

# Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model

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**Abstract** | Gram-negative bacteria have a double-membrane cellular envelope that enables them to colonize harsh environments and prevents the entry of many clinically available antibiotics. A main component of most outer membranes is lipopolysaccharide (LPS), a glycolipid containing several fatty acyl chains and up to hundreds of sugars that is synthesized in the cytoplasm. In the past two decades, the proteins that are responsible for transporting LPS across the cellular envelope and assembling it at the cell surface in *Escherichia coli* have been identified, but it remains unclear how they function. In this Review, we discuss recent advances in this area and present a model that explains how energy from the cytoplasm is used to power LPS transport across the cellular envelope to the cell surface.

Gram-negative bacteria have an inner membrane (IM), which surrounds the cytoplasm, and an outer membrane (OM), which is exposed to the environment. The OM of Gram-negative bacteria is essential, and its correct assembly is required for bacterial survival in harsh environments<sup>1</sup>. The OM is also the first point of contact with the environment that surrounds the bacterial cell, and subtle changes to this membrane affect fundamental bacterial processes, such as motility, attachment and pathogenesis<sup>2–6</sup>.

Similar to most membranes, the hydrophobic nature of the lipidic bilayer of the OM prevents the passage of large polar molecules through electrostatic repulsion. However, the OM of many Gram-negative bacteria also prevents small hydrophobic molecules from entering the cell<sup>1</sup>. This unusual barrier function of the OM is a consequence of its structure. In most Gram-negative bacteria, the OM is an asymmetric lipid bilayer with lipopolysaccharide (LPS) in the outer leaflet and phospholipids in the inner leaflet<sup>7–9</sup> (FIG. 1). Although LPS is present in most Gram-negative bacteria, bacterial cell surfaces have a great deal of structural diversity and LPS is not produced by some bacteria that have an OM, such as *Borrelia burgdorferi*, the causative agent of Lyme disease. The diversity in cellular envelopes exemplifies how bacteria have evolved to meet unique challenges in different environments and to adapt to new external pressures<sup>10</sup>.

LPS is an amphipathic molecule that contains fatty acyl chains attached to a polysaccharide containing up to 200 sugars<sup>6</sup> (FIG. 1). Some of these sugars contain

phosphate groups that mediate interactions with divalent metal ions (for example, Mg<sup>2+</sup>), which enables LPS molecules to pack tightly. The assembled LPS structure creates a highly ordered network of sugar chains on the cell surface that makes the partitioning of hydrophobic molecules into this well-packed material unfavourable. The hydrophobicity of LPS is directly responsible for why the development of new antibiotics to treat infections with Gram-negative bacteria has been so difficult, as many drugs are relatively hydrophobic.

Although the composition and structure of the OM prevents the access of antibiotics and other molecules to the cytoplasm, this barrier also presents challenges for the transport of bacterial components that are produced inside the cell. For example, LPS molecules are synthesized in the cytoplasm, and the transport of these large amphipathic molecules that contain many fatty acyl chains and hundreds of sugars across the IM, the periplasm and the OM, poses major challenges. Indeed, for more than a decade, the major question in the field of LPS biogenesis was what proteins are responsible for the transport of LPS across the cellular envelope and the assembly of LPS at the cell surface. Several of these LPS transport (Lpt) proteins have since been identified, and the history of their identification using multidisciplinary approaches has been comprehensively discussed (see REF. 11). However, although we now think that we know the essential players that are involved in the transport and assembly of LPS at the OM, detailed information about the function of these proteins is still lacking.

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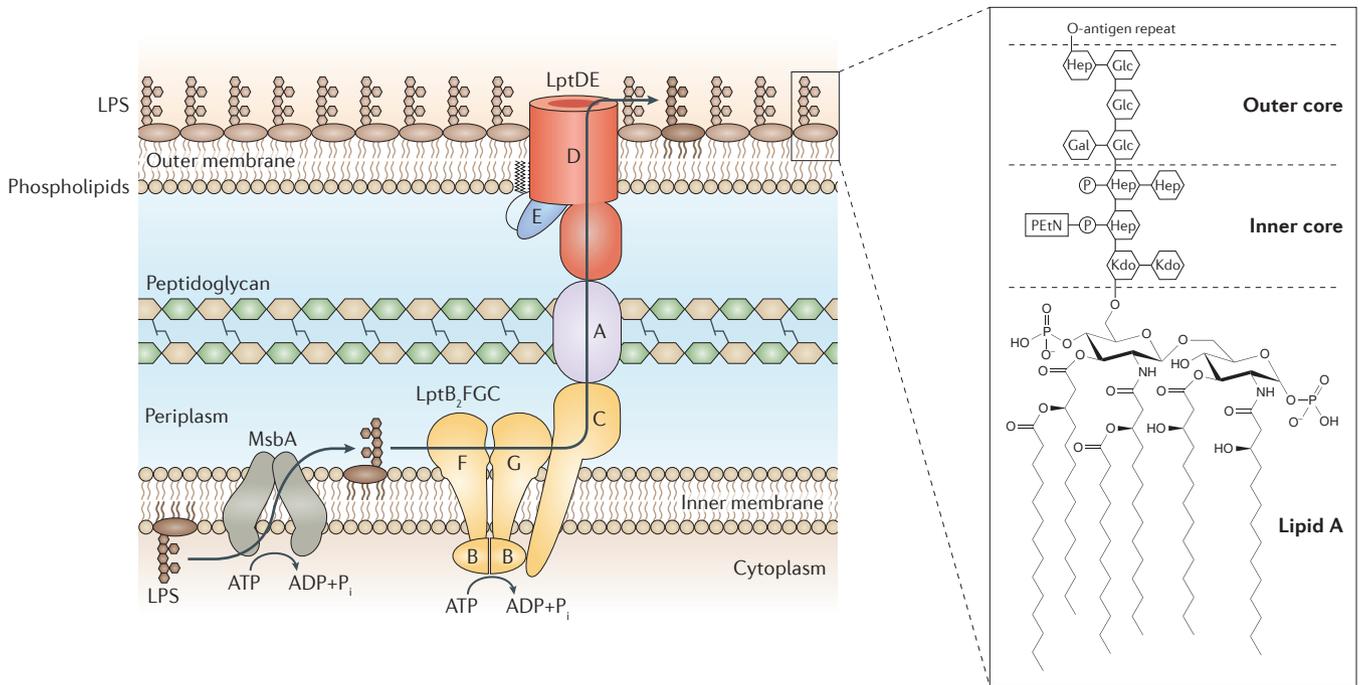
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**Figure 1 | LPS transport pathway in *Escherichia coli*.** Lipopolysaccharide (LPS) is synthesized on the cytoplasmic side of the inner membrane (IM) and flipped to the periplasmic side by the ATP-binding cassette (ABC) transporter MsbA. LPS is then transported to the cell surface by the LPS transport (Lpt) pathway. This pathway consists of seven essential proteins, LptA, LptB, LptC, LptD, LptE, LptF and LptG. LPS is extracted from the IM in an ATP-dependent manner by the ABC transporter LptB<sub>2</sub>FG and transferred to LptC, which forms a complex with LptB<sub>2</sub>FG. LptC consists of a single membrane-spanning domain and a large periplasmic domain, which forms a periplasmic bridge with the soluble protein LptA and the amino-terminal region of LptD. LPS transverses the aqueous periplasmic space through this protein bridge and reaches the cell surface with the aid of the carboxy-terminal domain of LptD, which forms a β-barrel structure that is plugged by the outer membrane (OM) lipoprotein LptE. LPS is composed of lipid A, the inner and outer core oligosaccharides, and the O antigen, which is highly variable and absent in *Escherichia coli* K-12. The letters A–G in the figure correspond to the respective Lpt protein in the transport pathway. EtN, ethanolamine; Gal, D-galactose; Glc, D-glucose; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid; P, phosphate; P<sub>i</sub>, inorganic phosphate.

**Periplasm**

An aqueous, densely packed compartment between the Gram-negative inner membrane and outer membrane. The periplasm has a unique assortment of proteins and also contains a thin layer of peptidoglycan.

**Lipid A**

The hydrophobic glucosamine-based phospholipid anchor of lipopolysaccharide molecules. Lipid A is also known as endotoxin.

**O antigen**

Attached to the core oligosaccharide, this repetitive glycan is the outermost part of the lipopolysaccharide molecule and is a target of the host immune system.

**ATP-binding cassette transporter (ABC transporter)**

A transmembrane protein complex that uses the energy derived from ATP binding and hydrolysis to transport various substrates. These proteins are members of one of the largest protein superfamilies and consist of transmembrane domains and conserved nucleotide-binding domains.

**Bitopic membrane protein**

A type of membrane protein that contains only one transmembrane helix.

