



Lipid trafficking across the Gram-negative cell envelope

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The outer membrane (OM) of Gram-negative bacteria exhibits its unique lipid asymmetry, with lipopolysaccharides (LPS) residing in the outer leaflet and phospholipids (PLs) in the inner leaflet. This asymmetric bilayer protects the bacterium against intrusion of many toxic substances, including antibiotics and detergents, yet allows acquisition of nutrients necessary for growth. To build the OM and ensure its proper function, the cell produces OM constituents in the cytoplasm or inner membrane and transports these components across the aqueous periplasmic space separating the two membranes. Of note, the processes by which the most basic membrane building blocks, *i.e.* PLs, are shuttled across the cell envelope remain elusive. This review highlights our current understanding (or lack thereof) of bacterial PL trafficking, with a focus on recent developments in the field. We adopt a mechanistic approach and draw parallels and comparisons with well-characterized systems, particularly OM lipoprotein and LPS transport, to illustrate key challenges in intermembrane lipid trafficking. Pathways that transport PLs across the bacterial cell envelope are fundamental to OM biogenesis and homeostasis and are potential molecular targets that could be exploited for antibiotic development.

Gram-negative bacteria are distinctively characterized by the presence of a complex cell envelope comprising an inner membrane (IM),³ a thin layer of cell wall (or peptidoglycan), and an outer membrane (OM) (1). The IM marks the boundary of the bacterial cytoplasm, and the OM defines a second aqueous compartment known as the periplasm. This double-membrane structure effectively protects the cell from external insults. In particular, the OM restricts the entry of large hydrophobic molecules, in part conferring Gram-negative bacteria intrinsic resistance against many antibiotics and detergents (1).

The OM is an essential lipid bilayer that contains integral proteins (mostly β -barrel OMPs) and peripherally-anchored

lipoproteins. It is also highly asymmetric (2–4), whereas the inner leaflet of the OM is composed of typical phospholipids (PLs), the outer leaflet contains tightly-packed lipopolysaccharides (LPS), which impart low fluidity and permeability to the OM (1). This asymmetric arrangement of lipids is critical for proper barrier function. To build a stable and functional OM, the respective components have to be transported from their sites of synthesis at the cytoplasm or IM, across the periplasm, and to the OM (5). Assembly of this second bilayer is extremely challenging not only because of the need for tight coordination between the various transport processes, but also because there is no obvious energy source, such as ATP, in the periplasmic space. Furthermore, all the major components of the OM are strongly amphipathic by nature, which necessitates distinct mechanisms for shielding at least the hydrophobic portions of these molecules from the aqueous environment as they transit the periplasm. In this regard, the transport pathways for OMPs (6), LPS (7), and lipoproteins (8) have been relatively well-characterized. Despite recent advances, however, our understanding of PL transport across the cell envelope is still lacking. In this review, we summarize the current knowledge on possible PL transport pathways that may contribute to OM biogenesis and homeostasis in Gram-negative bacteria. Through discussion of PL trafficking from a mechanistic viewpoint, and in the context of known transport pathways for other lipidated molecules (*i.e.* lipoproteins and LPS), we hope to highlight outstanding questions in this field and pave a path toward a better understanding of bacterial lipid transport.

Mechanistic models for lipid transport across the periplasm

There are three major steps to be considered for lipid transport between the IM and the OM: (i) release from the first membrane; (ii) transit across the aqueous periplasm; and (iii) insertion into the target membrane. Largely because of the hydrophobic effect, it is most energetically favorable to have the acyl tails of a lipid molecule sequestered in a hydrophobic environment (9). As one can imagine, it would be extremely difficult to pull a lipid molecule out of the membrane and transport it with its acyl tails exposed to aqueous solution; lipid transport across the periplasm therefore does not occur unassisted. A solution to this problem has been proteins that can bind to the acyl tails and shield them from water (Fig. 1). These come in two flavors, those that form soluble lipid–protein complexes as exemplified in lipoprotein transport (8), and those that physically bridge the two membranes to provide a hydrophobic path for lipids moving across the aqueous environment, such as that observed in LPS transport (7). In both of these strategies, the

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³ The abbreviations used are: IM, inner membrane; OM, outer membrane; OMP, outer membrane protein; PL, phospholipid; LPS, lipopolysaccharide; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; CL, cardiolipin; CDP-DAG, cytidine-diphosphate diacylglycerol; LOS, lipooligosaccharide; pmf, proton motive force; ABC, ATP-binding cassette; MCE, mammalian cell entry.

