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

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Abstract

Gram-negative bacteria contain a double-membrane cellular envelope that enables them to colonize harsh environments and prevents entry of many clinically available antibiotics. A main component of most outer membranes is lipopolysaccharide (LPS), a glycolipid containing multiple fatty acyl chains and up to hundreds of sugars that is synthesized in the cytoplasm. In the last two decades, the proteins 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 explaining how energy from the cytoplasm is used to power LPS transport across the cellular envelope to the cell surface.

Gram-negative bacteria possess an inner membrane (IM), which surrounds their cytoplasm, and an outer membrane (OM), which contacts the environment. The OM of Gram-negative bacteria is essential, and its proper assembly is required for bacterial survival in harsh environments[1]. The OM is also the first point of contact with the environment surrounding the bacterial cell, and subtle changes in this membrane affect fundamental bacterial processes such as motility, attachment, and pathogenesis[2–6].

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Like 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[1]. 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 lipopolysaccharides (LPS) on the outer leaflet and phospholipids on the inner leaflet[7–9] (Figure 1). Although LPS is present in most Gram-negative bacteria, bacterial cell surfaces display a great deal of structural diversity and LPS is not produced by some bacteria with an OM, such as *Borrelia burgdorferi*, a causative agent of Lyme disease. The diversity in cellular envelopes exemplifies how bacteria evolve to meet unique challenges in different environments and with new external pressures[10].

LPS is an amphipathic molecule containing fatty acyl chains attached to a polysaccharide containing as many as 200 sugars[6] (Figure 1). Some of these sugars contain phosphate groups that mediate interactions with divalent metal ions (e.g., Mg^{2+}), allowing for 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 unfavorable. The hydrophobicity of LPS is directly responsible for why it has been so difficult to develop new antibiotics to treat Gram-negative infections, 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 containing many fatty acyl chains and hundreds of sugars across the IM, the *periplasm* and the OM poses major challenges. Indeed, for over a decade, the major question in the field of LPS biogenesis was what proteins transport LPS across the cellular envelope and assemble it 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 believe that we know the essential players involved in the transport and assembly of LPS at the OM, detailed information about the function of these proteins is still lacking. 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, discussing 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 to be answered.

LPS biogenesis

The biosynthesis of LPS requires more than 100 genes and much is known about the molecular mechanisms of the biosynthetic enzymes[12–14]. LPS consists of a *lipid A* moiety, inner and outer core oligosaccharides, and the *O antigen*[6, 13] (Figure 1). The lipid A-core portion is synthesized on the cytoplasmic face of the IM and flipped to the periplasmic face by an *ABC transporter*, MsbA[15–19]. The O antigen, which is not present in some Gram-negative organisms, is ligated to the lipid A-core by an O antigen ligase, WaaL, following its independent synthesis in the cytoplasm and transport to the periplasm[20–23]. How LPS is subsequently transported to and assembled on the cell surface is much less understood.

In *Escherichia coli*, seven essential proteins, LptA-G, are required to transport LPS from the outer leaflet of the IM to the outer leaflet of the OM[11, 24–26] (Figure 1). Notably, all seven Lpt proteins are required, since depletion of individual Lpt components results in the accumulation of LPS on the periplasmic surface of the IM[27, 28]. An ABC transporter, $LptB_2FG$, associated with a *bitopic membrane protein*, LptC, is believed to extract LPS from the IM. The soluble protein LptA mediates transit of LPS across the aqueous periplasmic compartment. Finally, the membrane *β-barrel* protein LptD and the OM *lipoprotein* LptE form a heterodimeric OM translocon that somehow receives LPS from LptA and transports it to the cell surface, presumably without ever allowing LPS to reside in the inner leaflet of the OM.

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

Components of the Lpt pathway

LPS extraction from the IM by $LptB_2FGC$

In *E. coli*, LptC, LptA, and LptB (formerly known as YrbK, YhbN and YhbG, respectively) are encoded by the *lptCAB* locus, which is located downstream of genes involved in the biosynthesis of KDO (3-deoxy-d-manno-octulosonate), a component of the LPS inner core (Figure 1). *lptAB* were first identified as essential genes in a genetic screen designed to identify conditionally lethal *transposon mutants*[32]. The aforementioned *synteny* and phenotypes observed upon depletion of these factors facilitated the discovery of LptA, LptB and LptC as proteins required for LPS transport to the OM[27]. Through sequence homology, LptB was identified as a nucleotide-binding domain of an ABC transporter involved in the transport of LPS[31]. A *reductionist bioinformatics approach* led to the discovery of the transmembrane domains of this ABC transporter, LptF and LptG (formerly known as YjgP

and YjgQ, respectively)[28]. *In vivo* studies demonstrated the requirement of all these Lpt proteins in LPS transport and biochemical experiments confirmed the functional bioinformatics predictions. Furthermore, $LptB_2FG$ or $LptB_2FGC$ can be overexpressed and purified as a complex with ATPase activity. Interestingly, the ATPase activity of these complexes is much higher than that of LptB alone[33, 34], suggesting that LptF and LptG help to stabilize LptB to facilitate its dimerization, which is needed to hydrolyze ATP.

