Sugar-Coating Bacteria with Lipopolysaccharides

Bacterial lipopolysaccharide assembly requires more than 50 gene products, including several recently identified factors

Andrew C. McCandlish and Thomas J. Silhavy

Gram-negative bacteria produce lipopolysaccharide (LPS), the endotoxin that causes septic shock and is a leading cause of death in critically ill patients. Because LPS is the predominant surface-exposed lipid in Gram-negative bacteria, it is one of the first compounds to interact with host cells. Once LPS binds the Toll-like receptor-4 (TLR4) complex on mammalian host endothelial cells, a signaling cascade stimulates the innate immune response, recruiting macrophage cells and releasing cytokines at the site of infection, and also starting the adaptive immune response. Although these responses generally benefit the host, a high bacterial load may lead to severe inflammation, vasodilation, and potentially fatal septic shock.

For the bacteria, LPS is the major lipid molecular species in the outer leaflet of the outer membrane (OM). LPS molecules interact with one another, forming a tight matrix along the surface of the cell, preventing free diffusion of compounds to and from the environment. Owing to this barrier function, LPS is essential for viability for nearly all gram-negative bacteria. Because the bacterial outer membrane is assembled without an obvious biochemical energy source (the periplasm is devoid of adenosine triphosphate, ATP), understanding LPS biogenesis has proved a fascinating problem for us and other bacterial cell biologists. Also spurring us on is the possibility that, by better understanding the factors involved in assembling the LPS matrix, we will identify novel targets for antimicrobial therapies.

Summary

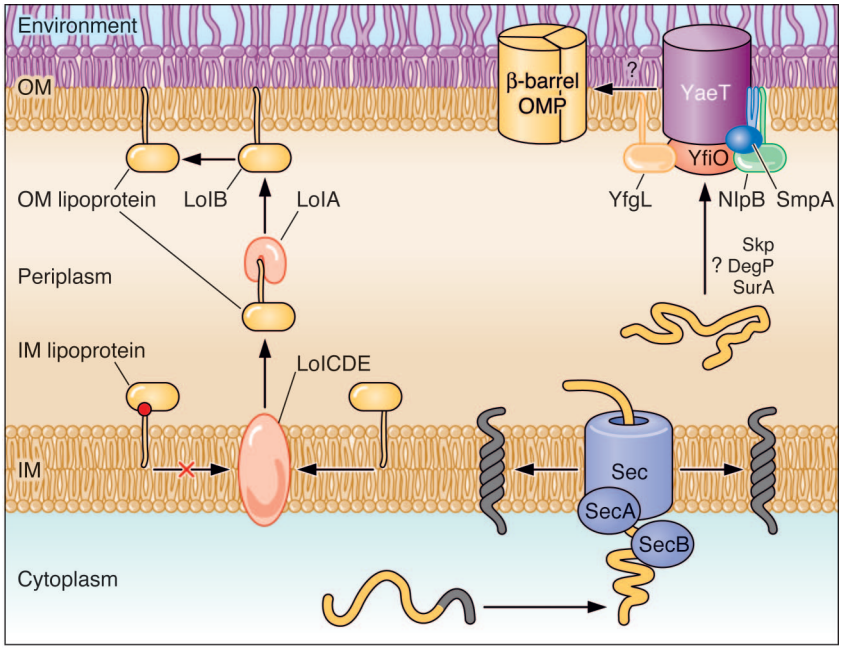
- Lipopolysaccharide (LPS) molecules interact in the outer membrane (OM) of gram-negative bacteria, preventing free diffusion of compounds from and into such cells.
- The SecYEG complex translocates both β-barrel proteins and lipoproteins across the inner membrane (IM) from the bacterial cytoplasm into the periplasm, where such proteins are shuttled to separate OM assembly sites.
- Either soluble proteins within the periplasm shuttle LPS from the IM to a docking site at the OM, or LPS travels through points of contact between the IM and OM.
- LPS is assembled at the cell surface either by a two-step process in which such molecules are first delivered to the inner leaflet of the OM and then flipped to the outer leaflet, or by a concerted process in which LPS molecules are inserted directly into the outer leaflet.