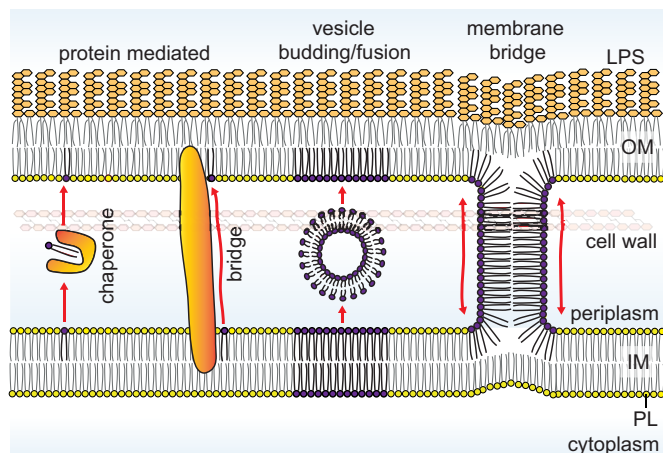


Figure 1. Mechanistic models for lipid trafficking across the periplasm in Gram-negative bacteria. The movement of lipids from the IM to the OM requires shielding of acyl chains from the aqueous periplasmic environment and can involve proteins that act either as chaperones or bridges or may theoretically occur via vesicular transport or hemifusion stalks at sites of membrane juxtaposition.

protein-bound lipid molecule is likely in a relatively stable state, thereby facilitating its transfer from the membrane into the aqueous periplasm, and back. External energy input may be required depending on whether it is more energetically favorable to have the lipid in the membrane or bound to transport proteins (Fig. 2).

A second possible solution to lipid transport across the periplasm may involve direct exchange of lipids between the two membranes, in ways such that the lipid molecules do not actually leave the membrane environment (Fig. 1). Theoretically, this can occur either via vesicles budding from one membrane and fusing with the target membrane, thereby transferring lipids, or via physical membrane bridges connecting the proximal leaflets of the two membranes, allowing free lipid diffusion. For the former, mechanisms that generate curvature leading to formation of vesicles would be required. For the latter, the two membranes would need to be brought into really-close proximity for hemifusion events to occur. Both pathways would require the assistance of proteins and likely external energy input. At present, there is no evidence for the existence of such transport pathways in bacteria. It has long been suggested that the presence of the peptidoglycan layer and the size of the periplasm (15–20 nm) are not compatible with vesicular transport. Although periplasmic vesicular structures have recently been observed in cryo-tomograms, they were largely found in cells showing signs of envelope stress and may have limited physiological relevance (10). It has also been hypothesized that lipid transport can occur in membrane adhesion zones between the IM and the OM (11). These regions, known as Bayer’s patches, could contain proteinaceous or membrane bridges used in lipid trafficking, but they have been highly controversial (12, 13). Recently, intercellular transfer of periplasmic and OM material occurring via transient fusion events between OMs on adjacent cells has been described in *Myxococcus* sp. (14). The existence of such a pathway for intercellular material exchange suggests that analogous mechanisms may be possible for lipid transport across the periplasm.

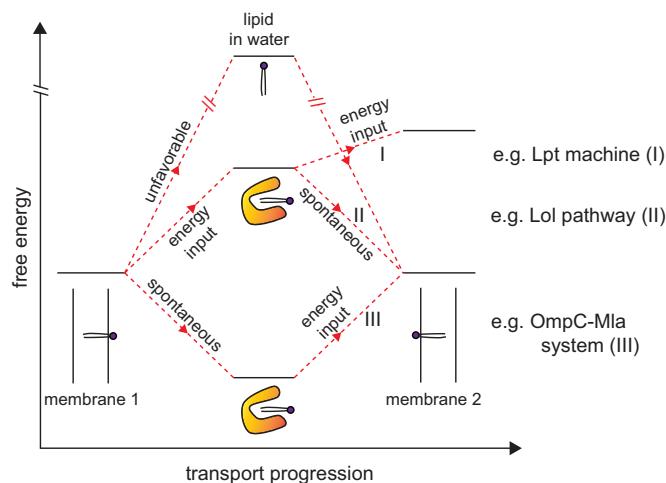


Figure 2. Possible free energy profiles for protein-mediated intermembrane lipid transport. Release of lipids from a membrane (donor) for unassisted diffusion across an aqueous environment to another membrane (recipient) is highly energetically disfavored. In known transport systems, lipid-binding proteins are central to shielding the acyl tails of lipid molecules upon release, giving rise to lipid–protein complexes with energy levels sufficiently close to those of lipids in the membrane environment; this renders lipid transport feasible with or without external energy input, e.g. derived from ATP hydrolysis. Shown here are three different scenarios with distinct energetic requirements at different stages: (I) energy is required to release the lipid molecule from donor membrane as well as to insert it into the recipient membrane, e.g. Lpt machine (Fig. 3); (II) energy is required to release the lipid molecule from the donor membrane only, whereas insertion into recipient membrane is spontaneous, e.g. Lol pathway (Fig. 3); and (III) release from donor membrane is spontaneous (due to high-affinity protein binding) but energy is then required to release the lipid molecule from protein for insertion into recipient membrane, e.g. OmpC–Mla pathway in the context of retrograde transport (Fig. 4). Donor and recipient membranes are labeled as membranes 1 and 2, respectively.