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 (Figure 1). Recent crystallographic evidence demonstrates that there is significant movement in the structure of LptB upon ATP hydrolysis, and it is predicted that this movement couples ATP hydrolysis in the cytoplasm with changes in the transmembrane domains of the ABC transporter[31]. Sites on LptFG responsible for interactions with other Lpt factors 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 $LptB_2FG$ with a weaker affinity than that of full-length LptC[35]. In addition, a single-residue substitution on the N-terminal edge of the β-*jellyroll* structure of LptC disrupts the formation of a complex with $LptB_2FG$[35]. Therefore, these observations suggest that the N-terminus of the β-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 β-jellyroll fold to LptA and LptC[28, 35]. 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 *E. coli* LPS are unlikely to cross the aqueous periplasmic compartment unaided. Therefore, the periplasmic component of the Lpt system, LptA, is believed to mediate the transport of LPS across the periplasm[32, 36]. By analogy to the transport of lipoproteins in *E. coli*, whose transit from the IM to the OM is mediated by the soluble *periplasmic chaperone* LolA[37–40], 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 like LolA, but rather by forming a transenvelope bridge that spans the periplasmic compartment (Figure 1 and Figure 2).

The first important observation challenging the notion that LptA works as a soluble chaperone was that LPS is not released from the IM when a concentrated periplasmic fraction is added to *spheroplasts*, while lipoproteins are[41]. This demonstrates that lipoproteins form a soluble complex with LolA, whereas LPS is never released from the membrane in a soluble form when the periplasmic fraction is added[37, 40]. Furthermore, pulse-chase experiments showed that LPS is transported to the OM even after the removal of soluble, periplasmic contents[41]. The first biochemical evidence for an “Lpt bridge” was the observation that the Lpt proteins, including LptA, co-fractionate within a distinct cellular fraction in sucrose gradients[42]. This fraction corresponds to a less-dense OM fraction known as OM_L in which newly synthesized LPS transiently accumulates during its transport from

the IM to the OM[43]. In addition, co-purification experiments using epitope-tagged versions of the inner membrane proteins LptB, LptC and LptF resulted in the co-purification of the periplasmic protein LptA and the OM proteins LptD and LptE[42]. 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 is unclear at this time.

The first clues about 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 belong to the OstA (Organic solvent tolerance protein A) superfamily and are all periplasmic[35, 44, 45] (Figure 1 and Figure 2). Understanding how the IM $LptB_2FGC$ complex is physically connected to LptA and to the OM LptDE complex was determined by *in vivo photo-cross-linking*[46–49] using the crystal structures of LptA and LptC as a guide[45]. Although the amino acid sequences of LptA and LptC are less than 10% identical, their structures are strikingly similar. Both LptA and LptC have slightly twisted β-jellyroll structures composed of 16 and 15 antiparallel β-strands, respectively[36, 50] (Figure 2). Interestingly, LptA crystallized as a filamentous oligomer in a head-to-tail fashion in the presence of LPS[36]. These observations led to the suggestion that LptC might be connected to LptD through one or more molecules of LptA. The continuous hydrophobic groove 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-cross-linking experiments in which variants of LptC, LptA and LptD containing an *unnatural amino acid* at different positions defined interaction sites between the OstA domains in these proteins. These experiments showed that the C-terminus of LptC interacts with the N-terminus of LptA, and that the C-terminus of LptA interacts with the N-terminus of LptD[45] (Figure 2). These interactions occur in a conserved manner involving the edges of the respective β-jellyrolls. Various *in vitro* binding experiments further supported the architecture of the transenvelope bridge 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 after *size-exclusion chromatography*[51]. In addition, alterations to the C-terminal domain of LptC or deletion of the N-terminal domain of LptD disrupt interactions with LptA, as expected based on the predicted transenvelope bridge structure[35, 45, 52]. Therefore, these experiments support the model proposing that head-to-tail oligomerization of these homologous OstA domains creates a transenvelope bridge that connects the IM Lpt complex ($LptB_2FGC$) and the OM Lpt complex (LptDE) via LptA (Figure 2). It is not known at this time how many LptA monomers comprise the bridge.

LPS transport across the OM by LptDE

One of the most intriguing questions concerning LPS biogenesis is how the OM components facilitate translocation of LPS across the OM. This process is mediated by an OM translocon containing two membrane proteins, LptD and LptE (formerly known as Imp/OstA and RlpB, respectively)[53–55]. LptD and LptE are targeted to the OM by separate pathways: LptD by the *Bam pathway*[56–63] and LptE by the *Lol pathway*[37, 38, 64–68]. In *E. coli*, both LptD and LptE are essential[54, 55] and biochemical studies have established that LptD and LptE interact with

each other very strongly[55, 69]. For example, LptD and LptE can be co-purified from solubilized membranes of cells over-producing these proteins, and they form a heterodimeric complex that resists dissociation except when subjected to heat. After size-exclusion chromatography, these two proteins co-migrate as a single band on a denaturing gel[69].