Therefore, over the past several years, biochemical, genetic and structural studies have focused on how individual Lpt components function and how they interact with each other. In addition, several intermediates of LPS transport have been observed *in vivo* and *in vitro*. In this Review, we summarize the current understanding of LPS transport and assembly at the OM, and discuss how recent studies have established the function of the Lpt machinery in directly facilitating the release of LPS from the IM and its transit across the periplasmic compartment. Furthermore, we propose a model that explains how energy from the cytoplasm powers LPS transport to the cell surface and highlight the most important questions in LPS transport and assembly that remain unanswered.

**LPS biogenesis**

The biosynthesis of LPS requires more than 100 genes and much is known about the molecular mechanisms of the biosynthetic enzymes<sup>12–14</sup>. LPS is composed of a lipid A moiety, inner and outer core oligosaccharides, and the O antigen<sup>6,13</sup> (FIG. 1). The lipid A–core oligosaccharide complex is synthesized on the cytoplasmic side of the IM and flipped to the periplasmic side by an ATP-binding cassette transporter (ABC transporter), MsbA<sup>15–19</sup>. The O antigen, which is not present in some

Gram-negative organisms, is ligated to the lipid A–core oligosaccharide complex by an O antigen ligase, Waal, following its independent synthesis in the cytoplasm and transport to the periplasm<sup>20–23</sup>. How LPS is subsequently transported to, and assembled on, the cell surface is much less understood.

In *Escherichia coli*, seven essential proteins, LptA (formerly known as YhbN), LptB (formerly known as YhbG), LptC (formerly known as YrbK), LptD (formerly known as Imp/OstA), LptE (formerly known as RlpB), LptF (formerly known as YjgP) and LptG (formerly known as YjgQ), are required for the transport of LPS from the outer leaflet of the IM to the outer leaflet of the OM<sup>11,24–26</sup> (FIG. 1). Notably, all seven Lpt proteins are required, as the depletion of individual Lpt components results in the accumulation of LPS on the periplasmic surface of the IM<sup>27,28</sup>. An ABC transporter, LptB<sub>2</sub>FG, in association with a bitopic membrane protein, LptC, is thought to extract LPS from the IM. The soluble protein LptA mediates the transit of LPS across the aqueous periplasmic compartment. Finally, the β-barrel membrane protein LptD and the OM lipoprotein LptE form a heterodimeric translocon in the OM that receives LPS from LptA and transports it to the cell surface, presumably without allowing LPS to reside in the inner leaflet of the OM.

**$\beta$ -barrel**

A class of integral membrane protein comprised of  $\beta$ -strands that satisfy their peptide backbone hydrogen bonds by forming a cylindrical barrel structure, which exposes hydrophobic side chains to the membrane and shields hydrophilic side chains.

**Lipoprotein**

A protein characterized by the presence of an amino-terminal lipid-modified cysteine that anchors the hydrophilic protein to the cell membrane.

**Transposon mutants**

Mutants that are created through the random insertion of a transposon (or transposable element) into a genome. In the cited study, the transposable element encoded an arabinose-inducible promoter that could drive the expression of chromosomal genes located immediately downstream of the transposon insertion site. It was used to identify essential genes by screening for transposon mutants that required the presence of arabinose in the medium for growth.

**Synteny**

The colocalization of genes in a genetic locus.

**Reductionist bioinformatics approach**

An approach to bioinformatics in which a complex biological system is studied through a comparative analysis of similar simpler systems. In this case, a comparative bioinformatics search was used to decrease the number of candidate genes of interest by comparing the genome of interest with other genomes of smaller size.

 **$\beta$ -jellyroll**

A structure in which anti-parallel  $\beta$ -strands are 'wrapped' into a cylindrical, barrel-like shape without necessarily maintaining a continuous hydrogen bonding network.

**Periplasmic chaperone**

A periplasmic protein that prevents macromolecules from aggregating and assists them in reaching their destination.

It has been suggested that 1–3 million molecules of LPS must be assembled on the bacterial cell surface per generation<sup>29</sup>. The transport and assembly of these large amphipathic LPS molecules presents several challenges, as LPS must efficiently cross three cellular compartments (the IM, the periplasm and the OM) unidirectionally, against a concentration gradient and without compromising the integrity of the cellular envelope. The energy responsible for driving LPS transport is derived from ATP hydrolysis catalysed by a cytoplasmic ATPase, LptB<sup>30,31</sup>. This energy must be coupled to movement across the periplasm and OM as there is no ATP in these compartments. To solve this problem while maintaining the integrity of the OM, the seven Lpt proteins form a trans-envelope protein bridge that spans from the cytoplasmic ATPase LptB to the OM translocon LptDE (FIG. 1).

**Components of the Lpt pathway**

**LPS extraction from the IM by LptB<sub>2</sub>FGC.** In *E. coli*, LptC, LptA and LptB are encoded by the *lptCAB* locus, which is located downstream of genes that are involved in the biosynthesis of KDO (3-deoxy-D-manno-octulosonic acid), a component of the LPS inner core (FIG. 1). *lptA* and *lptB* were first identified as essential genes in a genetic screen that was designed to identify conditionally lethal transposon mutants<sup>32</sup>. The aforementioned synteny and phenotypes that were observed on depletion of these genes facilitated the discovery of LptA, LptB and LptC as proteins that are required for the transport of LPS to the OM<sup>27</sup>. Through sequence homology, LptB was identified as a nucleotide-binding domain of an ABC transporter that is involved in the transport of LPS<sup>31</sup>. A reductionist bioinformatics approach led to the discovery of the transmembrane domains of this ABC transporter, LptF and LptG<sup>28</sup>. *In vivo* studies demonstrated that all of these Lpt proteins are required for the transport of LPS, and biochemical experiments confirmed the functional bioinformatics predictions. Furthermore, LptB<sub>2</sub>FG and LptB<sub>2</sub>FGC can be overexpressed and purified as complexes that have ATPase activity. Interestingly, the ATPase activity of these complexes is much higher than the activity of LptB alone<sup>33,34</sup>, suggesting that LptF and LptG help to stabilize LptB to facilitate its dimerization, which is required for ATP hydrolysis.

Based on these studies, it was proposed that LptB and LptFG are the ATPase and transmembrane components, respectively, of an ABC transporter that extracts LPS from the outer leaflet of the IM and interacts with LptC (FIG. 1). Recent crystallographic evidence demonstrates that there is substantial movement in the structure of LptB on ATP hydrolysis, and it is predicted that this movement couples ATP hydrolysis in the cytoplasm to changes in the transmembrane domains of the ABC transporter<sup>31</sup>. Sites on LptFG that are responsible for the interactions with other Lpt proteins have not been identified. By contrast, it is known that the transmembrane region of LptC is not essential for its function, and the soluble domain of LptC can form a complex with LptB<sub>2</sub>FG but with a weaker affinity than that of full-length LptC<sup>35</sup>. In addition, a single-residue

substitution on the amino-terminal edge of the  $\beta$ -jellyroll structure of LptC disrupts the formation of a complex with LptB<sub>2</sub>FG<sup>35</sup>. Therefore, these observations suggest that the N terminus of the  $\beta$ -jellyroll structure of LptC interacts with LptF and/or LptG. Both LptF and LptG are predicted to have six transmembrane regions and one large periplasmic domain, which may have a similar  $\beta$ -jellyroll fold to LptA and LptC<sup>28,35</sup>. Therefore, LptF and LptG are also speculated to contribute to the formation of a periplasmic Lpt bridge through these periplasmic domains (see below).

**LPS transport across the periplasm by the Lpt bridge.**

The six fatty acyl chains in the lipid A moiety of LPS in *E. coli* are unlikely to cross the aqueous periplasmic compartment unaided. Therefore, the periplasmic component of the Lpt system, LptA, is thought to mediate the transport of LPS across the periplasm<sup>32,36</sup>. By analogy to the transport of lipoproteins in *E. coli*, the transit of which from the IM to the OM is mediated by the soluble periplasmic chaperone LolA<sup>37–40</sup>, it was speculated that LptA could act as a soluble chaperone that shields the acyl chains of LPS during transport across the periplasm. However, the preponderance of evidence suggests that LptA does not facilitate LPS transport by acting as a soluble chaperone similar to LolA, but rather by forming a trans-envelope bridge that spans the periplasmic compartment (FIGS 1, 2).