Lipoprotein trafficking

The transport of lipoproteins from the IM to the OM is a classic example for lipid trafficking via soluble lipid–protein intermediates (8). Bacterial lipoproteins are produced with an N-terminal signal peptide that directs secretion across the IM (15). At the periplasmic leaflet of the IM, this signal peptide is processed (16, 17), and the eventual N-terminal cysteine residue is modified with three acyl tails derived from PLs (18–20); this triacyl moiety anchors typically soluble domains of lipoproteins firmly to one leaflet of the bilayer. Lipoproteins destined for the OM are transported across the aqueous periplasm via the Lol pathway, which in *Escherichia coli* comprises five essential proteins (Fig. 3). The periplasmic protein LolA serves as a lipid chaperone, shielding the acyl tails of lipoproteins from the aqueous environment and shuttling these lipoproteins from the IM to the OM (21). LolCDE constitutes an ATP-binding cassette (ABC) transporter that uses energy from ATP hydrolysis to release lipoproteins from the IM and pass them on to LolA (through LolC) (22, 23). Thus, lipoproteins bound to LolA appear to be in a less stable state compared to when anchored in the IM (Fig. 2). At the OM, LolA hands off the lipoprotein to LolB, itself an OM lipoprotein (Fig. 3) (24). LolA and LolB have similar structures, both containing a large hydrophobic cavity for binding the triacyl moiety of lipoproteins (25). Transfer of lipoprotein from LolA to LolB occurs spontaneously, indicating that the transfer is affinity-driven (26). Finally, LolB inserts the lipoprotein via its acyl tails into the inner leaflet of the OM (27,

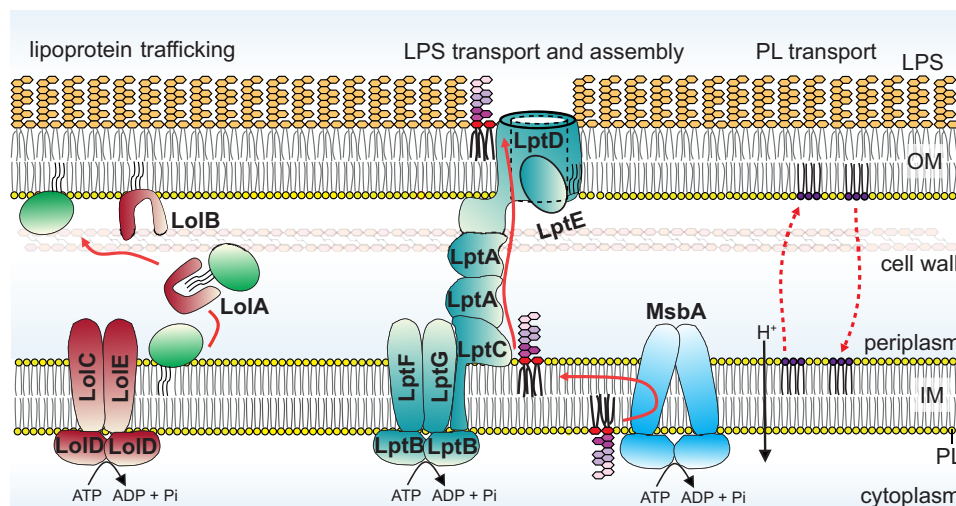


Figure 3. Transport pathways of OM lipoproteins and LPS across the cell envelope in Gram-negative bacteria. OM lipoproteins and LPS are transported via a chaperone (Lol pathway) and protein bridge (Lpt machine), respectively. In Lol-mediated transport, ATP hydrolysis is only required to release lipoproteins from the IM. In Lpt-mediated LPS transport, ATP hydrolysis powers LPS transport all the way from the IM to the OM. Bulk PL transport from the IM to the OM depends on the pmf. As opposed to lipoprotein and LPS transport, PL transport across the envelope is bidirectional.

28); the mechanism for this step is not known but is also energy-independent, consistent with the idea that the triacyl moiety anchored in the membrane would be the most stable state (Fig. 2).

Lipopolysaccharide transport and assembly

LPS transport from the periplasmic side of the IM to the outer leaflet of the OM occurs via a physical protein bridge connecting the two membranes (7). The structure of this glycolipid varies considerably across different Gram-negative species, but in *E. coli* it typically comprises up to hundreds of sugars anchored to the membrane by six acyl tails (29). LPS is synthesized at the cytoplasmic leaflet of the IM as “rough” LPS (Ra form: ~10 core sugars linked to lipid A), flipped across the IM by an ABC transporter MsbA (30, 31), and converted to “smooth” LPS via the addition of O-antigen polysaccharides at the periplasmic leaflet of the IM (32). The journey of this completed LPS structure to the cell surface is then mediated by seven essential Lpt proteins (Fig. 3) (7). Here, the IM ABC transporter LptBFGC (33–35) is physically connected to the OM translocon LptDE (36) through interactions with the periplasmic protein LptA (33, 37). The structurally homologous β -jelly roll domains of LptC (38), LptA (39), and the N-terminal domain of LptD (40) interact in a “head-to-tail” fashion to form a protein bridge (41, 42), providing a continuous hydrophobic groove to accommodate the multiple acyl tails of LPS during transit across the periplasm (39). With six acyl tails, *E. coli* LPS is presumably in its most stable state in a membrane. Therefore, LptBFGC harnesses energy derived from ATP hydrolysis to extract LPS from the IM and loads them onto LptC (43–45). Recent *in vitro* reconstitutions of this system demonstrate that LPS transfer from LptC to LptA, and then to LptD, along the hydrophobic groove also require ATP hydrolysis (46, 47). LptBFGC essentially powers direct transport of LPS from the IM all the way to the outer leaflet of the OM (Fig. 2). At the OM, the LptDE translocon assembles incoming LPS into the outer leaflet (Fig. 3). Here, the enormous polysaccharide