Recently, two x-ray crystal structures of the LptDE complex have been solved[70, 71]. One structure contains both an N-terminal periplasmic domain and a C-terminal β-barrel domain of LptD (Figure 3), whereas the second structure lacks the LptD N-terminal periplasmic domain[70]. These structures confirm earlier evidence predicting 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, suggesting that LptE resides within the C-terminal β-barrel of LptD[69]. Furthermore, *in vivo* photo-cross-linking using LptE variants with an unnatural amino acid at different positions showed that multiple residues located on the surface of LptE cross-link to LptD[72]. Moreover, a putative extracellular loop of the LptD β-barrel was identified by mass spectrometric analysis to be cross-linked with LptE[72], and the key role of this loop in the formation of the heterodimeric complex was confirmed by the recent LptDE crystal structures. However, despite these important advances in understanding the structure of the LptDE complex, how formation of this complex occurs at the OM is still unclear.

The two-protein plug-barrel conformation in which LptE is located inside the barrel of LptD is likely important for the mechanism of LPS transport across and insertion into the OM. Notably, LptD is the largest monomeric β-barrel so far identified in the OM of Gram-negative organisms, with a lumen large enough to permit LPS to cross the OM bilayer. LptD is also a *crenellated β-barrel* in which two adjacent strands of the β-barrel are not completely hydrogen-bonded. If these putative crenels were to open to the outer leaflet of the OM, they could serve as portals through which LPS molecules travel from the lumen of the LptD barrel to the cell surface[70–74]. This would be analogous to the way in which lipids present in the outer leaflet of the OM diffuse into the lumen of the crenellated OM β-barrels of FadL and PagP[75–80]. 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 somehow causes a conformational change of LptDE, allowing 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[70, 71, 73, 74]. Importantly, details of the conformational changes of 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 β-barrel domains of LptD, while the rest of the LPS molecule goes through the lumen of LptD[73, 74]. 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 serve 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[69, 81].

Regulation of Lpt bridge formation

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

LptD has four cysteine residues, two in the N-terminal periplasmic OstA domain (Cys31 and Cys173) and two in the C-terminal β-barrel domain (Cys724 and Cys725) (Figure 4). A functional LptDE translocon has two intramolecular disulfide bonds between nonconsecutive Cys residues (Cys31-Cys724 and Cys173-Cys725), which connect the N-terminal and C-terminal domains of LptD[83]. Interestingly, either of these two disulfide bonds is sufficient for LptD to be functional. However, this connectivity must be precise, even though the last Cys residues (Cys724 and Cys725) are adjacent, suggesting that proper oxidation is required to correctly position the N-terminal and C-terminal domains of LptD[83]. Indeed, this has been confirmed by the recent characterization of the complex mechanism of proper LptD folding (Figure 4). LptD is folded into a nonfunctional, rudimentary β-barrel structure at the OM by the Bam complex with the help of the periplasmic chaperone SurA[56, 84]. Genetic studies also suggest that LptE, which is targeted to the OM via the Lol pathway, interacts with LptD when LptD is bound to the Bam complex[85]. Furthermore, depletion of LptE results in incorrectly oxidized LptD, demonstrating that LptE is essential for the proper oxidization of LptD[82, 83, 85]. From these studies and pulse-chase experiments that have identified several intermediates along the LptD folding pathway, we know that assembly of the LptDE complex involves a nonfunctional LptD intermediate that contains non-native disulfide bonds (Cys31-Cys173) formed by the periplasmic oxidase DsbA, and a rudimentary β-barrel structure formed by Bam. 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 β-barrel into a stable structure, which requires LptE[82] (Figure 4). Importantly, we should point out 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 because oxidants present in the growth medium and oxygen can partially substitute for DsbA[83].

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 metalloprotease BepA and the disulfide isomerase DsbC have also been implicated in LptD biogenesis and disulfide rearrangement[86, 87]. BepA degrades misfolded LptD and its proposed chaperone function stimulates disulfide rearrangement in LptD through an unknown mechanism[87]. Overexpression of a variant of DsbC that traps substrates has shown that this variant interacts with LptD[86]. However, the biological significance of the DsbC-LptD interaction is unclear because deletion of *dsbC* has no detectable effect on the formation of properly oxidized LptD. Furthermore, disulfide-bond rearrangement intermediates of LptD have been shown to interact with DsbA, instead of DsbC[82, 83, 87].

Understanding the mechanism of assembly of the LptDE translocon in the OM has also elucidated how formation of the transenvelope Lpt bridge is regulated. Notably, *in vivo,* LptD variants lacking native disulfide bonds do not interact with LptA[45]. This finding suggests a model in which the disulfide bond rearrangement that occurs after proper assembly of the LptDE complex is needed to ensure that a functional LptDE translocon interacts with LptA, enabling the establishment of the transenvelope bridge. This LptDE-LptA interaction 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 also suggested to involve LptC, as impairing the interaction of LptC with $LptB_2FG$ destabilizes the entire Lpt transenvelope complex[35]. 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, we began to accumulate genetic and biochemical evidence supporting the model that the Lpt transenvelope 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[27, 28], 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. This transenvelope 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[50, 69, 88], a recent breakthrough was the detection of intermediate interactions between Lpt proteins and LPS in a cell[30, 74].