The first important observation that challenged the notion that LptA acts as a soluble chaperone was that LPS is not released from the IM when a concentrated periplasmic fraction is added to spheroplasts, whereas lipoproteins are<sup>41</sup>. This demonstrates that lipoproteins form a soluble complex with LolA<sup>37,40</sup>, whereas LPS is not released from the membrane in a soluble form when the periplasmic fraction is added. Furthermore, pulse-chase experiments showed that LPS is transported to the OM even after the removal of soluble periplasmic contents<sup>41</sup>. The first biochemical evidence for an 'Lpt bridge' was the observation that the Lpt proteins, including LptA, co-fractionate in a distinct cellular fraction in sucrose gradients<sup>42</sup>. This fraction corresponds to a less-dense OM fraction that is known as OM<sub>I</sub> in which newly synthesized LPS transiently accumulates during its transport from the IM to the OM<sup>43</sup>. In addition, co-purification experiments using epitope-tagged versions of the IM proteins LptB, LptC and LptF resulted in the co-purification of the periplasmic protein LptA and the OM proteins LptD and LptE<sup>42</sup>. Taken together, these experiments provided evidence for a direct interaction between the Lpt proteins to form a physical bridge between the IM and OM that transports LPS across the periplasmic compartment, but the nature of the LPS–Lpt protein interaction remains unclear.

The first clues regarding the architecture of the Lpt bridge came from homology and structural studies. The N-terminal soluble domain of the OM protein LptD is homologous to the soluble domain of LptC and to LptA. These three domains found in LptD, LptC and LptA are all periplasmic and belong to the organic solvent tolerance protein A (OstA) superfamily<sup>35,44,45</sup> (FIGS 1, 2).

**Spheroplasts**

Osmotically fragile bacterial cells that have had their outer membranes and peptidoglycan layers incompletely disrupted, which causes them to form a spherical shape.

**Photo-crosslinking**

The light-induced formation of a covalent bond between two molecules to detect molecular interactions.

**Unnatural amino acid**

Non-coded, non-proteinogenic amino acid that, when incorporated into proteins, enables various new functions.

**Size-exclusion chromatography**

A chromatographic technique that is used for preparative or analytical purposes to separate molecules (usually macromolecules) based on their size.

**$\beta$ -barrel assembly machine pathway**

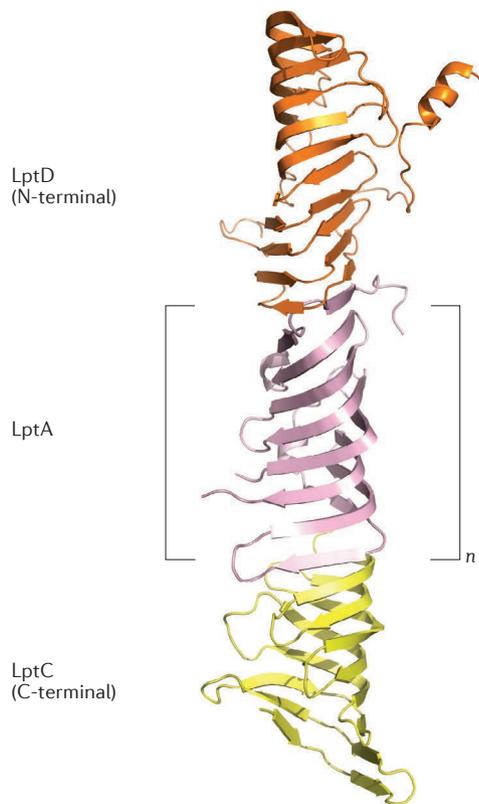
(Bam pathway). Following secretion from the inner membrane and translocation across the periplasm, the Bam complex is responsible for folding and inserting  $\beta$ -barrel proteins into the outer membrane. In *Escherichia coli*, the Bam complex is composed of one  $\beta$ -barrel protein, BamA, and four outer membrane lipoproteins, BamB, BamC, BamD and BamE.

**Localization of lipoproteins pathway**

(Lol pathway). A chaperone-based transport pathway that is involved in transporting outer membrane lipoproteins from the outer leaflet of the inner membrane to the inner leaflet of the outer membrane.

**Trypsin**

A serine protease that hydrolyses peptide bonds on the carboxy-terminal side of lysine and arginine residues, and is commonly used to determine the stability of proteins.



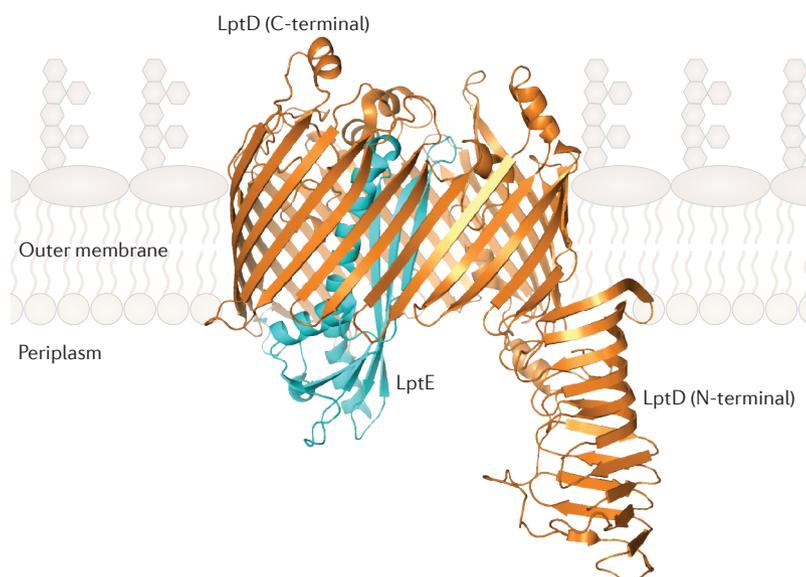
**Figure 2 | The periplasmic protein bridge.** The figure illustrates a model of the periplasmic protein bridge comprised of lipopolysaccharide (LPS) transport protein C (LptC), LptA and LptD. The carboxy-terminal periplasmic region of LptC (shown in yellow; RCSB Protein Data Bank (PDB) entry 3MY2), LptA (shown in pink; PDB entry 2R19) and amino-terminal region of LptD (shown in orange; PDB entry 4Q35) are stacked to illustrate the Lpt bridge. Two LptA molecules in the trigonal crystal form (PDB entry 2R1A) were replaced with the C-terminal domain of LptC and N-terminal domain of LptD. The number of LptA molecules in the bridge is unknown.

Understanding how the LptB<sub>2</sub>FGC complex in the IM is physically connected to LptA and to the LptDE complex in the OM was determined by *in vivo* photo-crosslinking<sup>46–49</sup> using the crystal structures of LptA and LptC as a reference<sup>45</sup>. Although the amino acid sequences of LptA and LptC are less than 10% identical, their structures are remarkably similar. Both LptA and LptC have slightly twisted  $\beta$ -jellyroll structures composed of 16 and 15 antiparallel  $\beta$ -strands, respectively<sup>36,50</sup> (FIG. 2). Interestingly, LptA crystallized as a filamentous oligomer in a head-to-tail fashion in the presence of LPS<sup>36</sup>. These observations led to the suggestion that LptC might be connected to LptD through one or more molecules of LptA. The continuous hydrophobic groove that is present in the OstA domains could then shield the lipid A portion of LPS molecules from the aqueous environment as they transverse the periplasm. This model was supported by *in vivo* photo-crosslinking experiments in which variants of LptC, LptA and LptD that contained an unnatural amino acid at different positions were used to define interaction sites between the OstA domains

in these proteins. These experiments showed that the carboxyl terminus of LptC interacts with the N terminus of LptA, and that the C terminus of LptA interacts with the N terminus of LptD<sup>45</sup> (FIG. 2). These interactions occur in a conserved manner and involve the edges of the respective  $\beta$ -jellyrolls. Various *in vitro* binding experiments further supported the architecture of the trans-envelope bridge that was observed *in vivo*. For example, purified LptA forms a complex with a soluble version of LptC that lacks its transmembrane region, and this complex co-purifies following size-exclusion chromatography<sup>51</sup>. In addition, alterations to the C-terminal domain of LptC or deletion of the N-terminal domain of LptD disrupt interactions with LptA, which is expected based on the predicted trans-envelope bridge structure<sup>35,45,52</sup>. Therefore, these experiments support the model proposing that head-to-tail oligomerization of these homologous OstA domains creates a trans-envelope bridge that connects the IM Lpt complex (LptB<sub>2</sub>FGC) and the OM Lpt complex (LptDE) through LptA (FIG. 2). It is not yet known how many LptA monomers comprise the bridge.

**LPS transport across the OM by LptDE.** One of the most intriguing questions regarding LPS biogenesis is how the OM components facilitate the translocation of LPS across the OM. This process is mediated by an OM translocon that contains two membrane proteins, LptD and LptE<sup>53–55</sup>. LptD and LptE are targeted to the OM by separate pathways: LptD is targeted to the OM by the  $\beta$ -barrel assembly machine pathway (Bam pathway)<sup>56–63</sup> and LptE is targeted to the OM by the localization of lipoproteins pathway (Lol pathway)<sup>37,38,64–68</sup>. In *E. coli*, both LptD and LptE are essential<sup>54,55</sup>, and biochemical studies have established that LptD and LptE interact with each other very strongly<sup>55,69</sup>. For example, LptD and LptE can be co-purified from the solubilized membranes of cells that overproduce these proteins, and they form a heterodimeric complex that is resistant to dissociation except when subjected to heat. Following size-exclusion chromatography, these two proteins co-migrate as a single band on a denaturing gel<sup>69</sup>.