chain of the LPS molecule likely crosses the OM via the large hydrophilic lumen of the LptD β -barrel domain, which is partially constricted by the lipoprotein LptE (48, 49), whereas its six acyl tails traverse along the side wall of the β -barrel directly into the outer leaflet (40, 50). The placement of LPS onto the cell surface occurs against a concentration gradient and comes at an entropic cost. Strong lateral interactions between LPS molecules may provide part of the driving force for establishing the resulting lipid asymmetry in the OM; however, because $\sim 10^6$ LPS molecules need to be assembled at the cell surface in every cell cycle (~ 20 min for *E. coli*), it is not surprising that the final step of LPS translocation across the OM also requires ATP hydrolysis at the IM (51).

Phospholipid transport across the cell envelope

Despite being the most basic building block of lipid bilayers, the transport of PLs from the IM to the OM is the least understood. In *E. coli*, the three major PL species are phosphatidylethanolamine (PE) 75%, phosphatidylglycerol (PG) 20%, and cardiolipin (CL) 5% (52). Some earlier studies suggest that PL compositions of the IM and the OM may be slightly different, with the OM enriched in PE (53, 54). Synthesis of PLs begins with phosphatidic acid (PA), which is converted to cytidine-diphosphate diacylglycerol (CDP-DAG), a common intermediate toward PE and PG (55, 56). CDP-DAG is either converted to phosphatidylserine (PS) before undergoing decarboxylation to give PE (57) or is converted to phosphatidylglycerol phosphate, which undergoes subsequent dephosphorylation to give PG (56, 58). CL is then produced from the condensation of two molecules of PG or one molecule each of PE and PG (59, 60). After their synthesis in the cytoplasmic side of the IM, PLs are presumably flipped across the IM by yet-to-be-identified transporters.

To get to the inner leaflet of the OM where they are predominantly located, PLs need to be extracted from the IM and transported across the aqueous periplasmic space (Fig. 3). As with lipoprotein and LPS transport, this process also requires energy

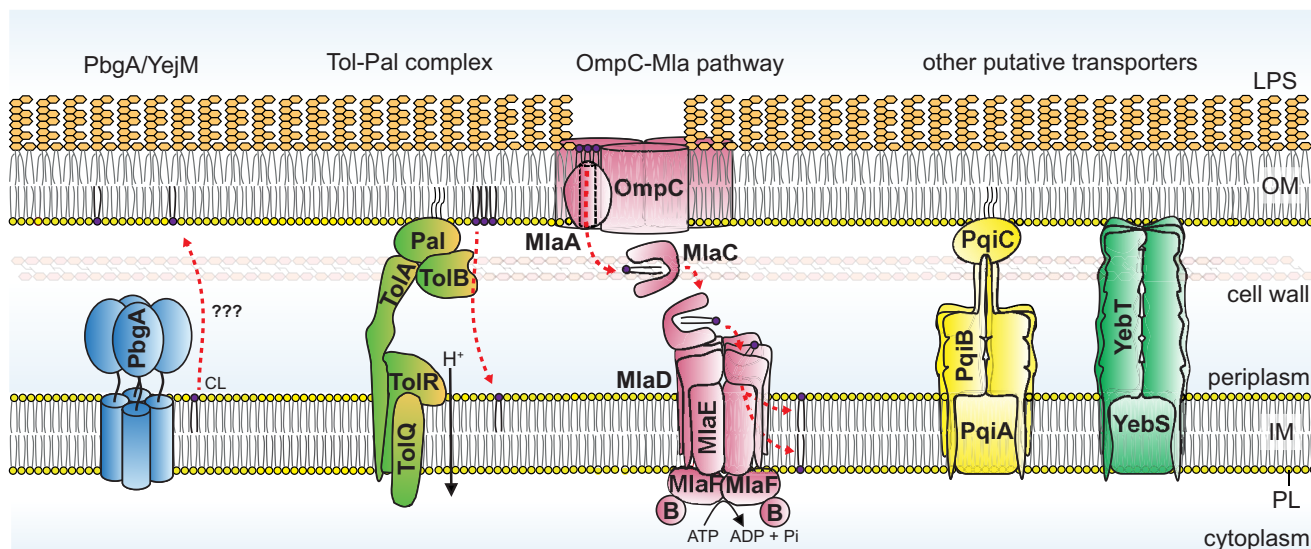


Figure 4. Protein systems implicated in PL transport in Gram-negative bacteria. Mechanism(s) for bulk anterograde PL transport are elusive, but the IM protein PbgA/YejM has been suggested to be involved in the transport of CLs to the OM. Both the Tol–Pal complex and the OmpC–Mla system are important for retrograde transport of PLs from the inner and outer leaflet of the OM, respectively. The directionality of the latter pathway has been a subject of controversy. Pqi and Yeb protein systems are putative PL transporters, but their biological relevant substrate(s) and functions are unknown. For each of these systems, additional experimental evidence is necessary to ascertain direct involvement in and/or directionality of PL transport.