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-cross-linking LPS to an unnatural amino acid located at specific positions in LptA and LptC. Trapping LPS in these Lpt proteins *in vivo* clarified that, during transport, LPS interacts with the hydrophobic groove of the β-jellyroll domain of LptA and LptC[30]. LPS accumulated in LptC or in LptA depending on the co-expression of $LptB_2FG$ or $LptB_2FGC$, respectively[30]. These results confirmed that the cross-linked products are intermediates of LPS transport by Lpt components, and that LptC and LptA need $LptB_2FG$ and $LptB_2FGC$, respectively, to receive LPS.

A similar strategy involving photo-cross-linking LPS to an unnatural amino acid was also used to demonstrate the predicted dependency of LPS transport on ATP hydrolysis[30]. For example, *right-side out (RSO) membrane vesicles*[89–91] containing an LptC variant known to cross-link to LPS *in vivo* were prepared with or without ATP. This LptC variant cross-linked to LPS in an $LptB_2FG$-dependent, time-dependent and ATP-dependent manner, suggesting that LPS is transported from the IM to LptC using energy provided by ATP hydrolysis by $LptB_2FG$. When LptA mutants known to cross-link with LPS or with LptC *in vivo* were added to the RSO membrane vesicles, the release of LPS from the IM to LptA was dependent on $LptB_2FGC$, time and ATP, whereas the interaction of LptA with LptC was not.

Strikingly, if LptA was added after LPS had already accumulated in LptC, transfer of LPS from LptC to LptA required additional ATP hydrolysis; this was shown by inhibiting the ATPase activity of the $LptB_2FGC$ complex with *vanadate*[30]. This observation suggested the existence of at least two ATP hydrolysis steps in LPS transport from the IM to the cell surface: the first step involves transfer of LPS from the IM to LptC; and the second step involves transfer of LPS from LptC to LptA. Moreover, in the absence of vanadate, the amount of LPS bound to LptC remained constant, even when LPS was transferred to LptA, suggesting that LPS-binding sites in the Lpt periplasmic bridge are always filled with LPS during transport.

Collectively, these data serve as the basis for the newly proposed "PEZ" model for LPS transport. This model proposes that Lpt proteins function similarly to a PEZ candy dispenser, in which PEZ candies filling 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 via the action of $LptB_2FG$, in a process that depends on ATP hydrolysis in the cytoplasm by LptB. Then, 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 $LptB_2FGC$. 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, while the sugar portion goes through the barrel; this 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 β-jellyroll of the N-terminus[73, 74]. The observation that LPS binding sites both in LptC and LptA are constantly occupied by LPS molecules, and the fact that there are LPS binding sites in 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 cytoplasmic ATP hydrolysis (Figure 5).

Outlook

In vitro assays using RSO membrane vesicles and photo-cross-linkable proteins enabled the study of the mechanism of LPS transport from the IM to the periplasmic protein LptA. We presume that the $LptB_2FG$ 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, $LptB_2FG$ is an unusual ABC transporter, since 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 $LptB_2FG$ may be gained by comparisons to $LolCD_2E$, 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[38, 66, 92–94]. In $LolCD_2E$, 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[65, 95, 96]. Therefore, LptF and LptG may also have distinct functions with respect to each other. In the future, a crystal structure of the periplasmic domains of LptF and LptG and *in vivo* photo-cross-linking studies might help us understand the functions of the membrane subunits of this LPS extractor.

Another important question that remains unanswered is how a molecule containing as many as 200 sugars crosses the OM. We speculate that LptE located inside the β-barrel of LptD has some function to assemble LPS at the cell surface in *E. coli*, due to its high affinity for LPS and its ability to disaggregate LPS *in vitro*[69, 81]. This functional role of LptE could differ in organisms in which LPS is not essential, such as *Neisseria meningitidis*[97]. The crystal structures of LptDE 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 proteoliposomes (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 LptD[98, 99]. This is based on the observations that a photoactive analog of L27-11 cross-links LptD and that mutations in *lptD* confer resistance to this compound, establishing 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 great target for novel antimicrobials.

The Lpt transenvelope 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, making it difficult to interfere with transport. However, the positions in LptA and LptC found to cross-link 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 plays in providing a barrier-like quality to the OM.

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Glossary

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

LIPID A
Also known as endotoxin, this is the hydrophobic glucosamine-based phospholipid anchor of LPS molecules.

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

ABC TRANSPORTER
(ATP-binding cassette transporter) A transmembrane protein complex that uses the energy derived from ATP binding and hydrolysis to transport a wide variety of 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 containing only one transmembrane helix.

β-BARRELS
A class of integral membrane protein comprised of β-strands that satisfy their peptide backbone hydrogen bonds by forming a cylindrical barrel structure, exposing hydrophobic side chains to the membrane and shielding hydrophilic side chains.

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

TRANSPOSON MUTANTS
Mutants obtained by the random insertion of a transposon (or transposable element) into the 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 co-localization of genes in a genetic locus.

REDUCTIONIST BIOINFORMATIC 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 reduce the number of candidate genes of interest by comparing the genome of interest to other genomes of smaller size.

β-JELLYROLL
A structure in which anti-parallel β-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 getting to their destinations.

SPHEROPLASTS
Osmotically fragile bacterial cells that have had their OMs and peptidoglycan layers incompletely disrupted, causing 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, allows for a variety of new functions.

