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Lipoproteins in Gram-negative bacteria: new insights into their biogenesis, subcellular targeting and functional roles☆

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Bacterial lipoproteins are globular proteins anchored to a membrane by a lipid moiety. By discovering new functions carried out by lipoproteins, recent research has highlighted the crucial roles played by these proteins in the cell envelope of Gram-negative bacteria. Here, after discussing the wide range of activities carried out by lipoproteins in the model bacterium *Escherichia coli*, we review new insights into the essential mechanisms involved in lipoprotein maturation, sorting and targeting to their final destination. A special attention will also be given to the recent identification of lipoproteins on the surface of *E. coli* and of other bacteria. The renewed interest in lipoproteins is driven by the need to identify novel targets for antibiotic development.

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Introduction

The cell envelope is the morphological hallmark of Gram-negative bacteria. It is composed of two concentric membranes: the inner membrane, which is in contact with the cytoplasm, and the outer membrane, which constitutes the interface with the environment [1]. The inner and outer membranes are separated by the periplasm, a compartment that represents 10–20% of the total cell volume and contains a thin layer of peptidoglycan [1]. The envelope is required for growth and survival, maintaining cell shape and providing osmotic protection to cells.

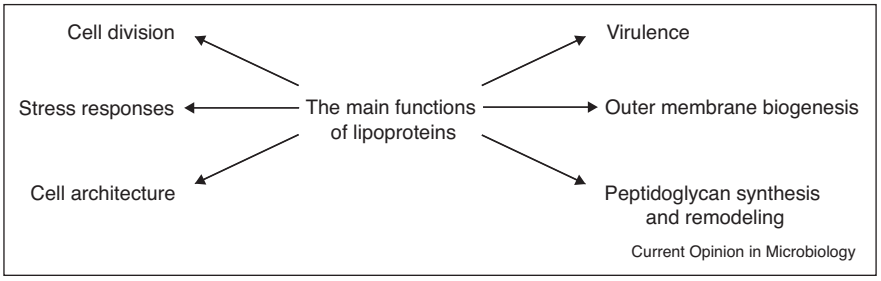
In the model bacterium *Escherichia coli*, about one-third of all proteins are targeted to the cell envelope [2]. Soluble proteins are present in the periplasm where they participate in a variety of processes including peptidoglycan assembly, disulfide bond formation and nutrient import. Integral membrane proteins are present in both the inner and outer membranes; in the outer membrane, they typically adopt a β-barrel conformation [3]. Another important group of envelope proteins is the lipoproteins, which are globular proteins anchored to a membrane by a lipid moiety [4]. Lipoproteins will be the focus of this article.

Lipoproteins exert diverse functions in the cell envelope

A bacterium like *E. coli* can produce and contains ~80 different lipoproteins in its cell envelope [5–7]. These proteins fulfill a variety of important functions (Figure 1). Whereas some, like the outer membrane stress sensors NlpE and RcsF, monitor envelope integrity [8], others play a role in virulence or in envelope biogenesis. For instance, the essential lipoproteins BamD and LptE are required for the assembly of the outer membrane [9,10]: BamD is one of the accessory lipoproteins of the β-barrel assembly machinery (BAM) [3,11] (see the review by Tomasek and Kahne in this issue), while LptE is involved in the trans-envelope complex that transports lipopolysaccharide molecules across the periplasm and inserts them into the outer membrane [12] (see the review by Wilson and Ruiz in this issue). DolP (YraP) is another example of a lipoprotein that is important for the integrity of the outer membrane [13] (Ranava *et al.*, bioRxiv doi: 10.1101/2020.09.23.308296), but its exact function remains to be established. The structure of DolP was recently reported, revealing that the protein is composed of two BON (Bacterial OsmY and Nodulation) domains [13]. Interestingly, the C-terminal BON domain binds anionic phospholipids, an interaction which is important for the function of DolP and its localization to the site of cell division [13].