input. Interestingly, even though a minimal requirement for ATP hydrolysis cannot be ruled out, it has been demonstrated that PE translocation from the IM to the OM (anterograde transport) requires the proton motive force (pmf) across the IM (54). Unlike lipoprotein and LPS transport, PL transport has also been shown to be bidirectional (54, 61, 62); PS specifically delivered to the OM, either endogenously via anterograde transport (62) or exogenously by vesicle fusion (61), are transported back to the IM (retrograde transport), where they become processed to give PE. To date, the pathway(s) for anterograde transport of bulk PLs (predominantly PE and PG in *E. coli*) has not been identified, whereas several systems have been implicated in retrograde PL transport.

Anterograde (IM–to–OM) phospholipid transport

The movement of PLs from the IM to the OM is rapid. It has been demonstrated ~40 years ago that PE pulse-labeled with radioactivity is translocated to the OM with a $t_{1/2}$ of minutes (54). This process depends on the presence of the pmf across the IM. Although no protein has been identified for this transport activity, it has been suggested that PL transport to the OM is fundamentally different from the processes identified for LPS and OM lipoproteins (63). Specifically, lipoproteins destined for the OM can be released from the IM when *E. coli* spheroplasts are exposed to LolA-containing periplasmic extracts. LPS transport in spheroplasts from the IM to remnants of the OM is also intact. In contrast, PLs are neither released from the IM in the presence of periplasmic extracts nor transported to the OM in spheroplasts. Given that *E. coli* spheroplasts maintain an intact pmf across the IM, thus allowing study of other known pmf-dependent processes (64–66), these observations suggest that anterograde PL transport is possibly independent of a soluble chaperone or a stable trans-envelope protein bridge.

Recently, a couple of studies suggested that an IM protein PbgA/YejM may be involved in CL translocation to the OM

(Fig. 4) (67, 68). Using quantitative lipidomics, it was shown that functional PbgA/YejM is required for the enrichment of CL in the OM in *Salmonella* Typhimurium, which occurs when the stress-response system PhoPQ is activated (67). Curiously, a similar trend of PbgA/YejM-dependent increase of CL was also observed in the IM. Although these results indicate perhaps an overall increase in cellular CL levels, it was thought that this IM protein functions to deliver CL to the OM. The same idea is supported by another study of PbgA/YejM in *Shigella flexneri* (68), albeit differential detergent extraction and nonquantitative TLC were employed here for membrane separation and lipid analysis, respectively, confounding interpretation. PbgA/YejM is a tetrameric protein with an essential five-helical-bundle transmembrane domain linked at the C terminus to a nonessential periplasmic domain (67, 69). Deletion of the periplasmic domain gives rise to OM permeability defects and reduced LPS levels (69). Why it is required for viability is not known, but overexpression of AcpT, a phosphopantetheinyl transferase, has been shown to suppress lethality in *E. coli* cells lacking YejM (69). Although the exact role of PbgA/YejM in PL transport still requires thorough investigation, it is likely that this protein plays a critical function in bacterial lipid biology.

Retrograde (OM–to–IM) phospholipid transport

It is known that PL transport across the cell envelope is bidirectional (54, 61, 62). By monitoring PS–to–PE conversion, a process that only takes place in the IM, it was demonstrated that radioactive PS accumulated in the OM can be translocated back to the IM (61, 62). However, despite making these observations close to 40 years ago, the significance and possible mechanism(s) of retrograde PL transport have not been revealed until recently. Below, we describe two molecular systems implicated in the retrograde transport of PLs (Fig. 4). The Tol–Pal complex is thought to be involved in the movement of bulk PLs presumably from the inner leaflet of the OM to the IM (70), whereas the OmpC–Mla system is believed to maintain

OM lipid asymmetry by transporting a small population of PLs that is mislocalized in the outer leaflet of the OM back to the IM (71, 72).

The Tol–Pal complex

The Tol–Pal complex is a trans-envelope system highly conserved in Gram-negative bacteria (Fig. 4) (73, 74). It comprises the TolQRA and TolB–Pal sub-complexes in the IM and the OM, respectively, and these complexes interact with each other in a manner dependent on the pmf (75). The Tol–Pal complex has always been thought to be important for maintaining OM integrity and stability, as mutants are highly sensitive to antibiotics and detergents, leak periplasmic contents, and produce large amounts of OM vesicles (73, 76). However, the exact function of this complex, and how its absence gives rise to the observed phenotypes, was not known. The Tol–Pal complex was also shown to be important for the invagination of the OM during cell division (77, 78).