Challenges Facing Bacteria in Assembling Outer Membrane Components

Gram-negative bacteria such as *Escherichia coli* are surrounded by two membrane bilayers. The OM, which serves as a permeability barrier to the outside milieu, is asymmetric. The characteristic lipid species of the outer leaflet of the OM is almost exclusively LPS, whereas the inner leaflet of the OM and both leaflets of the inner membrane (IM) of such cells are composed of phospholipids. The OM also contains lipoproteins, β-barrel proteins such as porins which allow selective passage of small, water-solu-

Andrew C. McCandlish is a recent Ph.D. graduate of the Silhavy Lab and is currently the Manager of Strategic Planning and Business Development at Flexible Medical Systems, Princeton, N.J., and Thomas J. Silhavy is the Warner Lambert-Parke Davis Professor of Molecular Biology, Princeton University, Princeton, N.J.

FIGURE 1



Lipoprotein and β-barrel protein (OMP) biogenesis. Lipoproteins and OMPs are synthesized in the cytoplasm with an amino-terminal signal peptide. SecA and SecB bind the nascent protein and direct it to the Sec machinery where it is translocated across the inner membrane. The signal sequence is cleaved by signal peptidases. Lipoproteins are modified by the addition of lipids which anchor them in the membrane. LolCDE facilitates the release of OM lipoproteins from the IM, while IM lipoproteins have a "Lol-avoidance" signal and thus remain in the IM. LolA shuttles the lipoprotein across the periplasm to LolB where it is inserted into the inner leaflet of the OM. Several periplasmic factors are known to be involved OMP biogenesis, but their precise role (ie. as a shuttle analogous to LolA) is unclear. A complex consisting of the essential OMP YaeT and four associated lipoproteins facilitates OMP insertion into the OM.

ble molecules in and out of the cell, and organelles such as pili. Immediately inside the OM is the periplasm, an aqueous compartment that is densely packed with proteins, including binding proteins that function in nutrient transport and degradative enzymes such as alkaline phosphatase.

The inner membrane of gram-negative bacteria is symmetric and, in addition to phospholipids, contains proteins involved in many functions, including energy production, active transport, and signal transduction. The IM separates the periplasm from the innermost compartment, the cytoplasm. The cytoplasm contains the *E. coli* chromosome and is where most cellular components are synthesized. Molecules destined for the OM must cross the IM and periplasm before inserting into the OM in their appropriate conformations. During the final stages of this process, they do so without an obvious source of biochemical energy such as ATP.

How Proteins Move to the Bacterial OM

Proteins destined for the outer membrane (OMPs) are synthesized in the cytoplasm with a short amino-terminal signal sequence containing about 25 amino acids (Fig. 1). Specialized proteins, designated SecA and SecB, bind these precursor proteins and direct them to the SecYEG channel in the inner membrane, through which they cross the IM, ultimately ending up in the periplasm. During this energy-dependent process, a peptidase along the periplasmic face of the IM cleaves the signal sequence on each translocated OM protein. Lipoproteins contain lipid moieties that anchor them in the membrane after the signal sequence is removed.

Lipoproteins that are destined for the OM, such as Braun's lipopoprotein (LPP), interact with an IM complex, designated LolCDE, that uses energy derived from ATP molecules to extract the lipoprotein from the IM. IM-destined lipoproteins have a "Lol-avoidance" signal (typically, an aspartate residue at the +2 position) that prevents them from interacting with LolCDE, thereby retaining them in the IM. The periplasmic chaperone protein LolA picks up OM-bound lipoproteins from LolCDE and shuttles them across the periplasm to an essential OM lipoprotein, LolB, where they are inserted into the inner leaflet of the OM.

Signal peptidase releases β-barrel OM proteins into the periplasm, where chaperone proteins such as SurA, Skp, and DegP prevent their misfolding and aggregating while delivering them to the OM. Recently, our group identified an essential OM complex that is required for assembling outer membrane proteins. This complex contains the β-barrel protein YaeT and four lipoproteins.

