incubated with the outer or inner membrane, the lipoprotein is transferred from LolA to the outer but not to the inner membrane, indicating that the outer membrane contains a factor catalyzing membrane anchoring of lipoproteins. The outer membrane was solubilized, fractionated, and reconstituted into proteoliposomes to identify this factor. LolB thus found is itself a lipoprotein anchored to the outer membrane. A LolB derivative, mLolB, lacking the N-terminal acyl chain was found to accept lipoproteins from LolA. The mLolB-lipoprotein complex thus formed represents the intermediate of the lipoprotein transfer reaction. LolA and mLolB therefore have common functions, viz., binding and transfer of lipoproteins, although their amino acid sequences are dissimilar.

3. *LolCDE*

LolA never releases lipoproteins anchored to the outer membrane. In contrast, LolA releases lipoproteins anchored to the inner membrane, suggesting that the inner membrane contains a factor involved in the release reaction. Indeed, the addition of LolA causes the release of lipoproteins anchored to right side-out membrane vesicles only when ATP is provided, suggesting that a novel ATPase in the inner membrane catalyzes lipoprotein release from the inner membrane. This ATPase was purified by monitoring the activity in reconstituted proteoliposomes. A complex composed of LolC, LolD, and LolE with a subunit stoichiometry of 1:2:1 was identified as this ATPase. This LolCDE complex was found to belong to the ABC transporter superfamily. LolC and LolE are membrane subunits each of which spans the membrane four times, while LolD is a nucleotide binding subunit that possesses Walker A and B motifs with an ABC signature motif. ABC transporters generally have more than 10 membrane spanning segments, while the LolCDE complex has only eight. Most ABC transporters catalyze the transmembrane movement of substrates, while the LolCDE complex catalyzes the release of lipoproteins from one side of the lipid bilayer. The LolCDE complex is thus quite different from most other ABC transporters.

Proteoliposomes reconstituted with LolCDE, *E. coli* phospholipids, and lipoproteins exhibit sorting signal-dependent release of lipoproteins in the presence of LolA. These results indicate that the sorting of lipoproteins from the inner to the outer membrane is catalyzed by the five Lol proteins (Fig. 4). All the Lol proteins are essential for *E. coli* growth. Translocation of lipoprotein precursors across the inner membrane and their processing to mature forms are not affected by inhibition of the function of LolCDE, LolA, or LolB. These results appear to indicate that the Sec translocon and the lipoprotein processing system do not exhibit functional coupling.

ABC transporters constitute a superfamily that is one of the largest protein families. Various eukaryotic ABC transporters are involved in drug export, while many bacterial ABC transporters mediate substrate import. The LolCDE complex is probably a novel version of an exporter, and its mechanism might be similar to that proposed based on the crystal structure of an ABC exporter, Sav1866, of *Staphylococcus aureus*. A number of ABC transporters are present in *E. coli*. It is noteworthy that the sorting of LPS to the outer membrane (Fig. 1) also involves ABC transporters, MsbA and the LptBFG complex. In addition to the LolCDE complex, these ABC transporters are essential for *E. coli*. Therefore, ABC transporters are important not only in substrate import but also in the biogenesis of the bacterial cell envelope.

4. Conservation of Lol proteins

Since Lol proteins are conserved in various Gram-negative bacteria, especially in *γ*-proteobacteria, the Lol pathway found in *E. coli* most likely functions as the major lipoprotein sorting system. However, the outer surface localization of *B. burgdorferi* lipoproteins occurs through an unknown mechanism. Moreover, LolB homologs are not found in some bacteria such
VI. Mechanisms of Lipoprotein Transfer by the Lol System

1. Lol avoidance signals

To determine why only Asp at position 2 functions as an inner membrane signal, lipoprotein release was examined in reconstituted proteoliposomes.43) The ATPase activity of LolCDE was stimulated by outer membrane-specific but not inner membrane-specific lipoproteins. Outer membrane-specific, not inner membrane-specific, lipoproteins inhibited the release of another outer membrane-specific lipoprotein. These results indicate that the inner membrane signal, Asp at position 2, acts as the LolCDE avoidance signal, preventing the recognition of lipoproteins by LolCDE and causing retention of lipoproteins in the inner membrane. It was further found that lipoproteins with Cys at position 2 are released from proteoliposomes even after the Cys residue is modified with SH-specific reagents, indicating that LolCDE does not recognize the residue at position 2.44) On the other hand, LolCDE does not recognize apolipoproteins lacking amino-linked acyl chains.45) Taken together, these results indicate that LolCDE recognizes only N-terminal Cys residues modified with three acyl chains, and that Asp at position 2 somehow inhibits the recognition of lipoproteins by LolCDE.