Recently, two X-ray crystal structures of the LptDE complex have been solved<sup>70,71</sup>. One structure contained both an N-terminal periplasmic domain and a C-terminal  $\beta$ -barrel domain of LptD<sup>71</sup> (FIG. 3), whereas the second structure lacked the N-terminal periplasmic domain of LptD<sup>70</sup>. These structures confirm previous evidence, which predicted that the C-terminal portion of LptD interacts with LptE. These earlier studies included the demonstration that LptE is protected from proteolytic degradation (using trypsin) only when co-purified with LptD, which suggests that LptE resides within the C-terminal  $\beta$ -barrel of LptD<sup>69</sup>. Furthermore, *in vivo* photo-crosslinking using variants of LptE with an unnatural amino acid at different positions showed that several residues located on the surface of LptE crosslink with LptD<sup>72</sup>. Moreover, a putative extracellular loop of the  $\beta$ -barrel of LptD was identified by mass spectrometry analysis to be crosslinked with LptE<sup>72</sup>, and the key role of this loop in the formation of the heterodimeric complex was confirmed by the recent crystal structures



**Figure 3 | The outer membrane translocon.** The figure illustrates a model of the two protein plug-and-barrel in the outer membrane (OM) comprised of lipopolysaccharide (LPS) transport protein D (LptD; shown in orange; RCSB Protein Data Bank (PDB) entry 4Q35) and LptE (shown in cyan; PDB entry 4Q35). C-terminal, carboxy-terminal; N-terminal, amino-terminal.

of LptDE. However, despite these important advances in our understanding of the structure of the LptDE complex, how the formation of this complex occurs at the OM remains unclear.

The two-protein plug-and-barrel conformation in which LptE is located inside the barrel of LptD is probably important for the mechanism of LPS transport across, and insertion into, the OM. Notably, LptD is the largest monomeric  $\beta$ -barrel protein identified so far in the OM of Gram-negative organisms, with a lumen sufficient in size to permit LPS to cross the OM bilayer. In addition, LptD is a crenellated  $\beta$ -barrel in which two adjacent strands of the  $\beta$ -barrel are not completely hydrogen-bonded. If these putative crenels were to open to the outer leaflet of the OM, they could act as portals through which LPS molecules could travel from the lumen of the LptD barrel to the cell surface<sup>70–74</sup>. This would be analogous to the way in which lipids that are present in the outer leaflet of the OM diffuse into the lumen of the crenellated OM  $\beta$ -barrels of FadL and PagP<sup>75–80</sup>. Accordingly, the current model for how the LptDE OM translocon functions to place LPS on the cell surface is that LPS molecules arrive from the IM and periplasm at the periplasmic N-terminal domain of LptD, and this causes a conformational change in LptDE, which enables LPS molecules to enter the interior of the barrel. LPS can then move through the lumen of LptD, selectively passing through the lateral opening of LptD into the extracellular leaflet of the OM<sup>70,71,73,74</sup>. Importantly, details of the conformational changes in the translocon and where the sugars and the fatty acyl chains of LPS are located during translocation are still missing, although it has been proposed that the hydrophobic lipid A moiety goes through the hydrophobic intramembrane opening between the N-terminal and

#### Crenellated $\beta$ -barrel

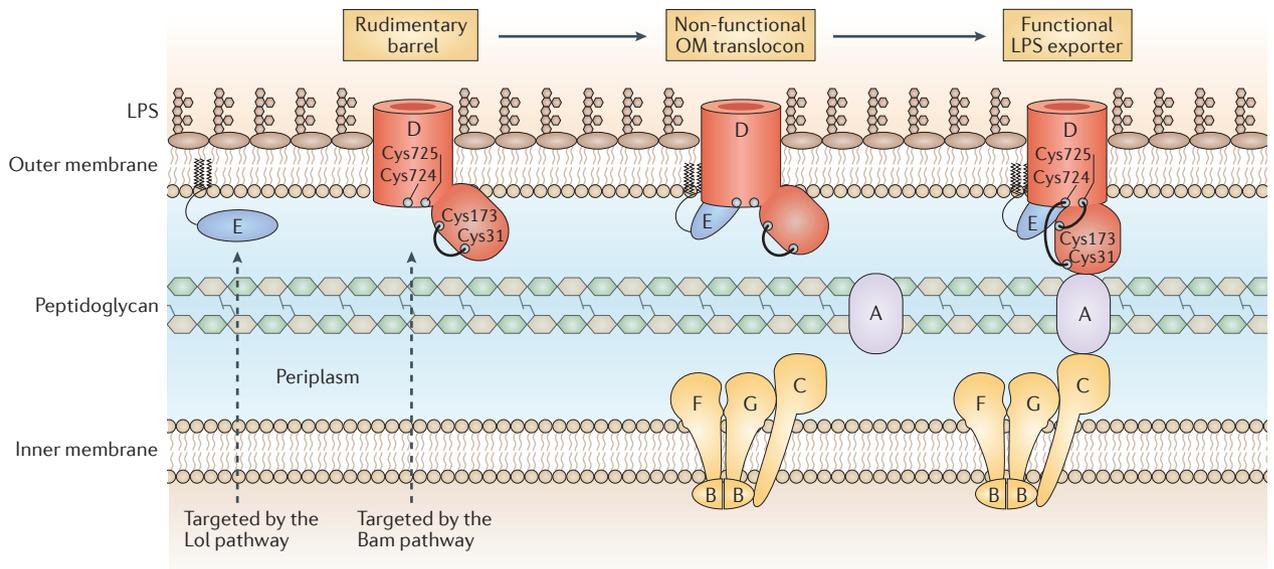
A  $\beta$ -barrel protein in which the formation of inter-strand hydrogen bonds is disrupted, creating openings similar to the crenels in the turret of a castle.

$\beta$ -barrel domains of LptD, whereas the rest of the LPS molecule goes through the lumen of LptD<sup>73,74</sup>. Furthermore, it is unclear whether there are direct interactions between the N-terminal portion of LptD and LptE during transport that might cause conformational changes in the translocon or promote interactions between LPS and LptE. LptE has been proposed to act as more than just a plug in the OM translocon, potentially having a role in the assembly of LPS at the cell surface by directly interacting with LPS<sup>69,81</sup>.

#### Regulation of Lpt bridge formation

How the cell assembles a trans-envelope complex of seven different Lpt proteins that are present in four separate cellular compartments (in the cytoplasm, IM, periplasm and OM) is an interesting question. Recent studies suggest that the cell determines whether a functional OM translocon has been assembled before docking the translocon to the periplasmic bridge, thereby coordinating the assembly of Lpt components<sup>35,45,82</sup>.

LptD has four cysteine residues, two in the N-terminal periplasmic OstA domain (Cys31 and Cys173) and two in the C-terminal  $\beta$ -barrel domain (Cys724 and Cys725; FIG. 4). A functional LptDE translocon has two intramolecular disulfide bonds between non-consecutive cysteine residues (Cys31–Cys724 and Cys173–Cys725), which connect the N-terminal and C-terminal domains of LptD<sup>83</sup>. Interestingly, either of these two disulfide bonds is sufficient for the function of LptD. However, this connectivity must be precise, even though the last cysteine residues (Cys724 and Cys725) are adjacent, which suggests that appropriate oxidation is required to correctly position the N-terminal and C-terminal domains of LptD<sup>83</sup>. Indeed, this has been confirmed by the recent characterization of the complex mechanism of correct LptD folding (FIG. 4). LptD is folded into a non-functional, rudimentary  $\beta$ -barrel structure at the OM by the Bam complex with the aid of the periplasmic chaperone SurA<sup>56,84</sup>. Genetic studies also suggest that LptE, which is targeted to the OM by the Lol pathway, interacts with LptD when LptD is bound to the Bam complex<sup>85</sup>. Furthermore, depletion of LptE results in incorrectly oxidized LptD, which demonstrates that LptE is essential for the correct oxidation of LptD<sup>82,83,85</sup>. From these studies and pulse-chase experiments that have identified several intermediates in the LptD folding pathway, we know that assembly of the LptDE complex involves a non-functional LptD intermediate that contains non-native disulfide bonds (Cys31–Cys173) formed by the periplasmic oxidase DsbA, and a rudimentary  $\beta$ -barrel structure formed by the Bam complex. The non-native disulfide bonds of this intermediate are then rearranged by DsbA into their native connectivity (Cys31–Cys724 and Cys173–Cys725), which is accompanied by folding of the  $\beta$ -barrel into a stable structure, which requires LptE<sup>82</sup> (FIG. 4). Importantly, it should be noted that even though at least one of the native disulfide bonds in LptD is required for viability, DsbA is not essential under the same conditions, as oxidants present in the growth medium and oxygen can partially substitute for DsbA<sup>83</sup>.