A common theme emerges from our knowledge of β-barrel and lipoprotein targeting and assembly in *E. coli*. In both pathways, the SecYEG complex translocates such proteins from

the cytoplasm. Following release from the IM, periplasmic proteins (LolA in the case of lipoproteins and either SurA, Skp, or DegP for β-barrel proteins), shuttle the OM-destined proteins across the periplasm to an OM assembly site, which is LolB for lipoproteins and the YaeT complex for β-barrel proteins, where they are inserted into the OM. Thus, although many details remain to be elucidated, we have some conception of how proteins move from the bacterial cytoplasm to the OM.

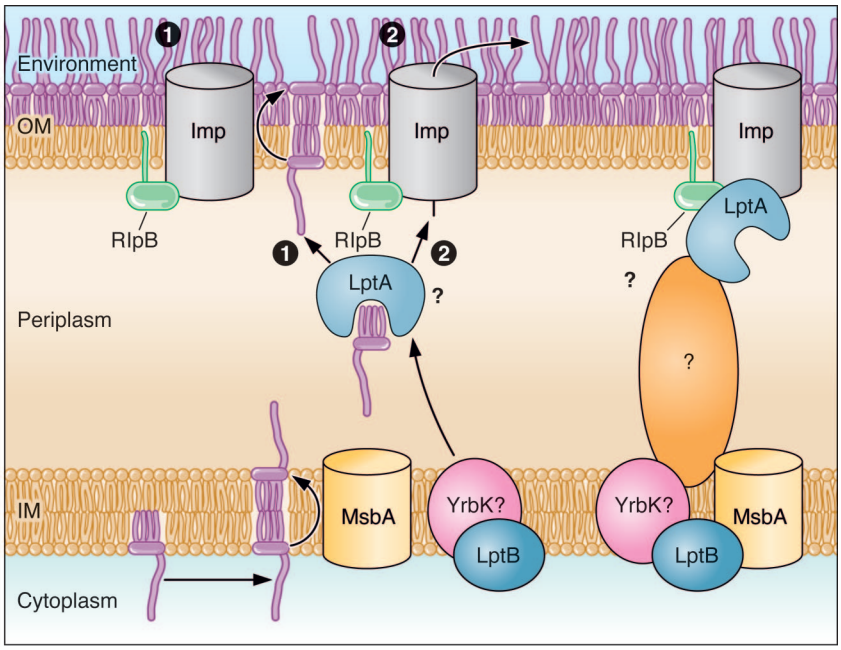
Two Competing Models To Explain How LPS Moves to the OM

Our understanding of how LPS molecules move into place in the OM is less well developed. LPS molecules consist of three distinct domains: lipid A, core oligosaccharide, and O-antigen. Though the precise structures of these domains vary, there are common features to each. For example, lipid A, which remains anchored inside the OM, generally consists of two diacylglucosamine residues that typically are substituted with six hydrocarbon chains. Those two diacylglucosamines link to residues of 3-deoxy-D-manno-oct-2-ulosonic acid (KDO) to serve as a scaffold, upon which the highly variable core oligosaccharide domain assembles.

There are at least five different conserved core structures in *E. coli* alone. In *E. coli* K-12, for example, the core region contains four heptose, three glucose, and one galactose residue. Additional sugars are added to form the O-antigen, a hypervariable polysaccharide chain. Because laboratory strains of *E. coli* have lost their capacity to synthesize the O-antigen, we use the term LPS to refer to the lipid A-core motif unless otherwise noted.

Most steps in assembling the lipid A-core LPS structure occur at the inner face of the IM, presenting LPS with the same challenge as OM proteins in reaching the OM. Thus LPS somehow must flip from the inner to the outer side of the IM, cross the periplasmic space, and insert from the inner into the outer side of the OM. Is the pathway for LPS biogenesis analogous to that of proteins? There are differing schools of thought on some of these details.