A recent systematic study by our group has now revealed that the Tol–Pal complex plays an important role in the maintenance of OM lipid homeostasis in *E. coli* (70). Using steady-state radioactive labeling of cellular lipids, it was demonstrated that cells lacking the Tol–Pal complex maintain WT levels of LPS but accumulate excess PLs in the OM. This molecular defect, which is also observed in *S. Typhimurium* (79), likely destabilizes the OM and can potentially account for the OM phenotypes described above. It was further established that PL buildup in the OM in *tol–pal* mutants is due to defects in retrograde PL transport. Here, by monitoring the turnover of pulse-labeled radioactive anionic PLs (PG/CL) in the OM through processes that occur only in the IM, it was shown that a functional Tol–Pal complex is required for efficient transport of bulk PLs from the OM back to the IM. This assay, which couples PL transport to turnover, is similar to those previously employed to demonstrate retrograde transport in cells (62). The Tol–Pal complex thus represents the first molecular machine implicated in bulk PL transport.

Even so, it is still not clear whether the Tol–Pal complex directly mediates PL transport between the two membranes. One possibility is that the Tol–Pal complex indirectly affects the function of a true PL transport system. Cells lacking the Tol–Pal complex exhibit delayed invagination of the OM during division (77); the resulting wider distance between the two membranes (around the division site) may somehow give rise to nonspecific effects on other processes, including retrograde PL transport. Alternatively, the Tol–Pal complex may physically move PLs, which could potentially happen in three ways. First, parts of the complex might bind PLs. However, PL-binding activities for various periplasmic domains of the complex have not been detected *in vitro*.⁴ Second, the complex may interact with a PL-binding protein, a yet-to-be-identified component of this system. Third, the Tol–Pal complex may bring the two membranes in close proximity to allow hemifusion and thus lipid diffusion to occur (*i.e.* Bayer's patch/bridge model). Each of these speculative transport mechanisms needs to account for the proposed directionality of transport and may require energy

input. In this regard, the TolQRA complex is homologous to the ExbBD–TonB (80, 81), MotAB (80, 82), and AglQRS (83) systems, which transduce energy from the pmf for the generation of force involved in siderophore uptake, flagella motility, and cell gliding, respectively. How this force is utilized in the Tol–Pal complex to maintain OM lipid homeostasis requires further investigation.

The OmpC–Mla system

The OmpC–Mla system has been shown to play an important role in the maintenance of OM lipid asymmetry in Gram-negative bacteria (71, 72). It comprises the OmpC–MlaA complex at the OM, a periplasmic protein MlaC, and an ABC transporter MlaFEDB at the IM (Fig. 4). Removing any member of the OmpC–Mla system results in aberrant accumulation of PLs at the outer leaflet of the OM in *E. coli*, thereby disrupting lipid asymmetry; this system is believed to remove the mislocalized PLs from the OM and transport them back to the IM (71, 72). Transport between the two membranes occurs via a soluble PL–protein intermediate. MlaC has been crystallized with a PL bound, revealing a hydrophobic pocket that shields both acyl tails of the PL molecule from the aqueous periplasm (84). In addition, MlaC has been shown to interact with both OM and IM complexes *in vitro* (84) and *in vivo* (85). Although removing MlaC does not cause observable defects in retrograde PL transport in cells, it was recently demonstrated that overproduction of MlaC together with the MlaFEDB complex is able to partially rescue defects in PL transport in cells lacking the Tol–Pal complex (70). Therefore, the OmpC–Mla system does in fact transport PLs in a retrograde fashion, even though it may only be important to remove a small subset of PLs from the OM, particularly those mislocalized to the outer leaflet.

The OM lipoprotein MlaA forms complexes with trimeric porins, including OmpC and OmpF, in the OM of *E. coli*, but it appears that only the OmpC–MlaA complex is important for the maintenance of lipid asymmetry (72). This complex is proposed to extract PLs from the outer leaflet of the OM presumably without external energy input. Despite being a lipoprotein, recent structural and biochemical studies revealed that MlaA is really an integral membrane protein that binds porin trimers within the bilayer, at one or more of its dimeric interfaces (86, 87). Interestingly, MlaA forms a hydrophilic channel across the OM, likely providing a path for PL translocation across the OM. In this context, even though a single amino acid change gives rise to OM lipid asymmetry defects, the exact function of OmpC within the complex remains unclear (87). Based on the architecture of MlaA, it has been proposed that PLs extracted from the outer leaflet of the OM do not enter the inner leaflet but are delivered directly to MlaC in the periplasm in an energy-independent fashion. Such a pathway circumvents the need to work against a PL concentration gradient at the OM; however, it necessitates that PLs in the outer leaflet of the OM are in a less stable state compared with those bound to MlaC. In support of this idea, molecular dynamic simulations have shown that the size of the hydrophobic pocket of MlaC can change with PL occupancy (88), suggesting MlaC may have conformational flexibility that possibly allows it to maximize its affinity for PLs via an induced fit mechanism. High affinity binding of PLs by

⁴ A. Z. H. Tan and S.-S. Chng, unpublished observations.