**Figure 4 | Regulation of the formation of the Lpt bridge.** The biogenesis of the functional lipopolysaccharide (LPS) transport (Lpt) outer membrane (OM) translocon, LptDE, requires disulfide bond rearrangements at the OM. LptD and LptE are targeted to the OM by the  $\beta$ -barrel assembly machine pathway (Bam pathway) and the localization of lipoproteins pathway (Lol pathway), respectively. LptD has four cysteines, two in the amino-terminal periplasmic region (Cys31 and Cys173) and two in the  $\beta$ -barrel domain (Cys724 and Cys725). LptD, with a disulfide bond between Cys31 and Cys173, forms a non-functional complex with LptE, followed by several disulfide bond rearrangements to produce a functional translocon with native disulfide bonds (Cys31–Cys724 and Cys173–Cys725). The formation of a functional translocon enables the N-terminal domain of LptD to interact with LptA, which results in a functional LPS transporter that includes the inner membrane (IM) complex, LptB<sub>2</sub>FGC. It is unknown how the interaction between LptA and LptC is regulated. The letters A–G in the figure correspond to the respective Lpt protein in the transport pathway.

These results explain how a functional OM translocon is assembled, but it is possible that other proteins are involved in the process. For example, the metallo-proteinase  $\beta$ -barrel assembly-enhancing protease (BepA) and the disulfide isomerase DsbC have also been implicated in the biogenesis of LptD and disulfide rearrangement<sup>86,87</sup>. BepA degrades misfolded LptD and its proposed chaperone function stimulates disulfide rearrangement in LptD through an unknown mechanism<sup>87</sup>. Overexpression of a variant of DsbC that traps substrates has shown that this variant interacts with LptD<sup>86</sup>. However, the biological significance of the DsbC–LptD interaction is unclear, as deletion of *dsbC* has no detectable effect on the formation of correctly oxidized LptD. Furthermore, disulfide-bond rearrangement intermediates of LptD have been shown to interact with DsbA, instead of DsbC<sup>82,83</sup>.

Understanding the mechanism of assembly of the LptDE translocon in the OM has also elucidated how the formation of the trans-envelope Lpt bridge is regulated. Notably, *in vivo*, variants of LptD that lack native disulfide bonds do not interact with LptA<sup>45</sup>. This finding suggests a model in which the disulfide bond rearrangement that occurs after the correct assembly of the LptDE complex is required to ensure that a functional LptDE translocon interacts with LptA, enabling the establishment of the trans-envelope bridge. The interaction between LptDE and LptA is thought to prevent mislocalization of LPS, which would occur if LptA interacted with a premature, improperly assembled LptDE complex. In addition,

the regulation of Lpt bridge formation was suggested to involve LptC, as impairing the interaction of LptC with LptB<sub>2</sub>FG destabilizes the entire Lpt trans-envelope complex<sup>35</sup>. Importantly, it is still unknown whether functional LptDE interacts with soluble LptA or with LptA that is already interacting with LptC.

### Molecular mechanism of LPS transport

As Lpt proteins were identified, genetic and biochemical evidence began to accumulate which supported the model that the Lpt trans-envelope bridge transports LPS from the outer leaflet of the IM to the outer leaflet of the OM. Furthermore, depletion of any of the Lpt proteins leads to the accumulation of LPS in the periplasmic leaflet of the IM<sup>27,28</sup>, which is consistent with a model in which all of the Lpt proteins are part of one single transport machine. According to this model, ‘breaking’ any component of this machine causes LPS to accumulate at the beginning of the process, in the outer leaflet of the IM. The trans-envelope bridge model also predicts that some Lpt proteins must interact directly with LPS. Although *in vitro* LPS-binding assays suggest that LptA, LptC and LptE interact with LPS<sup>50,69,88</sup>, a recent breakthrough involved the detection of intermediate interactions between Lpt proteins and LPS in a cell<sup>30,74</sup>.

To study the molecular mechanism of LPS transport, LPS binding to Lpt proteins first needed to be detected *in vivo*. This was possible by photo-crosslinking LPS to an unnatural amino acid located at specific positions in LptA and LptC. Trapping LPS in these Lpt proteins *in vivo*

**Right-side-out membrane vesicles**

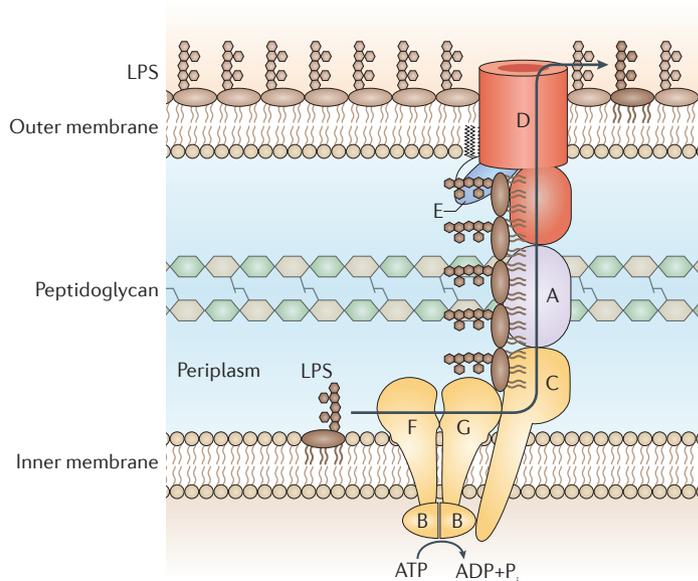
Membrane vesicles with a native orientation that are prepared by the osmotic lysis of spheroplasts.

**Vanadate**

Sodium orthovanadate ( $\text{Na}_2\text{VO}_4$ ) inhibits protein tyrosine phosphatases, alkaline phosphatases and many ATPases by acting as a phosphate analogue and binding in the active site in which phosphate usually binds.

clarified that, during transport, LPS interacts with the hydrophobic groove of the  $\beta$ -jellyroll domain of LptA and LptC<sup>30</sup>. LPS accumulated in LptC or in LptA depending on the co-expression of LptB<sub>2</sub>FG or LptB<sub>2</sub>FGC, respectively<sup>30</sup>. These results confirmed that the crosslinked products are intermediates of LPS transport by Lpt components, and that LptC and LptA require LptB<sub>2</sub>FG and LptB<sub>2</sub>FGC, respectively, to receive LPS.

A similar strategy that involved photo-crosslinking LPS to an unnatural amino acid was also used to demonstrate the predicted dependency of LPS transport on ATP hydrolysis<sup>30</sup>. For example, right-side-out membrane vesicles<sup>89–91</sup> containing an LptC variant that is known to crosslink to LPS *in vivo* were prepared with or without ATP. This LptC variant crosslinked to LPS in an LptB<sub>2</sub>FG-dependent, time-dependent and ATP-dependent manner, which suggests that LPS is transported from the IM to LptC using energy generated through ATP hydrolysis by LptB<sub>2</sub>FG. When LptA mutants that are known to crosslink with LPS or with LptC *in vivo* were added to the right-side-out membrane vesicles, the release of LPS from the IM to LptA was dependent on LptB<sub>2</sub>FGC, time and ATP, whereas the interaction of LptA with LptC was not.



**Figure 5 | The PEZ model.** The transport of lipopolysaccharide (LPS) from the inner membrane (IM) to LPS transport protein C (LptC), and from LptC to LptA, requires energy that is derived from the hydrolysis of ATP. LPS binding sites present in both LptC and LptA are constantly occupied by LPS molecules. The observation that several rounds of ATP hydrolysis are required to transport LPS to the cell surface, and that LPS binding sites in LptC and LptA are always filled, suggests that ATP is required to push a continuous stream of LPS through the Lpt bridge. Therefore, the PEZ model suggests that the transport of LPS occurs by analogy to a PEZ candy dispenser, in which PEZ candies that fill the dispenser are pushed by a spring at the bottom of the dispenser. In this model, LPS molecules in the outer leaflet of the IM are pushed towards LptC through the action of LptB<sub>2</sub>FG, in a process that is dependent on the hydrolysis of ATP in the cytoplasm, which is mediated by the ATPase LptB in the LptB<sub>2</sub>FG complex. LPS is then pushed from LptC to LptA and across the Lpt periplasmic bridge towards the LptDE translocon, in a process that also involves ATP hydrolysis mediated by LptB<sub>2</sub>FGC. LPS is then proposed to cross the translocon with the lipid portion of LPS being directly inserted into the outer leaflet of the outer membrane (OM) without entering the lumen of the LptD barrel, whereas the sugar portion of LPS travels through the barrel. The letters A–G in the figure correspond to the respective Lpt protein in the transport pathway. P<sub>i</sub>, inorganic phosphate.