☆ Given his role as Guest Editor, Jean-Francois Collet had no involvement in the peer-review of this article and has no access to information regarding its peer-review. Full responsibility for the editorial process for this article was delegated to Angelika Gründling.

Figure 1



Lipoproteins are involved in a wide range of vital processes in Gram-negative bacteria.

Lipoproteins also play important roles in peptidoglycan synthesis and remodeling. Examples include LpoA and LpoB, two outer membrane lipoproteins that regulate the transpeptidase activity of the peptidoglycan synthases PBP1A and PBP1B, respectively [14,15] (see the review by Pazos and Vollmer in this issue), and NlpD, which controls the activity of AmiC, one of the amidases that contribute to septum cleavage during cell division [16,17]. The list of lipoproteins involved in peptidoglycan remodeling keeps growing: the outer membrane lipoprotein YddW (renamed DigH) was recently identified as a divisome-localized glycosyl hydrolase able to cleave denuded peptide-free glycan strands [18], while NlpI was reported to scaffold peptidoglycan hydrolases and to contribute to complexes active in peptidoglycan synthesis during cell elongation and division [19].

Lipoproteins also are important for controlling the architecture of the cell envelope, for instance by connecting the outer membrane to the peptidoglycan. To that respect, we will now briefly discuss recent studies that brought new insights into two of the most studied *E. coli* lipoproteins, Lpp and Pal.

Lpp, the numerically most abundant protein in *E. coli* with 1 million copies per cell [20], functions as an α-helical tether covalently anchoring the outer membrane to the peptidoglycan: in its absence, the outer membrane pulls away from the cell body and forms blebs [21–23]. Lpp was also shown to dictate the size of the periplasm: increasing the length of Lpp augments the distance between the inner and outer membranes [22,23], which has functional consequences. First, it impacts the size of structures such as the flagellum rod spanning the intermembrane space [22]. Second, it renders cells unable to detect envelope alterations and to trigger the Rcs stress system as a response [23]. Given the crucial role played by Lpp in *E. coli*, it was intriguing that this protein is only present in a small number of γ-proteobacteria. Recent work addressed this problem by discovering that in several Gram-negative species, the outer membrane is covalently attached to the peptidoglycan by β-barrel proteins and, in *Coxiella burnetii*, by the lipoprotein LimB [24••,25••].

New insights have also been reported on Pal, the outer membrane component of the highly conserved Tol-Pal system [26]. The core of Tol-Pal consists of five proteins, 3 in the inner membrane (TolA, TolQ and TolR), one in the periplasm (TolB) and Pal. Like Lpp, Pal links the outer membrane to the peptidoglycan by binding to free diaminopimelic acid residue in the peptidoglycan stem peptides [27]; this interaction is however non-covalent. Tol-Pal is important in cell division, for maintaining OM integrity and for the uptake of colicins and of filamentous phage DNA [26]. In cell division, Tol-Pal stabilizes the outer membrane-peptidoglycan connection during constriction. Interestingly, the de Boer laboratory reported that Tol-Pal proteins, which are dispersed along the cell envelope in non-dividing cells, are dynamically recruited to the divisome near the time of constriction initiation [28]. The relocation of Pal at the division site, which was recently confirmed using functional, chromosomally-encoded proteins fused to fluorescent markers [29,30•], was puzzling given that the interaction of Pal with the cell wall limits its ability to laterally diffuse. De Boer and colleagues speculated that TolB, which binds Pal in a mutually exclusive manner with peptidoglycan, was involved in Pal's relocation by forming mobile TolB-Pal complexes [28]. Recent work experimentally confirmed this hypothesis and proposed an elegant mechanism [30•] for the mobilization of Pal by TolB and its deposition at the site of constriction in a process fueled by the proton motor force [29,30•].

