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High-resolution views of lipopolysaccharide translocation driven by ABC transporters MsbA and $LptB_2FGC$

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Abstract

Gram-negative bacteria possess a dual-membrane envelope, which provides defense against environmental assault, as well as formidable resistance against antibiotics. Lipopolysaccharide (LPS) is the primary lipid component in the outermost membrane leaflet of most Gram-negative bacteria, and plays critical roles in cell envelope formation. Newly synthesized LPS at the cytoplasmic side of the inner membrane is flipped across the inner membrane and pushed across the periplasm by two ATP-binding cassette transporters: MsbA and $LptB_2FGC$. Both transporters represent promising targets for developing new classes of antibiotics. In this review, we discuss recent advances in understanding the mechanism of LPS translocation driven by MsbA and $LptB_2FGC$, with a particular focus on the new findings from structural studies.

Keywords

MsbA; $LptB_2FGC$; lipopolysaccharide; ABC transporter; antibiotics

Introduction

Pathogenic Gram-negative bacteria are the major cause of antibiotic-resistant infection[1]. They produce a unique dual-membrane envelope. While the inner membrane (IM) is composed of phospholipids in both membrane leaflets, the outer membrane (OM) is an asymmetric lipid bilayer with the inner leaflet of phospholipids and the outer leaflet predominantly of lipopolysaccharide (LPS). The IM and OM have distinct permeation properties, and together they prevent many antibiotics from entering the cells. LPS is composed of three portions: lipid A, core oligosaccharide, and O-antigen (Fig. 1a). LPS

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recognition by Toll-like receptor 4 (TLR4) initiates an inflammatory response and in some cases leads to septic shock[2,3]. LPS also stimulates immune cells by binding to a cytoplasmic non-canonical inflammasome complex[4]. Bacteria modify LPS in a variety of ways to alter the OM properties, innate immune stimulation, and pathogenesis[5].

During their growth, Gram-negative bacteria assemble millions of LPS molecules in the outermost membrane leaflet, at a speed estimated to be 70,000 molecules per minute[6]. An LPS molecule newly synthesized at the cytoplasmic surface of the IM must cross three cellular compartments (IM, periplasm and OM) before reaching its destination. This remarkable journey of LPS is powered by two ATP-binding cassette (ABC) transporters, MsbA and $LptB_2FGC$[7]. Using the energy of ATP binding and hydrolysis, MsbA flips LPS across the IM and $LptB_2FGC$ pushes LPS towards the OM (Fig. 1b).

ABC transporters exist in all kingdoms of life and translocate a multitude of chemically diverse substrates across cell membranes[8,9]. ABC transporters have two transmembrane domains (TMDs), which form the substrate translocation pathway, and two nucleotide binding domains (NBDs), which bind and hydrolyze ATP. Whereas the conformational changes during the functional cycle have been extensively studied for a number of ABC transporters[10–12], much less is known for specific determinants of substrate selectivity and the mechanism underlying tight coupling between substrate translocation and ATP hydrolysis. In this regard, the two ABC transporters (MsbA and $LptB_2FGC$), which work on the same substrate (LPS) but perform completely different tasks, provide an ideal case study for understanding how ABC transporters achieve their extraordinary versatility, specificity and efficiency.

In this review, we summarize recent progress in understanding the mechanistic details of MsbA and $LptB_2FGC$. We highlight the structural analyses and accompanying biochemical data which have revealed the basis of LPS translocation driven by these two ABC transporters, and discuss how these insights may help develop novel antibiotics to combat important bacterial pathogens.

MsbA and LPS flipping: a well-studied protein and an unsolved mystery

MsbA has long been a popular model system for studying ABC transporters, due to its excellent stability and biochemical behavior, as well as to its structural similarity with human multidrug efflux pumps such as P-glycoprotein. As a result, much of the current paradigm of ABC exporter function has been established using results from MsbA. The first set of structures of MsbA in detergent were obtained using X-ray crystallography over a decade ago[13]. These include two different inward-facing open conformations of nucleotide-free MsbA, and one outward-facing open conformation for MsbA bound to AMPPNP (5′-adenylyl-β,γ-imidodiphosphate) or ADP-vanadate. These crystal structures and following spectroscopy studies[14–18] proposed an alternating access transport mechanism, in which LPS from the cytoplasmic leaflet of the IM binds to the inner cavity of MsbA in its inward-facing open conformation, and the subsequent ATP binding shifts MsbA to an outward-facing state in which LPS is released to the periplasmic side. However,

because of the absence of LPS in these structures, how MsbA specifically recognizes LPS and drives its 180° rotation remained largely unknown.

MsbA-LPS interaction

By combining single-particle cryo-electron microscopy (cryo-EM)[19–21] and lipid nanodisc technology[22], a breakthrough was made in understanding MsbA-driven LPS flipping[23]. The cryo-EM structure of MsbA in a native-like lipid bilayer clearly resolved a co-purified LPS molecule between the two TMDs of MsbA (Fig. 2a, b). This is the first visualization of LPS in the context of its transport protein machinery, revealing how the inner cavity of MsbA forms highly specific contact with the unique chemical signatures in LPS. Later a crystal structure of an inhibitor-bound MsbA also reveals a similarly bound LPS[24]. Surrounding the negatively charged phosphorylated glucosamines of LPS, a ring of positively charged amino acid residues from both MsbA subunits forms strong electrostatic interactions with LPS (Fig. 2c). Above the glucosamines, the lipid acyl chains are accommodated in a large hydrophobic pocket, which is sealed off from the aqueous periplasm. Notably, the size and shape of the inner cavity of MsbA are complementary to the corresponding portions in LPS such that MsbA would preferentially accommodate the 12–14 carbon chains of LPS acyl tails over a longer phospholipid (i.e. ‘hydrocarbon ruler’)[24]. Below the glucosamines, the inner core of LPS is directly exposed in the cytoplasm, showing no clear contact with MsbA. Taken together, the negatively charged electrostatic surface and the hydrophobic pocket in the inner cavity of MsbA simultaneously interact with two regions of LPS (glucosamines and acyl chains) with distinct surface properties, thus contributing to stable and highly specific LPS binding. These features are highly similar to those seen in the structure of the TLR4–MD-2–LPS complex[25], indicating that proteins with completely different folds use the same strategy to recognize LPS.