MlaC is also corroborated by the observation that MlaC does not spontaneously transfer PLs to the second lipid-binding protein in this system MlaD *in vitro* (85).

PLs are delivered from MlaC into the IM via the MlaFEDB complex, which is an ABC transporter (89). MlaF and MlaE represent the nucleotide-binding domain and transmembrane domain of the transporter, respectively. Biochemical characterization of the complex elucidated functions of auxiliary proteins MlaB and MlaD (89). MlaB is important for the assembly of the transporter and its ATP hydrolytic activity, whereas MlaD forms stable hexamers that bind PLs *in vitro* (84, 89). The crystal structure of the periplasmic domain of MlaD reveals six protomers organized in a donut-shaped architecture containing a central hydrophobic pore (84), presumably for interactions with the acyl tails of PLs. MlaD also interacts directly with MlaC in cells (85), indicating that MlaC transfers PLs to MlaD within the MlaFEDB complex. Here, because MlaC has high affinity for PLs (85), the energy derived from ATP hydrolysis in the complex may then be required to release the bound PLs and/or activate transfer to MlaD and then into the IM. Whether these PLs get flipped back to the cytoplasmic leaflet of the IM is not known.

There has in fact been some controversy regarding the directionality of lipid transport for the OmpC–Mla system. ABC transporters found in chloroplasts (TGD2) and mycobacteria (Mce1/4 complexes) but homologous to the MlaFEDB complex are known to be involved in PA and fatty acid/cholesterol uptake, respectively (90, 91). In *E. coli*, the OmpC–Mla system was thus initially annotated to function in retrograde PL transport, especially given that overexpressing OM phospholipase PldA rescues asymmetry defects in the OM of *ompC–mla* mutant strains (71, 72); in this scenario, if outer leaflet PLs in the OM cannot be removed (by transport), they can be degraded instead. More recently, evolution experiments revealed that removing the Mla system in *Acinetobacter baumannii* improves growth and restores OM barrier function in strains that do not make lipooligosaccharides (LOS) (92); this makes sense in the context of retrograde PL transport given that cells would require sufficient PLs in the outer leaflet of the OM when no LOS is made. Although these studies inferred function solely from genetic interactions, it has also been shown that overexpressing the *mfaFEDCB* operon partially rescues retrograde PL transport defects in *tol–pal* mutants (70). Therefore, both genetics and biochemical data support a role of the OmpC–Mla system in retrograde PL transport. Interestingly, there have also been some recent observations supporting anterograde PL transport. A separate group characterizing *mfa* mutants in *A. baumannii* found that these strains have severely compromised OM function due to reduced PL content in the OM. By monitoring the rate of appearance of newly-synthesized PLs relative to existing PLs in the IMs and OMs using MS, the authors propose a role for Mla proteins in anterograde PL transport (93). Unfortunately, because existing PL levels are supposedly lower in the OMs of *mfa* mutants, it may not be straightforward to infer about overall changes in PL transport from the rates of change of new *versus* existing PLs in these strains (as compared with that in WT cells). *In vitro* reconstitution experiments also suggest a role for the OmpC–Mla sys-

tem in anterograde transport (94). It has been shown that the complete MlaFEDB complex may transfer PLs spontaneously to MlaC *in vitro*. However, proper inactive enzyme controls were lacking. Furthermore, this *in vitro* reaction did not appear to be dependent on or modulated by ATP hydrolysis, which is quite puzzling indeed for an ABC transporter. Overall, additional studies are definitely needed to provide more clarity to this problem.

Other putative lipid transporters

The periplasmic domain of MlaD contains the mammalian cell entry (MCE) domain, which is widely conserved in proteobacteria and actinomycetes (95). Based on homology, two other MCE domain proteins have been described in *E. coli*, and they have recently been proposed to also be involved in lipid transport (Fig. 4) (95, 96). PqiB and YebT contain three and seven MCE domains, respectively, and both have been co-purified with PLs (84). Structural characterization of these proteins revealed that they form hexameric assemblies via their MCE domains, giving rise to structures that can span the entire periplasmic space between the IM and the OM (84), potentially facilitating transport of lipid substrates or other hydrophobic molecules. Even though cells lacking both proteins have some perturbations to the OM (95, 96), the true functions of these proteins are not yet clear.

OM lipid homeostasis via retrograde PL transport

The processes of lipid transport are inherent for the synthesis and maintenance of the OM in Gram-negative bacteria. Anterograde PL transport is essential for OM biogenesis. In contrast, the role(s) of retrograde PL transport pathways is less clear until recently, where they have been implicated in OM lipid homeostasis (70). The current model (proposed by our group) suggests cells transport more PLs than required to the OM to fill up spaces that may arise from changes in the OM; this process ensures that the bilayer is always complete. To maintain OM stability, however, excess PLs must then be continuously removed, particularly via retrograde transport to the IM in a manner dependent on the Tol–Pal complex. This model makes sense in the context of unidirectional (IM–to–OM) transport of LPS, β -barrel proteins, and lipoproteins and directly alleviates the need for fine control over the levels of these components in the OM.