The journey of lipoproteins in the envelope

The maturation of lipoproteins

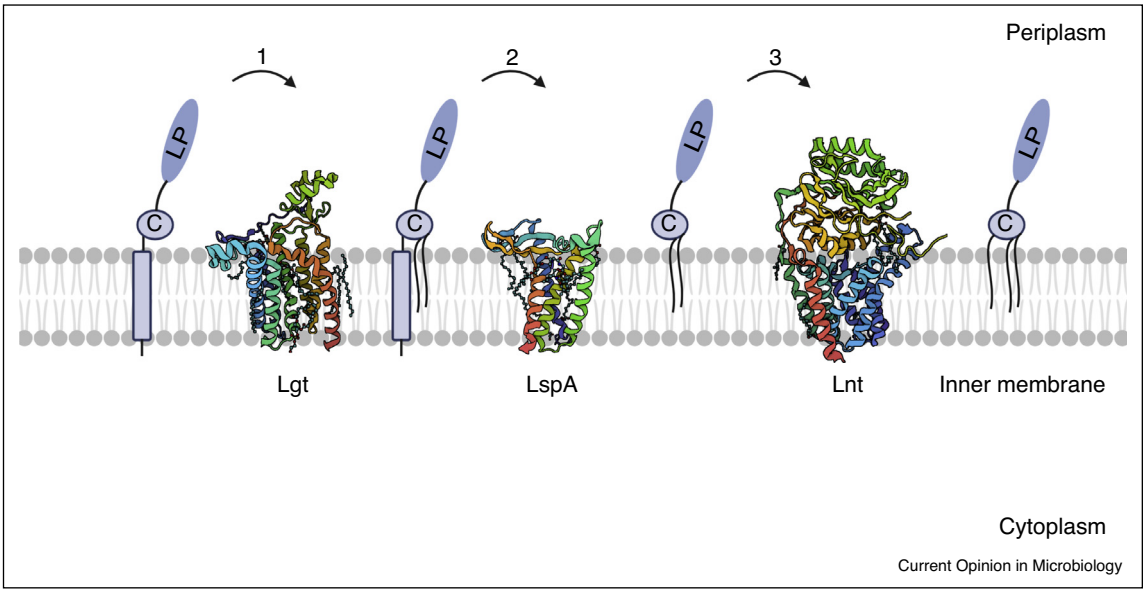
Lipoproteins are synthetized in the cytoplasm as precursors. They display an N-terminal signal peptide that is ~25 amino acids long and generally presents a short, positively charged region followed by a longer stretch of hydrophobic residues [31,32]. The last four residues of the signal peptide form a conserved ([LVI][ASTVI][GAS]C) motif referred to as the lipobox [32]. The

lipobox functions as a molecular determinant of lipid modification which is unique to bacteria; it can be used to predict genes encoding lipoproteins in bacterial genomes [32]. Only the cysteine at the last position of the lipobox is strictly conserved: this cysteine will later become the first residue of the mature protein.

The vast majority of lipoproteins cross the inner membrane in an unfolded state using the general secretory system (Sec) [33]. A few, however, cross the inner membrane in a folded state through the twin-arginine translocation (TAT) machinery [34]. After translocation, the lipoprotein remains anchored to the inner membrane by its N-terminal signal peptide and undergoes several maturation steps. Three inner membrane enzymes participate in this process [35] (Figure 2). The first enzyme is the prolipoprotein diacylglyceryl transferase (Lgt), which catalyzes the covalent attachment of a diacylglyceryl moiety to the cysteine of the lipobox by a thioether linkage. After addition of the diacylglyceryl moiety, the signal peptide is cleaved by the signal peptidase II (LspA), which generates an apolipoprotein, with a cysteine as first residue. The third and last step of lipoprotein maturation consists in acylating the N-terminal amino group of the cysteine, yielding a mature lipoprotein with three acyl chains. This reaction is catalyzed by the apolipoprotein N-acyltransferase (Lnt). Lgt, LspA and Lnt are conserved and essential in the majority of Gram-negative organisms, including pathogenic species [35–38]; they are therefore attractive targets for the design of new antimicrobials.