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

BAM PATHWAY
(β-barrel assembly machine pathway) Following secretion from the IM and translocation across the periplasm, the Bam complex is responsible for folding and inserting OM β-barrel proteins into the membrane. In *E. coli*, the Bam complex is composed of one β-barrel protein, BamA, and four OM lipoproteins, BamB-E.

LOL PATHWAY
(localization of lipoproteins pathway) Chaperone-based transport pathway for OM lipoproteins from the outer leaflet of the IM to the inner leaflet of the OM.

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

CRENELLATED β-BARREL
A β-barrel protein in which formation of inter-strand hydrogen bonds is disrupted, creating openings like the crenels in the turret of a castle.

RIGHT-SIDE OUT (RSO) MEMBRANE VESICLES
Membrane vesicles with a native orientation, prepared by the osmotic lysis of spheroplasts.

VANADATE
Sodium orthovanadate (Na_3VO_4) inhibits protein tyrosine phosphatases, alkaline phosphatases, and many ATPases by acting as a phosphate analog and binding in the active site where phosphate should bind.

PEPTIDOMIMETIC
Chemical compounds that mimic natural peptides because their essential property is a protein-like chain.

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Biographies

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Natividad Ruiz received her Ph. D. from Washington University in St. Louis, USA, where she studied bacterial pathogenesis in the laboratory of Michael Caparon. As a postdoctoral researcher in the laboratory of Thomas Silhavy at Princeton University, New Jersey, USA, she studied gene regulation and envelope biogenesis in *Escherichia coli*. She is an Associate Professor at The Ohio State University, USA, and her laboratory studies peptidoglycan and outer membrane biogenesis in Gram-negative bacteria.

Daniel Kahne is the Higgins Professor of Chemistry and Chemical Biology and of Molecular and Cellular Biology at Harvard University, Massachusetts, USA, as well as Professor of Biological Chemistry and Molecular Pharmacology at Harvard Medical School. He has longstanding interests in understanding the biogenesis of the cell envelope of Gram-negative bacteria, in particular peptidoglycan biosynthesis and outer membrane assembly. His research focuses on identifying and understanding the machinery necessary for proper assembly of this membrane barrier, as well as the mechanisms that lead to defects.

Online summary

- The outer membrane (OM) of most Gram-negative bacteria contains lipopolysaccharide (LPS), a large molecule containing multiple fatty acyl chains and up to hundreds of sugars, in its outer leaflet, creating a barrier that prevents entry of both large polar and small hydrophobic molecules.
- The transport of millions of LPS molecules from the IM, across the aqueous periplasmic compartment, and across the OM to the cell surface was not well understood, except that the process is mediated by seven essential and conserved LPS transport (Lpt) proteins.
- LPS extraction from the IM is mediated by an ABC transporter, $LptB_2FG$, and an associated membrane protein, LptC. These proteins couple ATP hydrolysis in the cytoplasm by LptB to movement to LptC; the $LptB_2FG$ and $LptB_2FGC$ protein complexes have been purified and demonstrate ATPase activity *in vitro*.
- LPS is believed to transit the periplasm by a bridge between LptC and the OM mediated by the periplasmic protein LptA. The bridge is formed by structurally homologous domains of LptC, LptA, and the OM protein LptD, and it helps mediate transit of the hydrophobic acyl chains of LPS through an aqueous compartment.
- The OM β-barrel protein LptD and OM lipoprotein LptE form a two-protein plug-and-barrel complex responsible for transporting LPS from the periplasmic bridge across the OM to the cell surface. A current model is that the OM translocon changes its conformation, allowing LPS molecules to enter the barrel of LptD and move to the cell surface through lateral openings, without ever residing in the inner leaflet of the OM.
- LptD is a large β-barrel protein that contains two nonconsecutive disulfide bonds, either of which is sufficient for LptD function. Proper rearrangement of the disulfides to the final configuration is required for LptA to interact with LptD, preventing mislocalization of LPS when the OM translocon is not properly assembled.
- Identification of LPS transport intermediates in *E. coli* cells allowed for the development of a system to study the ATP requirement for LPS transport out of membrane vesicles to soluble LptA. Using this system, the PEZ model was developed to describe how ATP hydrolysis by LptB in the cytoplasm “pushes” LPS molecules in a continuous stream out of the IM toward the cell surface through the periplasmic bridge built of LptC, LptA and LptD.