Remarkably, if LptA was added after LPS had already accumulated in LptC, the transfer of LPS from LptC to LptA required additional hydrolysis of ATP; this was shown by inhibiting the ATPase activity of the LptB<sub>2</sub>FGC complex using vanadate<sup>30</sup>. This observation suggested the existence of at least two ATP hydrolysis steps during the transport of LPS from the IM to the cell surface: the first step involves the transfer of LPS from the IM to LptC; and the second step involves the transfer of LPS from LptC to LptA. Moreover, in the absence of vanadate, the amount of LPS that was bound to LptC remained constant, even when LPS was transferred to LptA, which suggests that LPS-binding sites in the Lpt periplasmic bridge are always filled with LPS during transport.

Collectively, these data act as the basis for the newly proposed ‘PEZ’ model of LPS transport. This model proposes that Lpt proteins function similarly to a PEZ candy dispenser, in which PEZ candies that fill the dispenser are pushed by a spring at the bottom of the dispenser. In this model, LPS molecules in the outer leaflet of the IM are pushed towards LptC by the action of LptB<sub>2</sub>FG, in a process that depends on ATP hydrolysis by LptB in the cytoplasm. Subsequently, LPS is pushed from LptC to LptA and across the Lpt periplasmic bridge towards the LptDE translocon, in a process that also involves ATP hydrolysis mediated by LptB<sub>2</sub>FGC. It has been suggested that the lipid portion of LPS is then directly inserted into the outer leaflet of the OM without entering the lumen of the LptD barrel, whereas the sugar portion passes through the barrel; this suggestion is based on molecular dynamics simulations, mutagenesis experiments and the observation that LPS interacts with various positions in LptD, including the hydrophobic groove of the  $\beta$ -jellyroll at the N terminus<sup>73,74</sup>. The observation that LPS binding sites in both LptC and LptA are constantly occupied by LPS molecules, and the fact that there are LPS binding sites at the N terminus of LptD, suggest that there is a continuous stream of LPS from the IM to the cell surface, with the energy for transport being sequentially provided by the cytoplasmic hydrolysis of ATP (FIG. 5).

**Outlook**

*In vitro* assays using right-side-out membrane vesicles and photo-crosslinkable proteins enabled the study of the mechanism of LPS transport from the IM to the periplasmic protein LptA. We presume that the LptB<sub>2</sub>FG complex extracts LPS from the outer leaflet of the IM and transports it to the periplasmic domain of LptC, and then to LptA; however, the roles of LptF and LptG are unclear. Notably, LptB<sub>2</sub>FG is an unusual ABC transporter, as it mediates the transport of its substrate from the membrane to the periplasm, whereas most ABC transporters mediate the transport of substrates across the membrane. Some insight into LptB<sub>2</sub>FG may be gained by comparisons with LolCD<sub>2</sub>E, which is a similar type of ABC transporter that extracts lipoproteins from the outer leaflet of the IM and transfers them to the periplasmic chaperone LolA<sup>38,66,92–94</sup>. In LolCD<sub>2</sub>E, each of the membrane subunits, LolC and LolE, is predicted to have a large periplasmic domain that functions as a scaffold for LolA and as a binding site for the substrate, respectively<sup>65,95,96</sup>. Therefore, LptF and

## Peptidomimetic

Chemical compounds that mimic natural peptides because of their small protein-like chains.

LptG may also have distinct functions with respect to each other. In the future, crystal structures of the periplasmic domains of LptF and LptG and *in vivo* photo-crosslinking studies might help us to understand the functions of the membrane subunits of this LPS extractor.

Another important question that remains unanswered is how a molecule containing up to 200 sugars crosses the OM. We speculate that LptE, when located inside the  $\beta$ -barrel of LptD, has some function in assembling LPS at the cell surface of *E. coli*, owing to its high affinity for LPS and its ability to disaggregate LPS *in vitro*<sup>69,81</sup>. This functional role of LptE could differ in organisms in which LPS is not essential, such as *Neisseria meningitidis*<sup>97</sup>. The crystal structures of the LptDE complex have been helpful in interpreting biochemical and genetic data related to the later stages of LPS transport. However, a reconstitution from pure components is necessary to determine the details of how the Lpt proteins work together to efficiently transport LPS. This is technically challenging because it requires a method to stably bridge two different types of proteoliposome (containing separate IM and OM Lpt components) with soluble LptA.

Importantly, elucidating the molecular mechanisms of LPS biosynthesis and transport has the potential

to inform the development of novel therapies against Gram-negative bacteria that require LPS for viability. For example, the peptidomimetic antibiotic L27-11, which kills *Pseudomonas aeruginosa*, was recently reported to bind to LptD<sup>98,99</sup>. This is based on the observations that a photoactive analogue of L27-11 crosslinks LptD and that mutations in *lptD* confer resistance to this compound, which establishes LptD as the target of L27-11. This small molecule is the first compound known to target the Lpt proteins, and its discovery suggests that the LPS transport pathway can be a useful target for novel antimicrobials.

The Lpt trans-envelope complex presents a challenge for rational drug development because the PEZ model suggests that a large area of the bridge contacts LPS at any given time, which makes it difficult to interfere with transport. However, the positions in LptA and LptC found to crosslink with LPS represent sites at which LPS is bound for longer periods of time. These binding sites could be targeted in the development of inhibitors, which might be antibiotics themselves or might make the cell more susceptible to existing antibiotics because of the central role that LPS has in providing a barrier-like quality to the OM.