In the last few years, important achievements have been made in the understanding of the catalytic mechanisms of Lgt, LspA and Lnt. First, the crystal structure of *E. coli* Lgt was solved (Figure 2) in complex with either phosphatidylglycerol or palmitic acid [39], allowing potential mechanisms for the transacylation reaction between phosphatidylglycerol and the preprolipoprotein to be proposed [39,40]. Second, the structure of LspA from two different bacteria, the Gram-negative *Pseudomonas aeruginosa* (Figure 2) and the Gram-positive organism *Staphyloccocus aureus* were reported [41,42•]. In both organisms, LspA appears as a monomer with four transmembrane α-helices and a periplasmic domain with an amphiphilic β-sheet that rests on the membrane. The structures were solved in complex with antibiotics (globomycin for *Pa*LspA and *Sa*LspA, and myxovirescin for *Sa*LspA), which were found to block access to a region identified as the catalytic site. The active site contains two aspartate residues proposed to represent a catalytic dyad with a general acid–base role; the catalytic aspartates are located in the transmembrane domain and point towards the outer surface of the membrane. Finally, recent publications reported the crystal structures of *E. coli* [43•,44–46] and *P. aeruginosa* Lnt [45]. Both enzymes are remarkably similar and consist of a transmembrane domain with eight α-helices providing support to a

Figure 2



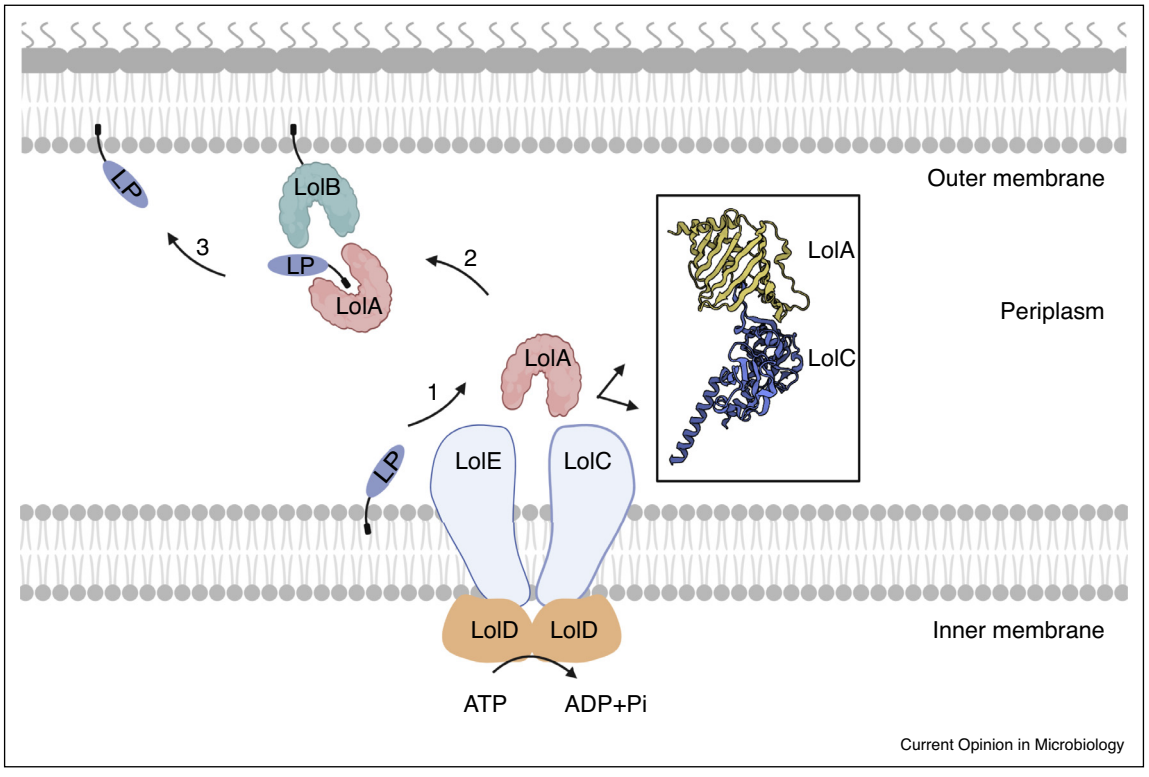
Three inner membrane proteins catalyze lipoprotein maturation. Following translocation of the preprolipoprotein across the inner membrane, Lgt catalyzes the addition of diacylglyceryl to the thiol group of the cysteine residue in the lipobox, generating "prolipoprotein" (1). The signal peptide is then cleaved by the peptidase LspA (2), giving rise to "apolipoprotein". The last step of the maturation process, the acylation of the amino group of the now N-terminal cysteine, is catalyzed by Lnt (3). The crystal structures of the three enzymes are shown: Lgt in complex with phosphatidylglycerol (pdb: 5AZC), LspA in complex with globomycin (pdb: 5DIR) and Lnt (pdb: 5VRG). LP: Lipoprotein; C: Cysteine.