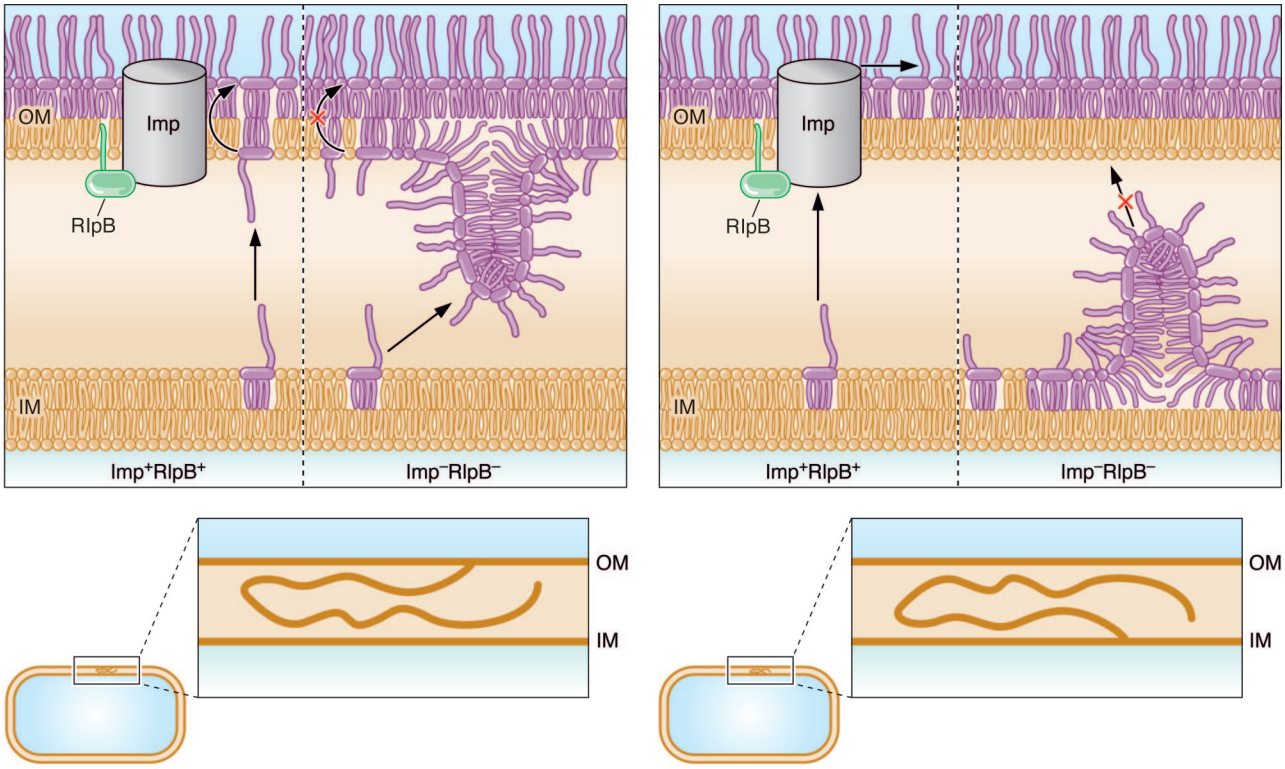
FIGURE 2



Current model of LPS biogenesis. LPS is synthesized on the inner face of the IM then flipped to the outer leaflet of the IM by MsbA. As the IM-associated protein LptB and the periplasmic protein LptA are known to be involved in LPS biogenesis, it is assumed they play a role in shuttling LPS from the IM to the OM, but their precise function remains unknown. The gene encoding YrbK is located immediately upstream of the *lptAB* operon, suggesting a possible role in LPS assembly, but its function is unknown. The Imp/RlpB complex resides in the OM and targets LPS to its final destination in the outer leaflet of the OM. Though the precise mechanism of Imp/RlpB is not known, two possibilities are depicted here. In one case, LPS is inserted into the inner leaflet of the OM then flipped to the outer leaflet. In the other case, LPS is received from the periplasm and inserted directly into the outer leaflet. Either function could be performed by Imp and/or RlpB. Several lines of evidence suggest that LPS travels through zones of adhesion between the IM and OM. The factors involved in LPS biogenesis could be independent entities associated with a larger lipid or protein "Bayer's Bridge," or they could form a periplasm-spanning complex as shown on the right.