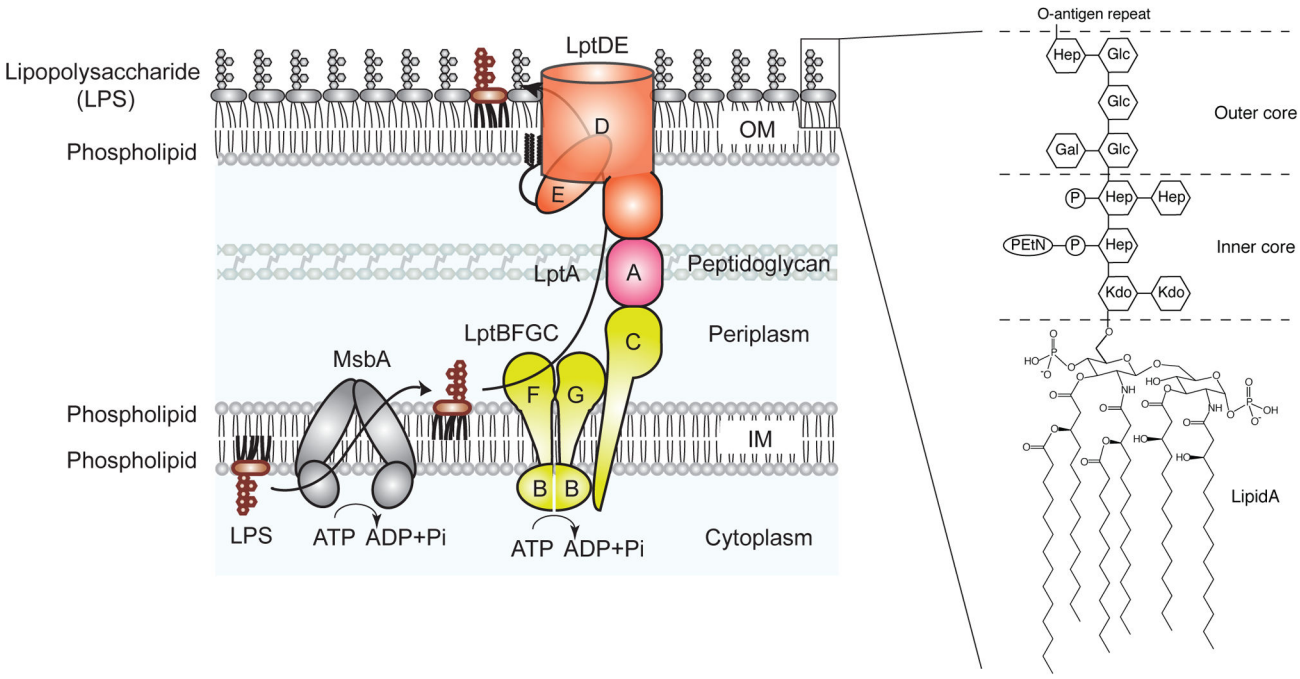

Fig. 1. LPS transport pathway in *E. coli*

LPS is synthesized on the cytoplasmic side of the inner membrane (IM) and flipped to the periplasmic side by an ABC transporter, MsbA. LPS is then transported to the cell surface via the Lpt pathway. This pathway consists of seven essential proteins, LptA-G. LPS is extracted from the IM in an ATP-dependent manner by the ABC transporter $LptB_2FG$ and transferred to LptC, which forms a complex with $LptB_2FG$. LptC consists of a single membrane spanning domain and a large periplasmic domain, which forms a periplasmic bridge with soluble protein LptA and the N-terminal region of LptD. LPS transverses the aqueous periplasmic space through this protein bridge and reaches the cell surface with the help of the C-terminal domain of LptD, which forms a β-barrel structure 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 *E. coli* K-12.

EtN, ethanolamine; Gal, D-galactose; Glc, D-glucose; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; P, phosphate.