Lipid homeostasis in the OM also impacts structural organization such as lipid asymmetry. It is perhaps logical to assume that both bulk anterograde and retrograde PL transport delivers PLs to and removes them from the inner leaflet of the OM, respectively. In the absence of the Tol–Pal complex, the cell would accumulate a large excess of PLs in the inner leaflet of the OM, which generates instability in the bilayer. This presumably allows PLs to flip across the OM, leading to subsequent accumulation in the outer leaflet and thus loss of lipid asymmetry (70). Even in a WT cell (with an intact Tol–Pal complex), new OM needs to be continuously synthesized for growth and division. As such, it is conceivable that anterograde PL transport would still be faster than retrograde transport; this scenario may inevitably lead to a slight buildup of PLs in the inner leaflet of the OM and subsequently the outer leaflet. Besides employ-

ing PL-degrading enzymes (PldA (97) and PagP (98)) to correct such perturbations in OM lipid asymmetry, retrograde PL transport mediated by the OmpC–Mla system additionally contributes to OM homeostasis by removing this small amount of PLs that ended up aberrantly in the outer leaflet of the OM (71, 72).

Conclusions and outlook

There are still huge gaps in our knowledge of OM biogenesis. Although significant advances have been made in understanding OM lipoprotein trafficking, LPS transport and assembly, and β -barrel protein folding over the past 2 decades, we have made little progress in deciphering PL transport across the cell envelope. This contrasts with the major advances made toward elucidating nonvesicular PL transport pathways within single organelles (*i.e.* chloroplast (90) and mitochondria (99)) or between separate ones (*i.e.* membrane contact sites (100)) in eukaryotic cells. The obvious major mystery in Gram-negative bacteria cell envelope biology is anterograde PL transport. Why these systems have remained elusive may be due to the lack of targeted genetic approaches to discover factors. One component of the β -barrel protein-folding machine (BamB) was identified in a chemical genetic selection as a mutation that rescues OM leakiness in an *lptD* mutant (101), which has now been shown to be a β -barrel assembly defect (102, 103). One may therefore argue that a genetic selection targeted at correcting OM lipid dyshomeostasis, such as those found in *tol*–*pal* strains (70) or gain-of-function *mfaA* mutants (104), could instead lead to new information regarding PL transport across the cell envelope. Genetic screens based specifically on direct detection of defects in OM lipid homeostasis and/or lipid asymmetry may also aid in the identification of PL transport pathways. It is especially intriguing what the (main) anterograde PL transport system could be, given that the process differs from OM lipoprotein and LPS transport in its requirement for energy derived from the pmf (54).

The functions of various proposed PL transport systems should also be thoroughly investigated. It is not yet clear whether the Tol–Pal complex directly mediates retrograde PL transport nor is it known whether PbgA/YejM, PqiB, and YebT are in fact lipid transporters. The transport directionality of the OmpC–Mla system also requires further clarification. In general, functional assignment of PL transport systems should include both cell-based and *in vitro* assays monitoring PL movement between the two membranes. The cell-based PL transport assays are clearly nontrivial (even in model organisms like *E. coli*), and extra caution should be taken to ensure proper separation of and minimal lipid mixing between IM and OM fragments during analysis. In this regard, current studies, including ours, employ IM and OM integral membrane proteins and LPS (very different properties from PLs) as molecular markers in membrane separation, which cannot truly inform the extent of PL mixing, if any. Therefore, methods that may influence the propensity of PL mixing between membranes should be avoided. Specifically, removal of the cell wall layer and divalent cations by use of lysozyme (2) and EDTA (106), respectively, is known to disrupt lipid asymmetry in the OM, and it could have other unexpected effects on membrane sta-

bility and thus PL mixing between membranes. In addition, cell lysis by sonication or French press at high pressures (>12,000 p.s.i.), albeit after spheroplasting, has been shown to cause significant membrane mixing or cross-contamination (107). Careful evaluation of protocols for cell lysis and/or membrane separation would go a long way to ensure more robust and reliable tracking of PL movement across the cell envelope. Beyond these intricate cell-based assays, *in vitro* reconstitutions of each putative PL transport system would ultimately be required for definitive assignment of function. *In vitro* approaches to study lipid transfer between two membranes are likely highly challenging yet feasible, as exemplified by the recent elegant demonstration of intermembrane LPS transport using purified Lpt proteins and artificial membranes (46).

Gram-negative bacteria are intrinsically resistant to many clinically-used antibiotics due in part to the OM permeability barrier. The OM is also essential for bacterial growth and therefore is a great molecular target for novel antibiotic intervention. To this end, small molecule inhibitors against the Lol (108, 109) and MsbA/Lpt pathways (105, 110) have already been identified. Continued efforts in deciphering PL-trafficking processes will ultimately yield new targets that can be exploited for combating Gram-negative bacterial pathogens.

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