periplasmic nitrilase-like domain (Figure 2). A catalytic triad consisting of a cysteine, a glutamate and a lysine is present in the periplasmic domain, residing slightly above the membrane surface. The available biochemical and structural data support a two-steps ping-pong mechanism for the N-acyltransferase reaction [46–48]. First, an acyl chain is transferred from a phospholipid substrate (preferably phosphatidylethanolamine) to form a thioester linkage with the catalytic cysteine of Lnt. In a second step, the acyl chain is transferred from the cysteine to the α-amino group of the N-terminal cysteine of the lipoprotein. Altogether, the high-resolution structures of Lgt, LspA and Lnt set the stage to the identification of novel antibiotics targeting lipoprotein maturation [49•,50•]; novel fluorescent-based assays that were developed to monitor the activity of Lnt and LspA *in vitro* will facilitate the search for specific inhibitors of these enzymes [51,52].

Dispatching lipoproteins to their final destination

Once matured, lipoproteins either remain in the inner membrane or are exported to the outer membrane. In *E. coli*, most lipoproteins (~90%) are targeted to the outer membrane [53,54]. Lipoprotein extraction from the inner membrane and transport to the outer membrane is carried out by the Lol system (Figure 3). Lol consists of the inner membrane ABC transporter LolCDE, which extracts lipoproteins from the inner membrane at the expense of ATP hydrolysis and transfers them to the soluble periplasmic chaperone LolA [55]. LolA then shuttles the lipoprotein across the periplasm for delivery to LolB, an outer membrane lipoprotein in charge of outer membrane insertion [55]. In *E. coli*, the current view is that Lol uses the residue adjacent to the lipidated cysteine to sort inner and outer membrane lipoproteins. If the residue at position +2 is an aspartate, the lipoprotein remains in the inner membrane [56,57], most likely because of strong electrostatic interactions between this residue and the headgroups of anionic phospholipids that prevent lipoproteins from interacting with LolCDE [58]. Noteworthy, the residue at position +3 also plays a role: aspartate, glutamate and glutamine strengthen inner membrane retention, whereas histidine and lysine weaken it [56,57]. Different mechanisms have been described in other bacteria. In *P. aeruginosa* for instance, an aspartate is rarely found at position +2 and inner membrane retention appears to be determined by residues at position +3 and +4 [59,60], while in *Borrelia burgdorferi*, the causative agent of Lyme disease in humans, lipoproteins are

Figure 3



The Lol system transports lipoproteins to the outer membrane. Lipoproteins which are destined to the outer membrane are extracted from the inner membrane by LolCDE, an ABC transporter, and delivered to the periplasmic chaperone LolA (1). The mouth-to-mouth interaction between LolA and LolB delivers lipoproteins to LolB (2), which finally inserts lipoproteins in the outer membrane by a poorly known mechanism (3). The crystal structure of LolA bound to the periplasmic domain of LolC (pdb: 6F3Z) is shown. LP: Lipoprotein.