Researchers tend to agree on the early steps of this process. For instance, following synthesis, LPS is flipped to the outer leaflet of the IM in an ATP-dependent step mediated by the essential IM protein, MsbA (Fig. 2). MsbA is a member of the ABC-transporter family of proteins and shares homology with mammalian multidrug transporters. In *E. coli* strains containing temperature-sensitive *msbA* alleles, LPS accumulates in the inner leaflet of the IM at nonpermissive temperatures, appearing in electron micrographs as invaginations of membranous material into the cytoplasm. Such *msbA* mutants also display altered membrane density, while LPS can be isolated in IM fractions using biochemical methods.

FIGURE 3



Models for Imp/RlpB function. If Imp/RlpB flips LPS from the inner leaflet of the OM to the outer leaflet, then depleting Imp/RlpB will cause accumulation of LPS in the OM (left panel). If Imp/RlpB receives LPS and inserts it directly into its final destination at the cell surface, then LPS will accumulate in the IM upon Imp/RlpB depletion (at the last completed step in biogenesis, right panel). Upon Imp/RlpB depletion, "extra" membrane material accumulates in the periplasm as visualized in electron micrographs. When MsbA function is disrupted, LPS accumulates in the inner leaflet of the IM, and membranes invaginate into the cytoplasm. Thus, by determining whether the "extra" membranes originate from the OM or the IM in Imp/RlpB depleted cells, we could differentiate between the proposed models of Imp/RlpB action. The bottom panels depict this strategy diagrammatically.

However, there are two competing models for how LPS is transported from the IM to the OM. According to one, LPS travels through points of contact between the IM and OM (Fig. 2, right). The validity of such zones of adhesion, which were identified by Manfred Bayer about 40 years ago and came to be called Bayer's bridges, remains controversial. They appear at several points along the envelope, where the IM and OM appear to contact or come very close to one another—at least, when depicted in thin-section electron micrographs. However, some researchers continue to argue that these contact sites are artifacts that disappear when cryofixation techniques are used instead of chemical fixation methods to visualize bacterial cellular structures, an idea first suggested by the late Eduard Kellenberger.

In any case, newly synthesized LPS appears at discrete points along the bacterial cell surface. When radiolabeling is used, LPS appears transiently in membranous fractions of novel density, according to Larry Rothfield of the University of Connecticut Health Center in Farmington, Conn., and his collaborators, and these may be the adhesion zones seen in electron micrographs. Moreover, according to Jan Tommassen at the University of Utrecht in Utrecht, the Netherlands, and his collaborators, radiolabeled LPS reaches the OM even when ordinary cells are converted into spheroplasts, draining those cells of their periplasmic contents. Adhesion zones could be what provides the structural continuity in spheroplasts that enables LPS to move between the IM and OM.