E. coli phospholipids are comprised of 70–75% PE, 20–25% PG, and about 5% CL. The Lol avoidance function of Asp at position 2 is significantly affected by the phospholipid composition in proteoliposomes.44,46) It is completely abolished in proteoliposomes reconstituted with phosphatidylcholine alone. In contrast, PE was found to be critically important to the Lol avoidance function. E. coli can grow in the absence of PE, a non-bilayer phospholipid, if a high level of magnesium is added to the medium.47) The sorting of lipoproteins is completely normal in cells growing under PE-deficient conditions.46) It has been speculated that CL forms a non-bilayer structure in the presence of high magnesium levels.48) The release of lipoproteins from CL-proteoliposomes is significantly stimulated by magnesium. However, not only outer membrane-specific but also inner membrane-specific lipoproteins are efficiently released from CL-proteoliposomes in the presence of a high magnesium level.49) The release of lipoproteins from proteoliposomes reconstituted with PG and CL decreases with increases in the amount of PG. When the PG content is more than 50%, the release of inner membrane-specific lipoprotein is preferentially inhibited. As a result, the release of lipoproteins becomes sorting signal-dependent in proteoliposomes reconstituted with 75% PG and 25% CL.46) Taken together, these results suggest that phospholipids have diverse effects on the release of lipoproteins. An electrostatic interaction between the positive charge of PE and the negative charge of Asp is important to the Lol avoidance function.44) Moreover, acidic phospholipid PG appears to be important for correct functioning of the LolCDE complex.

As mentioned above, lipoprotein sorting signals function at the release step catalyzed by the LolCDE complex. A LolC(A40P) mutant carrying an Ala to Pro mutation in the first transmembrane segment causes the release and mislocalization of a portion of the lipoprotein with the strong inner membrane signal, Asp at position 2 with Gln at position 3, to the outer membrane,49) indicating that both LolA and LolB interact with lipoproteins possessing inner membrane retention signals once they are released by mutant LolCDE.

2. Structures of LolA and LolB with hydrophobic cavities

Consistently with their analogous functions, the crystal structures of LolA and LolB were found to be very similar to each other (see Fig. 6) but this was unexpected, because their amino acid sequences are dissimilar. The unique structures of LolA and LolB represent a novel fold, comprising 11 antiparallel β-strands folded into an incomplete β-barrel, and two loops covering the barrel.50) The barrel and the loops containing three α-helices form a hydrophobic cavity inside. Although the overall structures are very similar, the following two structures are characteristic only of LolA: (i) LolA has an extra C-terminal loop containing β-strand 12, and (ii) the hydrophobic cavity of LolA is closed by hydrogen bonding between Arg at position 43 located in the β-barrel and residues in the loops. The C-terminal loop of LolA is important to prevent re-localization of lipoproteins to the inner membrane after their release.51) In addition to closure of the hydrophobic cavity, Arg at position 43 plays an important role in the efficient transfer of lipoproteins to LolB. Replacement of Arg by Leu causes almost complete inhibition of lipoprotein transfer to LolB, whereas this derivative, LolA(R43L), is active in the lipoprotein binding,52) causing an accumulation of the LolA(R43L)-lipoprotein complex in the periplasm. Various outer membrane lipoproteins are found associated with LolA(R43L). To understand more about the role of Arg at position 43, this residue was mutated to other residues.53) Many mutants caused a periplasmic accumulation of lipoproteins. The extent of lipoprotein accumulation varied depending on the species of residue, and LolA(R43L) accumulated the highest amounts of lipoproteins. The strength of the hydrophobic interaction between LolA mutants and lipoproteins can be determined from the resistance to a detergent, DDM. It was found that the LolA(R43L)-lipoprotein complex is the most resistant to DDM. The hydrophobic interaction of this complex is as strong as that of the mLoIB-lipoprotein complex, causing an inhibition of lipoprotein transfer to mLoIB.53) These results indicate that Arg at position 43 plays a critical role in the efficient transfer of lipoproteins by decreasing the strength of the hydrophobic interaction with lipoproteins.

The crystal structure of LolA(R43L) revealed that Arg 43 is indeed important to close the hydrophobic cavity. Two crystal forms of LolA(R43L) were obtained.54) One represented a closed form that was essentially identical to the structure of free wild-type LolA. The other one
Fig. 4. Lipoprotein Transfer by the Lol System.