- Nikaido, H. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol. Mol. Biol. Rev.* **67**, 593–656 (2003).
- Berg, H. C. The rotary motor of bacterial flagella. *Annu. Rev. Biochem.* **72**, 19–54 (2003).
- Nan, B., McBride, M. J., Chen, J., Zusman, D. R. & Oster, G. Bacteria that glide with helical tracks. *Curr. Biol.* **24**, R169–R173 (2014).
- Lai, Y., Rosenshine, I., Leong, J. M. & Frankel, G. Intimate host attachment: enteropathogenic and enterohaemorrhagic *Escherichia coli*. *Cell. Microbiol.* **15**, 1796–1808 (2013).
- Laverty, G., Gorman, S. P. & Gilmore, B. F. Biomolecular mechanisms of *Pseudomonas aeruginosa* and *Escherichia coli* biofilm formation. *Pathogens* **3**, 596–632 (2014).
- Raetz, C. R. & Whitfield, C. Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**, 635–700 (2002).
- Garten, W., Hindennach, I. & Henning, U. The major proteins of the *Escherichia coli* outer cell-envelope membrane. Cyanogen bromide fragments of protein I, composition and order. *Eur. J. Biochem.* **60**, 303–307 (1975).
- Kamio, Y. & Nikaido, H. Outer membrane of *Salmonella typhimurium*: accessibility of phospholipid head groups to phospholipase c and cyanogen bromide activated dextran in the external medium. *Biochemistry* **15**, 2561–2570 (1976).  
**This study is the first to demonstrate that phospholipids are not exposed on the cell surface of Gram-negative bacteria.**
- Osborn, M. J., Gander, J. E., Parisi, E. & Carson, J. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. *J. Biol. Chem.* **247**, 3962–3972 (1972).  
**This study demonstrates that LPS fractionates almost exclusively with the OM following sucrose-density gradient centrifugation.**
- Sutcliffe, I. C. A phylum level perspective on bacterial cell envelope architecture. *Trends Microbiol.* **18**, 464–470 (2010).
- Ruiz, N., Kahne, D. & Silhavy, T. J. Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *Nat. Rev. Microbiol.* **7**, 677–683 (2009).
- Raetz, C. R., Reynolds, C. M., Trent, M. S. & Bishop, R. E. Lipid A modification systems in Gram-negative bacteria. *Annu. Rev. Biochem.* **76**, 295–329 (2007).
- Whitfield, C. & Trent, M. S. Biosynthesis and export of bacterial lipopolysaccharides. *Annu. Rev. Biochem.* **83**, 99–128 (2014).
- Osborn, M. J., Gander, J. E. & Parisi, E. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Site of synthesis of lipopolysaccharide. *J. Biol. Chem.* **247**, 3973–3986 (1972).  
**This paper establishes that LPS is synthesized inside the cell, raising the question of how LPS is transported to its final destination.**
- Karow, M. & Georgopoulos, C. The essential *Escherichia coli msbA* gene, a multicopy suppressor of null mutations in the *htrB* gene, is related to the universally conserved family of ATP-dependent translocators. *Mol. Microbiol.* **7**, 69–79 (1993).
- Polissi, A. & Georgopoulos, C. Mutational analysis and properties of the *msbA* gene of *Escherichia coli*, coding for an essential ABC family transporter. *Mol. Microbiol.* **20**, 1221–1233 (1996).
- Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C. & Raetz, C. R. Function of *Escherichia coli* MsbA, an essential ABC family transporter, in lipid A and phospholipid biosynthesis. *J. Biol. Chem.* **273**, 12466–12475 (1998).
- Doerfler, W. T., Gibbons, H. S. & Raetz, C. R. MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. *J. Biol. Chem.* **279**, 45102–45109 (2004).
- Ward, A., Reyes, C. L., Yu, J., Roth, C. B. & Chang, G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proc. Natl Acad. Sci. USA* **104**, 19005–19010 (2007).
- McGrath, B. C. & Osborn, M. J. Localization of terminal steps of O-antigen synthesis in *Salmonella typhimurium*. *J. Bacteriol.* **173**, 649–654 (1991).
- Whitfield, C. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu. Rev. Biochem.* **75**, 39–68 (2006).
- Cuthbertson, L., Kos, V. & Whitfield, C. ABC transporters involved in export of cell surface glycoconjugates. *Microbiol. Mol. Biol. Rev.* **74**, 341–362 (2010).
- Greenfield, L. K. & Whitfield, C. Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. *Carbohydr. Res.* **356**, 12–24 (2012).
- Sperandeo, P., Deho, G. & Polissi, A. The lipopolysaccharide transport system of Gram-negative bacteria. *Biochim. Biophys. Acta* **1791**, 594–602 (2009).
- Simpson, B. W., May, J. M., Sherman, D. J., Kahne, D. & Ruiz, N. Lipopolysaccharide transport to the cell surface: biosynthesis and extraction from the inner membrane. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **370**, 20150029 (2015).
- May, J. M., Sherman, D. J., Simpson, B. W., Ruiz, N. & Kahne, D. Lipopolysaccharide transport to the cell surface: periplasmic transport and assembly into the outer membrane. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **370**, 20150027 (2015).
- Sperandeo, P. *et al.* Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J. Bacteriol.* **190**, 4460–4469 (2008).
- Ruiz, N., Gronenberg, L. S., Kahne, D. & Silhavy, T. J. Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **105**, 5537–5542 (2008).
- Rietschel, E. T. *et al.* Bacterial endotoxin: molecular relationships of structure to activity and function. *FASEB J.* **8**, 217–225 (1994).
- Okuda, S., Freinkman, E. & Kahne, D. Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science* **338**, 1214–1217 (2012).  
**This study describes the first observation of intermediate LPS transport states in living cells.**
- Sherman, D. J. *et al.* Decoupling catalytic activity from biological function of the ATPase that powers lipopolysaccharide transport. *Proc. Natl Acad. Sci. USA* **111**, 4982–4987 (2014).  
**This paper provides the first biochemical and genetic evidence that LPS transport is powered by ATP hydrolysis by LptB.**
- Sperandeo, P. *et al.* Characterization of *lptA* and *lptB*, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. *J. Bacteriol.* **189**, 244–253 (2007).
- Narita, S. & Tokuda, H. Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides. *FEBS Lett.* **583**, 2160–2164 (2009).  
**This study describes the purification of the LptBFGC complex at the IM.**
- Sherman, D. J., Okuda, S., Denny, W. A. & Kahne, D. Validation of inhibitors of an ABC transporter required to transport lipopolysaccharide to the cell surface in *Escherichia coli*. *Bioorg. Med. Chem.* **21**, 4846–4851 (2013).
- Villa, R. *et al.* The *Escherichia coli* Lpt trans-envelope protein complex for lipopolysaccharide export is assembled via conserved structurally homologous domains. *J. Bacteriol.* **195**, 1100–1108 (2013).
- Suits, M. D., Sperandeo, P., Deho, G., Polissi, A. & Jia, Z. Novel structure of the conserved Gram-negative lipopolysaccharide transport protein A and mutagenesis analysis. *J. Mol. Biol.* **380**, 476–488 (2008).