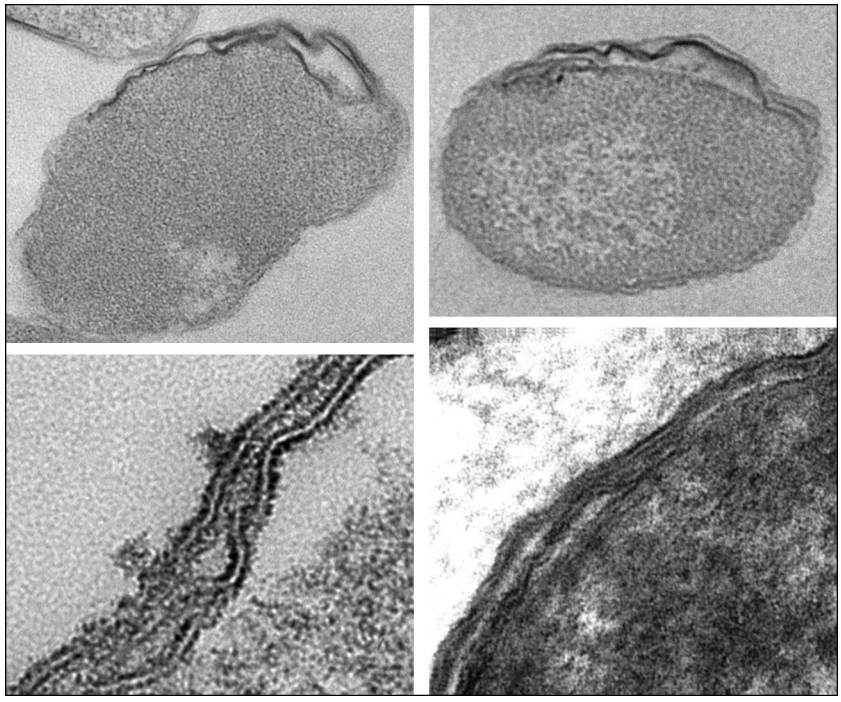
Meanwhile, according to the alternative

model, soluble proteins within the periplasm shuttle LPS from MsbA in the IM to a docking site along the OM (Fig. 2, left). This model closely parallels what we know about the targeting of β-barrel proteins and lipoproteins to the OM. Further and also consistent with this view, two genes, *lptB* and *lptA*, apparently play key roles in a periplasmic intermediate-based pathway for transporting LPS to the OM, according to Alessandra Polissi of Università degli Studi in Milano, Italy, and her collaborators.

Through a large-scale genomic mutagenesis approach, the Italian researchers determined that these two genes, which are located near KDO synthesis genes along the *E. coli* chromosome, are essential for *E. coli* viability. LptB protein appears to be a member of the ABC-transporter family, while LptA is periplasmic, according to Polissi and her collaborators. Cells depleted of either gene product cannot properly assemble LPS. In particular, these strains accumulate misassembled LPS in the IM.

These two models may not be mutually exclusive. Periplasmic factors may transport LPS across the periplasm while still requiring zones of adhesion between the inner and outer membranes. For instance, Bayer's Bridges might be periplasm-spanning proteins that act as a scaffold for soluble periplasmic shuttles.

FIGURE 4



Electron micrographs of Imp- and RlpB-depleted cells. The top panels show the "extra" membrane material in the periplasm of cells depleted of Imp or RlpB. These cells have higher levels of LPS than wild-type cells. Presumably the "extra" membranes originate at the site of LPS accumulation as described in Fig. 3; that is, there is such a high level of local LPS accumulation in the OM or IM that LPS-rich membranes are protruding into the periplasm from the site of accumulation. The bottom panels present high-resolution electron micrographs initiated to determine whether the membrane protrusions originate from the IM or the OM. Although discontinuities are apparent at some points along the IM and OM which might suggest the novel membranes are protruding from these points, this method does not support visualization at high enough resolution to determine where LPS is accumulating definitively.

LPS Assembly in the OM

We recently identified two factors that are involved in assembling LPS at the cell surface—namely, the β-barrel protein Imp and the lipoprotein RlpB. The *imp* gene was first identified in a screen for genes that allow *E. coli* strains lacking maltoporin (the *lamB* gene product) to use maltodextrins as a carbon source. The first *imp* mutants were in-frame deletions that disrupt the barrier function of the OM (hence, the term *imp*, for increased membrane permeability). These mutants are used in the pharmaceutical industry to test the intracellular effectiveness of novel compounds with antibacterial activity, and thus allow the postponement of efforts to deal with permeability problems that those candidate antibiotics might have.

Imp is a large OMP with β-barrel topology. Using a strain in which Imp could be depleted, we found that the protein is essential for *E. coli* viability. To study Imp further, we radiolabeled proteins and lipids after the mutants were depleted of Imp, and then we collected membrane fractions by sucrose density gradient centrifugation. Almost all proteins and lipids that are made when Imp is depleted accumulate in a fraction that is denser than is the wild-type OM. This result implicates Imp in OM biogenesis without, however, revealing its function.

LPS is essential for almost all gram-negative bacteria, but not *Neisseria meningitidis*. Similarly, *imp* is not essential in this bacterium.

Taking advantage of this fact, Tommassen's group created a mutant strain that is lacking *imp*. This strain has very low levels of LPS, and what little LPS remains in the cell does not reach the cell surface and is unavailable to an enzyme that is active on LPS in the OM.

Using an affinity-tagged version of Imp, we showed that it interacts with the lipoprotein RlpB in *E. coli*. Reciprocal purifications using affinity-tagged RlpB confirm this interaction, suggesting that Imp and RlpB interact in vivo.