LolCDE, LolA, and LolB cycle between liganded and free forms. ATP is required only for the transfer of lipoproteins from liganded LolCDE to free LolA. Lipoprotein transfer from liganded LolA to free LolB and from liganded LolB to the outer membrane is driven by differences in affinity for lipoproteins. Lipoprotein transfer is one-directional, from the inner to the outer membrane, and is very efficient. Thus liganded Lol proteins are not detectable under normal conditions.

Fig. 5. Detailed Molecular Events Involved in Lipoprotein Release from the Inner Membrane.

The scheme indicated was based on a single cycle of the lipoprotein transfer reaction examined in vitro with the liganded LolCDE complex. The release reaction starts with the recognition of outer membrane-specific lipoproteins by LolCDE. Formation of the LolCDE-lipoprotein complex increases the affinity of LolD for ATP (step 1). ATP binding to LolD causes a conformational change in LolCDE and decreases the strength of the hydrophobic interaction between LolCDE and lipoproteins (step 2). ATP hydrolysis presumably further weakens the hydrophobic interaction of the LolCDE-lipoprotein complex, but lipoproteins remain associated with LolCDE until LolA is added (step 3). The hydrophobic cavity of LolA opens on the binding of lipoproteins (step 4). Vanadate is an inorganic phosphate analog that inhibits the release of ADP from LolD but does not inhibit the transfer of lipoproteins from LolCDE to LolA, indicating that the conformational change in LolA is induced even by vanadate-trapped LolCDE. Release of inorganic phosphate and ADP leads to the original conformation of LolCDE (step 5). Mutations K48M and E171Q in LolD inhibit the steps of the reaction specified. The interaction between LolCDE and lipoproteins is resistant to 1% DDM before but not after ATP-binding (step 2).

represented a major and open form, in which the loop covering the hydrophobic cavity had moved outward by 5–6 Å. A fluorescent probe, bis-ANS, shows an increase in fluorescence when it binds to a hydrophobic environment. Bis-ANS fluorescence significantly increases in the presence of the LolA(R43L) derivative, whereas free wild-type LolA has little effect on this fluorescence, indicating that bis-ANS is accessible to the hydrophobic cavity of LolA(R43L) but not to that of wild-type LolA. The LolA(R43L)-lipoprotein complex is not formed when these two proteins are mixed, however, suggesting that the LolCDE function is required for complex formation even when the hydrophobic cavity of LolA(R43L) is open.

The LolA-lipoprotein complex is an intermediate of the lipoprotein transfer reaction but is not detectable in the periplasm under normal conditions because lipoproteins are immediately transferred from LolA to LolB. It was not possible to prepare a large amount of the LolA-lipoprotein complex until recently. It was found that co-overexpression of LolA and lipoprotein in the absence of the LolB function causes the accumulation of a significant amount of the LolA-lipoprotein complex in the periplasm. The LolA-Pal complex thus purified causes an increase in bis-ANS fluorescence to essentially the same level that LolA(R43L) does. Furthermore, free LolA generated from the LolA-Pal complex does not increase bis-ANS fluorescence, indicating that the hydrophobic cavity of LolA undergoes opening and closing on the binding and release of lipoproteins.

3. Single cycle of lipoprotein transfer from LolCDE to LolA

Detergents significantly affect the stability of the LolCDE complex after solubilization from membranes.
The complex disintegrates into subunits with octylglucoside. LolCDE is stable with sucrose monocaprate only when ATP or phospholipids are present. On the other hand, DDM can stably solubilize the LolCDE complex not only in the presence but also in the absence of ATP. The LolCDE complex solubilized with 1% DDM and purified in the presence of 0.01% DDM throughout in the absence of ATP was found to contain various outer membrane-specific, but not inner membrane-specific, lipoproteins. This liganded LolCDE was found to be an intermediate of the lipoprotein release reaction in the inner membrane. Homogenous LolCDE-Pal complex was prepared from cells overproducing both LolCDE and Pal. The purified LolCDE-Pal complex revealed that one molecule of Pal binds to one molecule of the LolCDE complex. Liganded LolCDE exhibited higher affinity for ATP than free LolCDE, whereas the maximum ATPase activity was similar for free and liganded LolCDE. The reason liganded LolCDE was not obtained in the presence of ATP was examined. Liganded LolCDE was incubated with various concentrations of DDM with and without ATP. When the DDM concentration was 0.01%, slightly higher than its critical micellar concentration (0.0087%), LolCDE remained liganded whether ATP was present or absent. On the other hand, ATP caused dissociation of lipoproteins from LolCDE in the presence of 1% DDM. ATP binding, but not hydrolysis, was required for this lipoprotein dissociation. Taken together, these results indicate that ATP binding to LolD weakens the hydrophobic interaction between LolCDE and lipoproteins and therefore causes the dissociation of lipoproteins from LolCDE in the presence of 1% DDM. It has been found by crystallographic study that ATP binding to the nucleotide binding subunits of ABC transporters induces an outward facing conformation and that ATP hydrolysis induces an inward facing conformation. The hydrophobic interaction between membrane subunits, LolC and/or LolE, and lipoproteins is likely to be weakened by the binding of ATP to the LolD subunit.