secreted to the outer membrane by default [61]. Interestingly, it was recently shown that lipoproteins are correctly sorted in *P. aeruginosa* cells expressing the *E. coli* LolCDE complex, despite differences in the retention signal between these two species [62•]. To explain this surprising result, a more general mechanism was proposed in which inner membrane retention results from the ability of the N-terminal domain of inner membrane lipoproteins to adopt a conformation favoring strong interactions with the phospholipid bilayer [62•]. Finally, in a recent study, we found that approximately half of lipoproteins destined to the *E. coli* outer membrane display an intrinsically disordered linker at their N-terminus and that these linkers are required for optimal trafficking by the Lol system and normal targeting to the outer membrane (IMN El Rayes & Szewczyk et al, bioRxiv doi: 10.1101/2021.01.05.425367). Clearly, further work remains to be done to fully comprehend how Gram-negative bacteria control the triage of lipoproteins between their two membranes.

When lipoproteins do not have an inner membrane retention signal, they are extracted from the membrane by LolCDE. LolCDE comprises a heterodimer of the integral membrane proteins LolC and LolE and a homodimer of the peripheral membrane protein LolD [63,64]. LolD, present on the cytoplasmic side of the inner membrane, has a nucleotide binding domain that is responsible for ATP hydrolysis, thus providing the energy necessary for lipoprotein extraction. The mechanism by which LolCDE couples ATP hydrolysis, lipoprotein extraction and delivery to LolA remains unclear. Current data support a model in which lipoprotein substrates bind to LolE, before being passed on to LolA, while LolC would act as a scaffold for LolA [65,66••]. On the basis of molecular dynamics simulations, LolC was also recently proposed to facilitate lipoprotein transfer to LolA by destabilizing the inner membrane [67•].

The structure of a complex between LolA and the periplasmic domain of LolC has been solved [66••], but the structure of the full LolCDE complex remains unknown. However, a model has been built on the basis of the structure of MacB, an antibiotic transporter that is homologous to LolCDE [66••]. This model shows LolCDE in two conformations, corresponding to the nucleotide-free state and the ATP-bound state [66••]. In both conformations, LolA is located on top of LolCDE, ~60 Å away from the inner membrane, thus suggesting that lipoproteins, with their hydrophobic lipid moiety, travel a relatively long distance before reaching LolA. The molecular details of this "migration" as well as the potential role of LolC in membrane deformation suggested by molecular dynamics simulations [67•] remain to be determined. It is interesting to note that some Gram-negative bacteria express a homodimer of LolF proteins instead of a heterodimer of LolC and LolE. Bacteria that express LolF, such as *Francisella tularensis*, *Neisseria meningitidis*, *Acinetobacter species* and *Helicobacter pylori* can tolerate Lnt deletion [68–71].

ATP hydrolysis by LolD causes lipoproteins to be released and delivered to the periplasmic chaperone LolA [65]. LolA folds into an incomplete β-barrel forming a hydrophobic cavity that binds the lipid moiety of substrate lipoproteins, thereby protecting the acyl chains from the hydrophilic periplasm. The hydrophobic cavity of LolA was considered large enough to only accommodate one of the acyl chains of the lipoprotein substrates [72], but recent molecular dynamics simulations questioned this idea, suggesting that it could in fact fit up to three [73]. After crossing the periplasm, LolA delivers its lipoprotein substrates to LolB by a mouth to mouth interaction [74]. LolB, which is a structural homolog of LolA, then anchors the lipoprotein into the inner leaflet of the outer membrane via an unknown mechanism [75]. LolA is well conserved in Gram-negative bacteria, but LolB is only present in γ- and β-proteobacteria [35,76], which indicates that other mechanisms mediating lipoprotein insertion into the outer membrane remain to be identified. In line with this idea, it was recently reported that LolA and LolB, but not LolCDE, could be deleted under certain specific conditions (including *lpp* deletion) in *E. coli*, which suggests that at least one alternative trafficking route to the outer membrane exists in this bacterium [77].