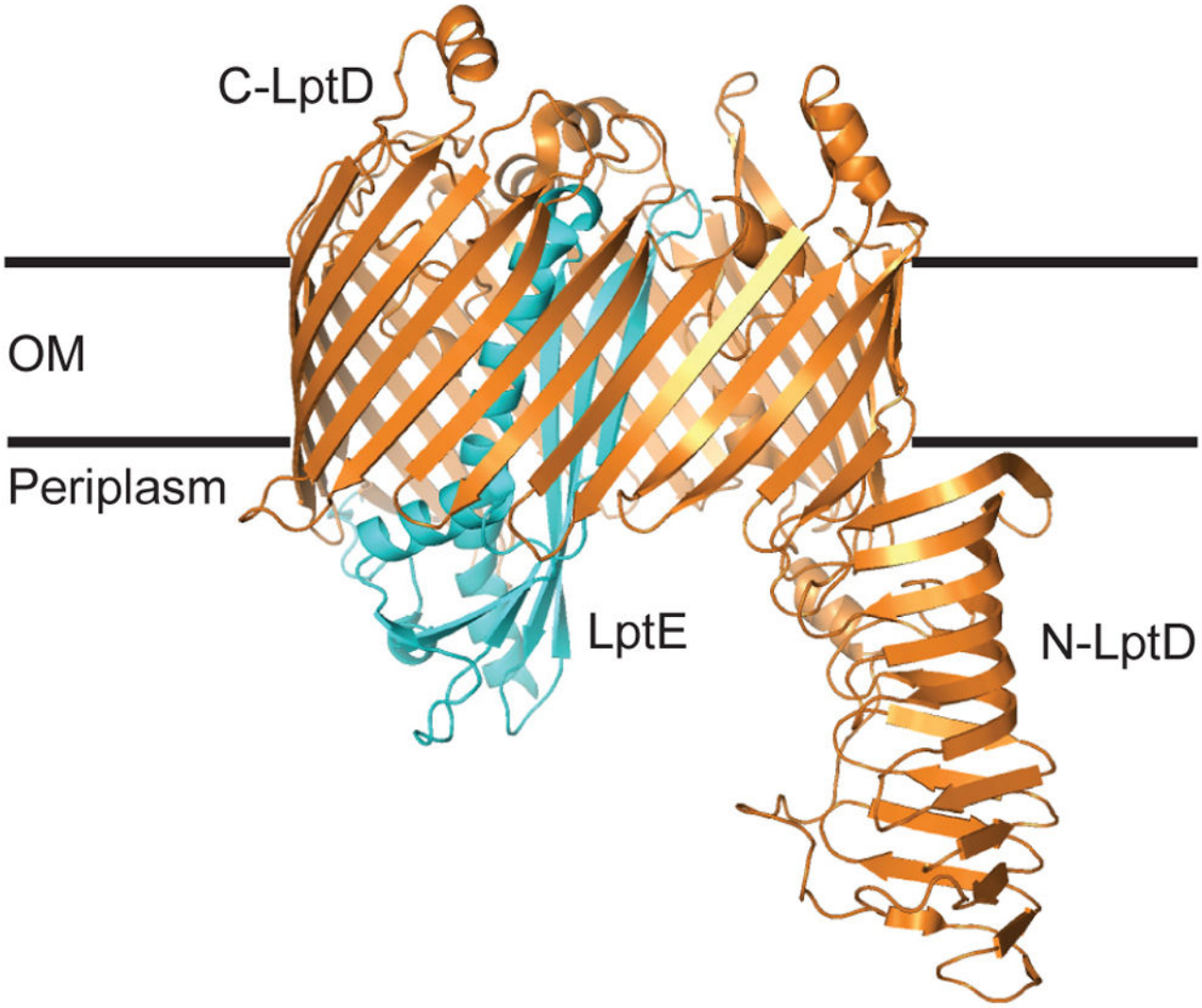

Fig. 2. The periplasmic protein bridge

The figure illustrates a model of the periplasmic protein bridge comprised of LptC, LptA and LptD. The C-terminal periplasmic region of LptC (yellow; PDB ID: 3MY2), LptA (pink; PDB ID: 2R19) and N-terminal region of LptD (orange; PDB ID: 4Q35) are stacked to illustrate the Lpt bridge. Two LptA molecules in the trigonal crystal form (PDB ID: 2R1A) were replaced by C-LptC and N-LptD. The number of LptA molecules in the bridge is unknown.

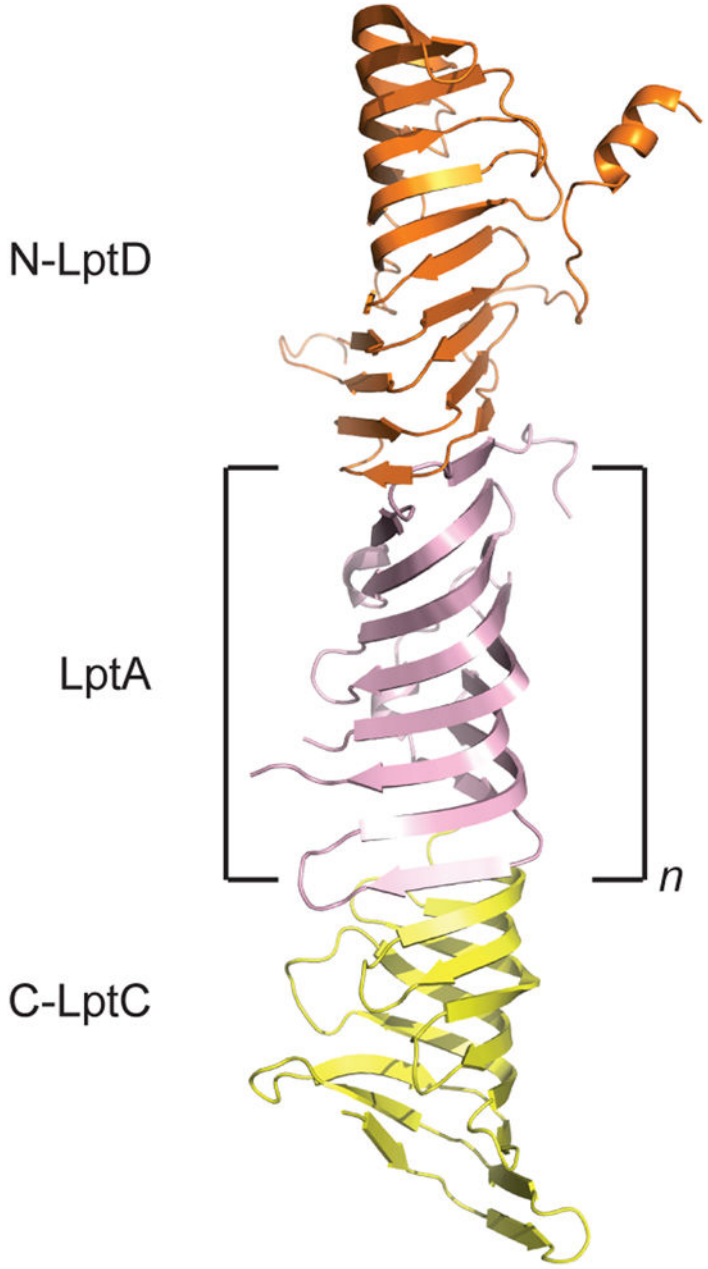

Fig. 3. The outer membrane translocon

The figure illustrates a model of the two protein plug-and-barrel in the OM comprised of LptD (orange; PDB ID:4Q35) and LptE (cyan; PDB ID:4Q35).