The opening of the hydrophobic cavity of LolA requires the LolCDE function and is coupled to the transfer of lipoproteins. As discussed above, ATP does not dissociate lipoproteins from LolCDE with 0.01% DDM. In marked contrast, ATP added in the presence of 0.01% DDM was found to cause lipoprotein transfer to LolA with 0.01% DDM. ATP hydrolysis is essential for this lipoprotein transfer reaction, indicating that it mimics a single cycle of the lipoprotein transfer reaction in vivo. Vanadate, an inorganic phosphate analog, inhibits many ABC transporters by stabilizing the ADP bound form of nucleotide binding subunits. Vanadate strongly inhibits the lipoprotein release reaction reconstituted in proteoliposomes, but not a single cycle of the lipoprotein transfer reaction. Moreover, addition of LolA induces the lipoprotein transfer reaction even after liganded LolCDE is completely inhibited by vanadate in the presence of ATP. Lipoprotein transfer to LolA accompanies the opening of the LolA hydrophobic cavity, as discussed above. Taking these results together, the molecular events in the initial lipoprotein transfer reaction are speculated to be as shown in Fig. 5.

VII. Problems to Be Solved

Recently we have learned about many aspects of the mechanisms underlying the biogenesis and maintenance of outer membranes but a number of problems remain unsolved. The Lol system is the paradigm of the transport of hydrophobic components to the outer membrane of Gram-negative bacteria. Moreover, LppX and the N-terminal domain of RseB, RseB(N), are known to have structures very similar to those of LolA and LolB (Fig. 6). These proteins are likely to be involved in the binding and/or transport of hydrophobic ligands on the outside of the cytoplasmic membrane. RseB constitutes an envelope stress response system leading to expression of the outer membrane of unfolded proteins. It is likely that the hydrophobic cavity of RseB(N) binds unfolded lipoproteins, which is critical to activation of the periplasmic quality-control mechanism. LppX is a lipoprotein presumably involved in the transport of complex lipids to the outer layers of the Gram-positive bacterium Mycobacterium tuberculosis. Further proteins having the LolA/LolB fold might exist in the periplasm and play important roles in the maintenance of the integrity of the bacterial cell envelope structure.

Periplasmic LptA, which is involved in the transport of LPS, appears to exhibit functional but not structural similarity with LolA. The mechanisms by which phospholipids are transported to the outer membrane remain to be clarified. So far as examined, the Lol system is unlikely to be involved in the transport of phospholipids to the outer membrane. MsbA was once reported to transport phospholipids to outer membranes but this is unlikely, as has been reported. An apparatus composed of BamA (YaeT) and four lipoproteins, BamB/C/D/E, has been found to be involved in the insertion of β-barrel proteins into outer membranes, although the details of the mechanisms are unknown.

Among the mechanisms underlying the transport of outer membrane components, the mechanism of the Lol system is most extensively clarified. However, it is not completely clear how phospholipid composition affects the Lol avoidance function of Asp at position 2. It was reported that the electrostatic interaction between Asp and PE is critical but the exact role of PG in this signal recognition remains unknown. The Lol system is likely to be the major system in the sorting of lipoproteins in Gram-negative bacteria. Some bacteria have lipoproteins on the outer surface of their outer membranes. This is catalyzed by an unknown mechanism other than the Lol system. Many inner membrane-specific lipoproteins without Asp at position 2 are found in bacteria other than E. coli. The inner membrane signals identified in Pseudomonas aeruginosa are diverse and do not immediately indicate a rule. It is also unclear why Asp at position 2 can function as an inner membrane signal but is not used for many inner membrane lipoproteins in P. aeruginosa. Finally, many lipoproteins still have no known function even in E. coli. Since lipoproteins are involved in various envelope activities, their structures and functions must be clarified in order to understand how bacterial envelopes are formed and maintained.
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