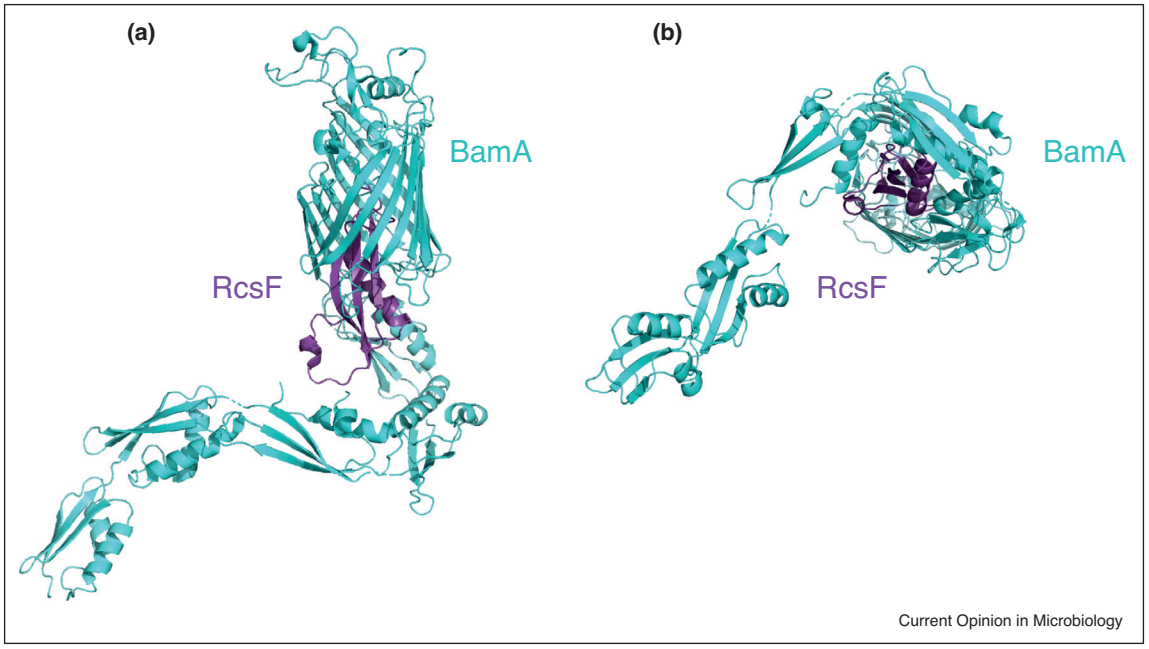
Monitoring lipoprotein trafficking

Given the crucial function played by many outer membrane lipoproteins, bacteria have evolved molecular systems to monitor their trafficking across the cell envelope. In *E. coli*, failure to target outer membrane lipoproteins to their final destination triggers two envelope stress responses, Rcs and Cpx [77–81]. While Rcs copes with outer membrane and peptidoglycan defects [82•], Cpx responds to a broad range of perturbations, including protein misfolding and inner membrane stress [83]. Both Rcs and Cpx can be triggered by an outer membrane lipoprotein, RcsF and NlpE, respectively [8]. When perturbations occur in lipoprotein trafficking, RcsF and NlpE accumulate in the inner membrane, where they can reach their downstream partner (IgaA for RcsF [80,81] and CpxA for NlpE [78,79]), thereby activating their respective signaling cascade. Although Rcs activation leads to higher *lolA* expression [84], which should help fix the damage, it does not provide a fitness advantage when trafficking is perturbed, in contrast to Cpx activation which is beneficial to mutants with defective lipoprotein biogenesis [77–79].