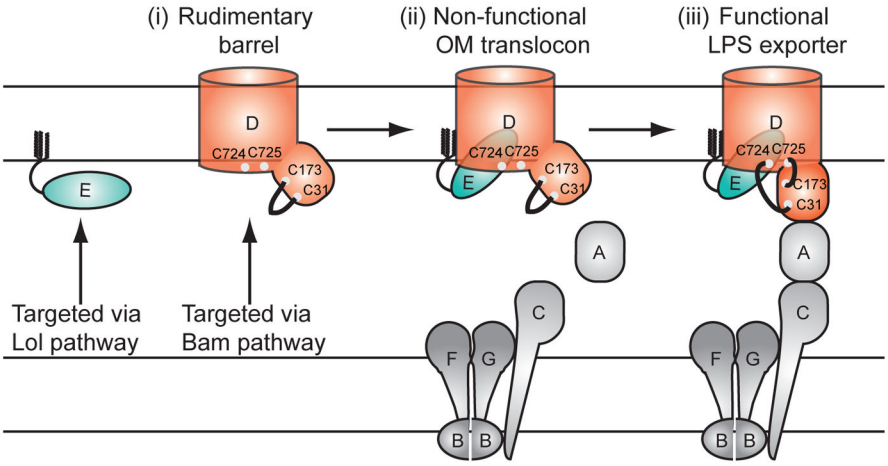

Fig. 4. Regulation of the formation of the Lpt bridge

The biogenesis of the functional LPS LptDE translocon requires disulfide bond rearrangements at the OM. LptD and LptE are targeted to the OM via the Bam and Lol pathways, respectively. LptD has four cysteines, two in the N-terminal periplasmic region (Cys31 and Cys173) and two in the β-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). Functional translocon formation permits N-LptD to interact with LptA, resulting in a functional LPS transporter including the IM complex, $LptB_2FGC$. It is unknown how the interaction between LptA and LptC is regulated.

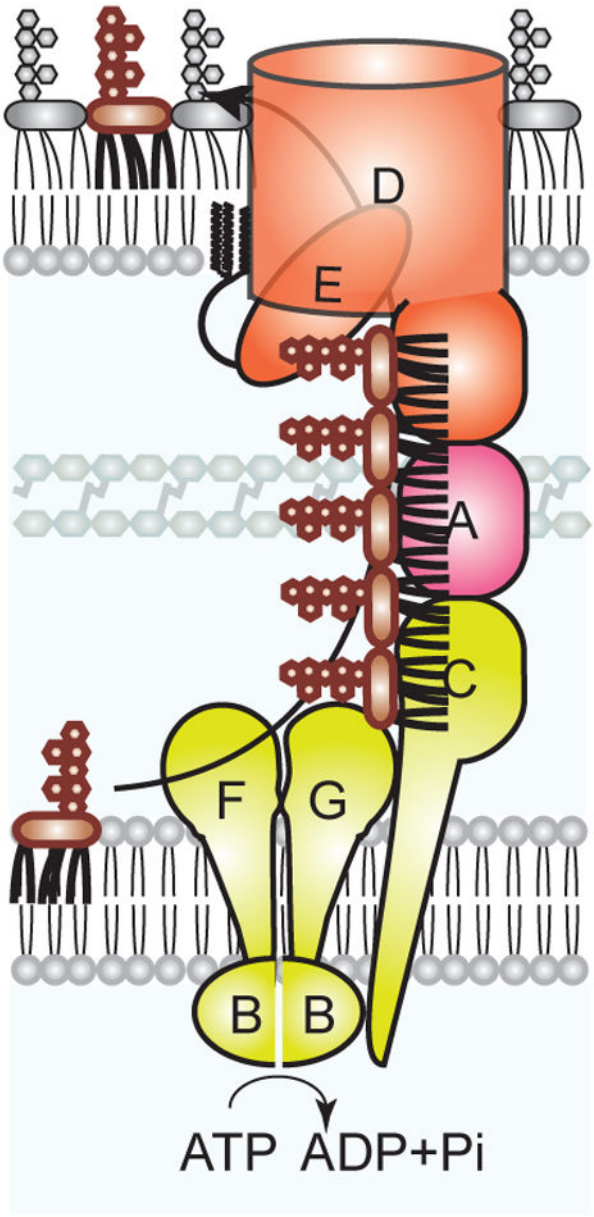

Fig. 5. The PEZ model

LPS transport from the IM to LptC, and from LptC to LptA, requires energy derived from ATP hydrolysis. LPS binding sites both in LptC and LptA are constantly occupied by molecules of LPS. The observation that multiple 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 needed to push a continuous stream of LPS through the Lpt bridge. Therefore, the PEZ model suggests that LPS transport occurs by analogy to a PEZ candy dispenser, in which PEZ candies filling 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 via the action of $LptB_2FG$, in a process that depends on ATP hydrolysis in the cytoplasm, which is

mediated by the ATPase LptB in the 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 $LptB_2FGC$. LPS is then proposed to cross the translocon with the lipid portion of LPS being directly inserted into the outer leaflet of the OM without entering the lumen of the LptD barrel, while the sugar portion of LPS goes through the barrel.