37. Matsuyama, S., Tajima, T. & Tokuda, H. A novel periplasmic carrier protein involved in the sorting and transport of *Escherichia coli* lipoproteins destined for the outer membrane. *EMBO J.* **14**, 3365–3372 (1995).
38. Okuda, S. & Tokuda, H. Lipoprotein sorting in bacteria. *Annu. Rev. Microbiol.* **65**, 239–259 (2011).
39. Takeda, K. *et al.* Crystal structures of bacterial lipoprotein localization factors, LolA and LolB. *EMBO J.* **22**, 3199–3209 (2003).
40. Okuda, S., Watanabe, S. & Tokuda, H. A short helix in the C-terminal region of LolA is important for the specific membrane localization of lipoproteins. *FEBS Lett.* **582**, 2247–2251 (2008).
41. Tefsen, B., Geurtsen, J., Beckers, F., Tommassen, J. & de Cock, H. Lipopolysaccharide transport to the bacterial outer membrane in spheroplasts. *J. Biol. Chem.* **280**, 4504–4509 (2005).  
**This study provides the first evidence that LPS transport does not involve a soluble periplasmic chaperone.**
42. Chng, S. S., Gronenberg, L. S. & Kahne, D. Proteins required for lipopolysaccharide assembly in *Escherichia coli* form a transenvelope complex. *Biochemistry* **49**, 4565–4567 (2010).  
**This paper provides evidence that LPS transport involves a trans-envelope bridge.**
43. Ishidate, K. *et al.* Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the inner and outer membranes and the murein skeleton of the cell envelope. *J. Biol. Chem.* **261**, 428–443 (1986).
44. Bos, M. P., Robert, V. & Tommassen, J. Biogenesis of the Gram-negative bacterial outer membrane. *Annu. Rev. Microbiol.* **61**, 191–214 (2007).
45. Freinkman, E., Okuda, S., Ruiz, N. & Kahne, D. Regulated assembly of the transenvelope protein complex required for lipopolysaccharide export. *Biochemistry* **51**, 4800–4806 (2012).
46. Chin, J. W., Martin, A. B., King, D. S., Wang, L. & Schultz, P. G. Addition of a photocrosslinking amino acid to the genetic code of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **99**, 11020–11024 (2002).
47. Liu, C. C. & Schultz, P. G. Adding new chemistries to the genetic code. *Annu. Rev. Biochem.* **79**, 413–444 (2010).
48. Ryu, Y. & Schultz, P. G. Efficient incorporation of unnatural amino acids into proteins in *Escherichia coli*. *Nat. Methods* **3**, 263–265 (2006).
49. Wang, L., Brock, A., Herberich, B. & Schultz, P. G. Expanding the genetic code of *Escherichia coli*. *Science* **292**, 498–500 (2001).
50. Tran, A. X., Dong, C. & Whitfield, C. Structure and functional analysis of LptC, a conserved export protein involved in the lipopolysaccharide export pathway in *Escherichia coli*. *J. Biol. Chem.* **285**, 33529–33539 (2010).
51. Bowyer, A., Baardsnes, J., Ajamian, E., Zhang, L. & Cygler, M. Characterization of interactions between LPS transport proteins of the Lpt system. *Biochem. Biophys. Res. Commun.* **404**, 1093–1098 (2011).
52. Sperandio, P. *et al.* New insights into the Lpt machinery for lipopolysaccharide transport to the cell surface: LptA–LptC interaction and LptA stability as sensors of a properly assembled transenvelope complex. *J. Bacteriol.* **193**, 1042–1053 (2011).
53. Bos, M. P., Tefsen, B., Geurtsen, J. & Tommassen, J. Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. *Proc. Natl Acad. Sci. USA* **101**, 9417–9422 (2004).
54. Braun, M. & Silhavy, T. J. Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*. *Mol. Microbiol.* **45**, 1289–1302 (2002).
55. Wu, T. *et al.* Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **103**, 11754–11759 (2006).
56. Hagan, C. L., Silhavy, T. J. & Kahne, D.  $\beta$ -barrel membrane protein assembly by the Bam complex. *Annu. Rev. Biochem.* **80**, 189–210 (2011).
57. Ruiz, N., Falcone, B., Kahne, D. & Silhavy, T. J. Chemical conditionality: a genetic strategy to probe organelle assembly. *Cell* **121**, 307–317 (2005).
58. Voulhoux, R., Bos, M. P., Geurtsen, J., Mols, M. & Tommassen, J. Role of a highly conserved bacterial protein in outer membrane protein assembly. *Science* **299**, 262–265 (2003).
59. Wu, T. *et al.* Identification of a multicomponent complex required for outer membrane biogenesis in *Escherichia coli*. *Cell* **121**, 235–245 (2005).
60. Sklar, J. G. *et al.* Lipoprotein SmpA is a component of the YaeT complex that assembles outer membrane proteins in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **104**, 6400–6405 (2007).
61. Ricci, D. P. & Silhavy, T. J. The Bam machine: a molecular cooper. *Biochim. Biophys. Acta* **1818**, 1067–1084 (2012).
62. Rigel, N. W. & Silhavy, T. J. Making a  $\beta$ -barrel: assembly of outer membrane proteins in Gram-negative bacteria. *Curr. Opin. Microbiol.* **15**, 189–193 (2012).
63. Eggert, U. S. *et al.* Genetic basis for activity differences between vancomycin and glycolipid derivatives of vancomycin. *Science* **294**, 361–364 (2001).
64. Matsuyama, S., Yokota, N. & Tokuda, H. A novel outer membrane lipoprotein, LolB (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer membrane of *Escherichia coli*. *EMBO J.* **16**, 6947–6955 (1997).
65. Okuda, S. & Tokuda, H. Model of mouth-to-mouth transfer of bacterial lipoproteins through inner membrane LolC, periplasmic LolA, and outer membrane LolB. *Proc. Natl Acad. Sci. USA* **106**, 5877–5882 (2009).
66. Yakushi, T., Masuda, K., Narita, S., Matsuyama, S. & Tokuda, H. A new ABC transporter mediating the detachment of lipid-modified proteins from membranes. *Nat. Cell Biol.* **2**, 212–218 (2000).
67. Tsukahara, J., Mukaiyama, K., Okuda, S., Narita, S. & Tokuda, H. Dissection of LolB function — lipoprotein binding, membrane targeting and incorporation of lipoproteins into lipid bilayers. *FEBS J.* **276**, 4496–4504 (2009).
68. Nakada, S. *et al.* Structural investigation of the interaction between LolA and LolB using NMR. *J. Biol. Chem.* **284**, 24634–24643 (2009).
69. Chng, S. S., Ruiz, N., Chimalakonda, G., Silhavy, T. J. & Kahne, D. Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. *Proc. Natl Acad. Sci. USA* **107**, 5363–5368 (2010).
70. Dong, H. *et al.* Structural basis for outer membrane lipopolysaccharide insertion. *Nature* **511**, 52–56 (2014).
71. Qiao, S., Luo, Q., Zhao, Y., Zhang, X. C. & Huang, Y. Structural basis for lipopolysaccharide insertion in the bacterial outer membrane. *Nature* **511**, 108–111 (2014).  
**Together with reference 70, this article reports the first crystal structures of LptDE.**
72. Freinkman, E., Chng, S. S. & Kahne, D. The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc. Natl Acad. Sci. USA* **108**, 2486–2491 (2011).  
**This paper establishes that LptD and LptE form a plug-and-barrel structure and suggests that membrane insertion proceeds through a lateral gate in a proline-rich region of the  $\beta$ -barrel of LptD.**
73. Gu, Y. *et al.* Lipopolysaccharide is inserted into the outer membrane through an intramembrane hole, a lumen gate, and the lateral opening of LptD. *Structure* **23**, 496–504 (2015).
74. Li, X., Gu, Y., Dong, H., Wang, W. & Dong, C. Trapped lipopolysaccharide and LptD intermediates reveal lipopolysaccharide translocation steps across the *Escherichia coli* outer membrane. *Sci. Rep.* **5**, 11883 (2015).
75. Ahn, V. E. *et al.* A hydrocarbon ruler measures palmitate in the enzymatic acylation of endotoxin. *EMBO J.* **23**, 2931–2941 (2004).  
**This study is the first to suggest a gating mechanism by which lipids pass into the lumen of a  $\beta$ -barrel through a lateral opening.**
76. Bishop, R. E. The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis. *Mol. Microbiol.* **57**, 900–912 (2005).
77. Khan, M. A. & Bishop, R. E. Molecular mechanism for lateral lipid diffusion between the outer membrane external leaflet and a  $\beta$ -barrel hydrocarbon ruler. *Biochemistry* **48**, 9745–9756 (2009).
78. Hearn, E. M., Patel, D. R., Lepore, B. W., Indic, M. & van den Berg, B. Transmembrane passage of hydrophobic compounds through a protein channel wall. *Nature* **458**, 367–370 (2009).
79. van den Berg, B. Going forward laterally: transmembrane passage of hydrophobic molecules through protein channel walls. *ChemBiochem* **11**, 1339–1343 (2010).
80. van den Berg, B., Black, P. N., Clemons, W. M. & Rapoport, T. A. Crystal structure of the long-chain fatty acid transporter FadL. *Science* **304**, 1506–1509 (2004).
81. Malojcic, G. *et al.* LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. *Proc. Natl Acad. Sci. USA* **111**, 9467–9472 (2014).
82. Chng, S. S. *et al.* Disulfide rearrangement triggered by translocon assembly controls lipopolysaccharide export. *Science* **337**, 1665–1668 (2012).
83. Ruiz, N., Chng, S. S., Hiniker, A., Kahne, D. & Silhavy, T. J. Nonconsecutive disulfide bond formation in an essential integral outer membrane protein. *Proc. Natl Acad. Sci. USA* **107**, 12245–12250 (2010).
84. Vertommen, D., Ruiz, N., Leverrier, P., Silhavy, T. J. & Collet, J. F. Characterization of the role of the *Escherichia coli* periplasmic chaperone SurA using differential proteomics. *Proteomics* **9**, 2432–2443 (2009).
85. Chimalakonda, G. *et al.* Lipoprotein LptE is required for the assembly of LptD by the  $\beta$ -barrel assembly machine in the outer membrane of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **108**, 2492–2497 (2011).
86. Denoncin, K., Vertommen, D., Paek, E. & Collet, J. F. The protein–disulfide isomerase DsbC cooperates with SurA and DsbA in the assembly of the essential  $\beta$ -barrel protein LptD. *J. Biol. Chem.* **285**, 29425–29433 (2010).
87. Narita, S., Masui, C., Suzuki, T., Dohmae, N. & Akiyama, Y. Protease homolog BepA (YfgC) promotes assembly and degradation of  $\beta$ -barrel membrane proteins in *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **110**, E3612–E3621 (2013).
88. Tran, A. X., Trent, M. S. & Whitfield, C. The LptA protein of *Escherichia coli* is a periplasmic lipid A-binding protein involved in the lipopolysaccharide export pathway. *J. Biol. Chem.* **283**, 20342–20349 (2008).
89. Kaback, H. R. Bacterial membranes. *Methods Enzymol.* **22**, 99–120 (1971).
90. Kaback, H. R. & Stadtman, E. R. Proline uptake by an isolated cytoplasmic membrane preparation of *Escherichia coli*. *Proc. Natl Acad. Sci. USA* **55**, 920–927 (1966).
91. Kim, Y. J., Rajapandi, T. & Oliver, D. SecA protein is exposed to the periplasmic surface of the *E. coli* inner membrane in its active state. *Cell* **78**, 845–853 (1994).
92. Masuda, K., Matsuyama, S. & Tokuda, H. Elucidation of the function of lipoprotein-sorting signals that determine membrane localization. *Proc. Natl Acad. Sci. USA* **99**, 7390–7395 (2002).
93. Ito, Y., Kanamaru, K., Taniguchi, N., Miyamoto, S. & Tokuda, H. A novel ligand bound ABC transporter, LolCDE, provides insights into the molecular mechanisms underlying membrane detachment of bacterial lipoproteins. *Mol. Microbiol.* **62**, 1064–1075 (2006).
94. Taniguchi, N. & Tokuda, H. Molecular events involved in a single cycle of ligand transfer from an ATP binding cassette transporter, LolCDE, to a molecular chaperone, LolA. *J. Biol. Chem.* **283**, 8538–8544 (2008).
95. Mizutani, M. *et al.* Functional differentiation of structurally similar membrane subunits of the ABC transporter LolCDE complex. *FEBS Lett.* **587**, 23–29 (2013).
96. Yasuda, M., Iguchi-Yokoyama, A., Matsuyama, S., Tokuda, H. & Narita, S. Membrane topology and functional importance of the periplasmic region of ABC transporter LolCDE. *Biosci. Biotechnol. Biochem.* **73**, 2310–2316 (2009).
97. Bos, M. P. & Tommassen, J. The LptD chaperone LptE is not directly involved in lipopolysaccharide transport in *Neisseria meningitidis*. *J. Biol. Chem.* **286**, 28688–28696 (2011).
98. Srinivas, N. *et al.* Peptidomimetic antibiotics target outer-membrane biogenesis in *Pseudomonas aeruginosa*. *Science* **327**, 1010–1013 (2010).
99. Werneburg, M. *et al.* Inhibition of lipopolysaccharide transport to the outer membrane in *Pseudomonas aeruginosa* by peptidomimetic antibiotics. *ChemBiochem* **13**, 1767–1775 (2012).

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**Competing interests statement**

The authors declare no competing interests.

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