Surface exposed lipoproteins and lipoprotein-OMP complexes

Some lipoproteins, after reaching the outer membrane, cross the asymmetric lipid bilayer and reach the cell

Figure 4



The structure of a complex between the lipoprotein RcsF and BamA suggests a push-and-pull mechanism for the export of lipoproteins to the cell surface. Ribbon diagram of the BamA-RcsF complex in side (a) and periplasmic (b) view. BamA, cyan; RcsF, purple. RcsF is located deep inside the lumen of the BamA β-barrel.

surface, using poorly characterized mechanisms [37,85]. Surface-exposed lipoproteins, which are attractive candidates for vaccine development [86] and often are required for virulence [87], are common in certain bacterial species such as *B. burgdorferi*, where as many as two-thirds of all lipoproteins are surface-exposed [88]. Other bacteria commonly displaying lipoproteins on their surface include species from the *Bacteroides* and *Neisseria* genus [89–91]. In *Neisseria*, surface exposure involves a Surface Lipoprotein Assembly Modulator (SLAM) [92], an outer membrane protein with an N-terminal domain and a 14-stranded β-barrel which is required for virulence [91]. In contrast, only a handful lipoproteins, including Lpp and Pal, have been identified on the *E. coli* cell surface [80,93–99]. Caution is required when considering lipoprotein surface exposure: unambiguously determining whether a lipoprotein crosses the outer membrane or not is technically challenging due to the high yields of false positive. The only *E. coli* lipoprotein that has been shown to reach the cell surface by different groups and complementary techniques is the stress sensor RcsF [80,96,100]. Therefore, RcsF has become a paradigm to investigate the mechanisms of lipoprotein surface exposure in Enterobacteriacea. According to the current model, RcsF is exported to the cell surface by the β-barrel assembly machinery (BAM) via the assembly of complexes with β-barrel proteins. The crystal structure of a complex between RcsF and BamA, the core BAM component, has recently been solved [101••]. In this complex, which is considered to be an intermediate in the formation of the RcsF-β-barrel complexes, RcsF binds inside the lumen of the BamA β-barrel (Figure 4), interacting with regions proposed to undergo a lateral opening during β-barrel insertion. This structure suggests a push-and-pull mechanism for the transfer of RcsF to its β-barrel partners [101••] and provides a molecular explanation to the observation that RcsF accumulation on BamA lethally blocks β-barrel assembly [102•,103•]. Interestingly, RcsF uses its interaction with BamA to detect stress in the cell envelope: when damage occurs in the peptidoglycan or the outer membrane, newly synthesized RcsF molecules fail to interact with BamA, they are not exported to the surface and remain exposed to the periplasm, which allows them to trigger the Rcs signaling cascade by reaching IgaA [80,81].

The abundant β-barrels OmpA, OmpC and OmpF were identified as potential vehicles for RcsF surface-exposure [80,96]. However, by dissecting the OmpA-RcsF interaction *in vivo* and *in vitro*, we found that RcsF interacts with the C-terminal, periplasmic domain of OmpA, not with the N-terminal β-barrel. We therefore concluded that OmpA does not mediate the surface exposure of RcsF and predicted that only OmpC and OmpF (which form large β-barrels) serve as vehicles for surface exposure [104].

Conclusions

The diverse roles played by lipoproteins in the cell envelope have been drawing a lot of attention lately,

revealing how crucial these proteins are in a wide range of vital processes. However, this is only the tip of the iceberg: even in the well-studied model organism *E. coli*, the function of most lipoproteins remains to be discovered. Future research will also establish whether lipoproteins commonly decorate the bacterial surface, which could change our view of the cell envelope, and determine whether surface-exposed lipoproteins use BAM as a general machinery to cross the outer membrane or if other machineries, like SLAM [105], are involved. Finally, future research will also identify potential alternative trafficking routes across the periplasm and address how lipoproteins are inserted in the outer membrane of bacteria missing LolB. Addressing these questions will open the way to novel antibiotics by providing both new molecular targets and crucial mechanistic information.

Acknowledgments

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- •• of outstanding interest

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