Although RlpB was previously known only as a low-abundance lipoprotein in *E. coli* (RlpB stands for rare lipoprotein B), we proved that it is essential for viability and that it localizes to the OM. To show that newly synthesized LPS never reaches the OM in Imp- or RlpB-depleted cells, we used PagP, an enzyme that modifies LPS in the OM by converting the hexa-acyl form of lipid A to the hepta-acyl form.

In cells depleted of Imp or RlpB, PagP can modify LPS that is synthesized before depletion, indicating that LPS is present in the outer leaflet of the OM. In contrast, PagP does not modify LPS synthesized after Imp or RlpB depletion, indicating that those LPS molecules never reached the outer leaflet. Because Imp targeting and folding appear unaltered in RlpB-depleted cells, the defects observed appear to be due to the role RlpB plays in LPS biogenesis.

The Mechanism of Imp/RlpB Action

We think that LPS reaches the outer leaflet of the OM either by a one- or two-step process. In the two-step hypothesis, LPS is inserted into the inner leaflet first, and then flipped to the outer leaflet. Imp and RlpB could each be the OM docking site, the inner-leaflet insertase, or the flippase, and they could perform these functions together or independently. In the one-step scenario, LPS is inserted directly into the outer leaflet. Here, Imp and RlpB would act together to receive LPS at the inner face of the OM, transport it across the OM, and release it into the outer leaflet. If depleting either Imp or RlpB blocks LPS delivery to the OM (i.e., LPS is never inserted into the inner leaflet if the two-step hypothesis is correct and never inserted into the outer leaflet in the one-step model), we would expect LPS to accumulate in the IM because it is energetically unfavorable for LPS to exist as a soluble entity in the periplasm. Alternatively, if depleting Imp or RlpB abrogates the hypothetical flipping function, LPS would accumulate in the inner leaflet of the OM (Fig. 3).

In *Neisseria meningitidis*, Imp null mutants have severely reduced LPS levels. However, the opposite is true in *E. coli*. Tracking immunoblots with an antibody that recognizes LPS, LPS levels increase as Imp and RlpB decrease. Consistent with this observation, electron micrographs reveal that membrane material accumulates in such cells (Fig. 4). These pictures look strikingly similar to those of *ftsH* mutants in which LPS levels increase.

In our electron micrographs of Imp- and RlpB-depleted cells, the extra membranes that we observe appear in the periplasm (Fig. 4). However, they could not be visualized at high-enough resolution to determine whether they originated from the IM or OM. Accordingly, we do not know whether LPS is accumulating in the IM or OM in these strains. Thus, while we know that the Imp/RlpB complex is required to assemble LPS at the cell surface, we still need to determine whether one or both of these proteins also functions as a delivery site for LPS in the OM.

SUGGESTED READING

Bayer, M. E. 1968. Areas of adhesion between wall and membrane of *Escherichia coli*. J. Gen. Microbiol. **53:**395–404.

Braun, M., and T. J. Silhavy. 2002. Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. Mol. Microbiol. **45:**1289–1302.

Narita, S., S. Matsuyama, and H. Tokuda. 2004. Lipoprotein trafficking in *Escherichia coli*. Arch. Microbiol. **182:**1–6.

Nikaido, H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol. Mol. Biol. Rev. **67:**593–656.

Ruiz, N., D. Kahne, and T. J. Silhavy. 2006. Advances in understanding bacterial outer-membrane biogenesis. Nature Rev. Microbiol. **4:**57–66.

Raetz, C. R., and C. Whitfield. 2002. Lipopolysaccharide biogenesis. Annu. Rev. Biochem. **71:**635–700.

Sperandeo, P., R. Cescutti, R. Villa, C. Di Benedetto, D. Candia, G. Deho, and A. Polissi. 2006. Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. J. Bacteriol. **189:**244–253.

Tefsen, B., J. Geurtsen, F. Beckers, J. Tommassen, and H. de Cock. 2005. Lipopolysaccharide transport to the bacterial outer membrane in spheroplasts. J. Biol. Chem. **280:** 4504–4509.
Wu, T., A. C. McCandlish, L. S. Groenberger, C. C. Chng, T. J. Silhavy, and D. Kahne. 2006. Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **103:**11754-**11759.**