Inside the MsbA central cavity, LPS acyl chains are located at the level of the periplasmic membrane leaflet (Fig. 2a and step 2 in Fig. 2d). Thus, the cryo-EM structure reveals a previously unrecognized functional state, in which LPS has accomplished much of the translocation across the IM, but without the upside-down rotation. This state is presumably followed by the actual flipping of LPS. We named this process as “trap and flip” (Fig. 2d). This proposed model emphasizes the finding that LPS first undergoes translation within the membrane bilayer without rotation, which had not been observed or speculated as a mechanism for lipid flippases. Interestingly, in a recent cryo-EM structure of a type IV P-type ATPase that translocates phosphatidylserine from the outer to the inner membrane leaflet, which is opposite to the direction of MsbA-mediated LPS flipping, the bound phospholipid has moved towards the target leaflet without flipping its acyl chains[26]. Thus, translation followed by rotation may be a general scheme of ATP-powered lipid flipping.

LPS interacts dynamically and intimately with MsbA throughout its functional cycle, and contributes positively to the high transport efficiency. The LPS binding to MsbA brings two NBDs in proper positions to facilitate ATP hydrolysis[23,24]. Comparison between the nucleotide-free and nucleotide-bound conformations of MsbA further demonstrates that ATP binding-induced movement of TMDs, as well as tight dimerization of NBD and ATP hydrolysis, would not occur unless the bound LPS is moved out of the central cavity (step 3–

4 in Fig. 2d). Thus, the translocation of LPS towards the periplasm, conformational changes of MsbA, and ATP hydrolysis must occur in a concerted process. Therefore, rather than a cargo being passively moved, LPS appears more like an essential constituent to maximize the efficiency of the MsbA molecular machine.

MsbA inhibitors

The first MsbA-specific inhibitors were not reported until mid-2018 by two different groups[24,27]. A quinoline class of molecules identified by Genentech selectively inhibits the activity of MsbA and displays bactericidal activity[24,28]. The crystal structures of inhibitor-bound MsbA show that these compounds bind the TMDs and lock MsbA in an inward-facing wide-open conformation. A copurified LPS is resolved inside the central cavity demonstrating similar interactions as those in the cryo-EM structure[23]. The other class of inhibitor was identified from a phenotypic screen, which was designed to target the proteins involved in LPS biogenesis[27]. Despite low bactericidal efficacy, this compound has an interesting property of stimulating the activity of MsbA for ATP hydrolysis while blocking LPS transport, suggesting an action mode distinct from the MsbA inhibitors from Genentech[24].

Lpt machinery with two mysterious pieces: LPS and LptC

After being flipped across the IM by MsbA, LPS in the periplasmic leaflet of the IM travels across the periplasm to the OM, a process mediated by the seven Lpt proteins (LptA through LptG) (Fig. 1b). The structures of individual Lpt proteins or domains have been determined[29–34], and reveal a common β-jellyroll like fold for essentially all the domains that are located in the periplasm, including LptA and the periplasmic domains from LptF, LptG, LptC, and LptD. Combined with this structural knowledge, biochemical experiments have supported what has been termed the “PEZ” model, in which Lpt proteins form a physically connected bridge spanning across the periplasm to shuttle LPS from the IM to the OM[35,36]. $LptB_2FGC$ is the sole energy source for LPS extraction out of the IM and LPS movement towards the OM. The concave inner surface of the β-jellyroll domains is hypothesized to protect the hydrophobic acyl chains of LPS during its translocation across the aqueous periplasm. However, the mechanistic details of LPS transport through Lpt machinery remained largely speculative, mainly due to the lack of LPS resolved in the structures of Lpt proteins.

The ABC transporter complex $LptB_2FGC$ has several distinct features compared to MsbA. First, $LptB_2FGC$ has various domains made of individual proteins. Second, in addition to two TMDs (TM helices from LptF and LptG) and two NBDs (two LptB subunits), it contains a stably associated LptC protein which has a single N-terminal transmembrane segment and a C-terminal β-jellyroll domain in the periplasm[34]. However, the function of LptC has been mysterious. The periplasmic domain of LptC is essential for LPS transport and bacterial growth[37,38]. The TM helix of LptC is dispensable for bacterial survival in laboratory conditions, even though all natural LptC proteins contain the TM helix[37].

$LptB_2FGC$-LPS interaction

The first glimpse of the $LptB_2FG$ complex (without LptC) was provided by two crystal structures of $LptB_2FG$ from *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*[31,32], which display a similar architecture. Between the TMDs formed by LptF and LptG there is a large hydrophobic pocket presumably for LPS binding. However, LPS was not resolved in either structure, and it was unclear what conformation of $LptB_2FG$ is competent for LPS binding. Later the cryo-EM structure of nucleotide-free *Escherichia coli* $LptB_2FG$ in nanodiscs resolved a co-purified LPS molecule[23] (Fig. 3a–c). The six lipid acyl chains are tightly packed inside the hydrophobic pocket, and their intimate contact with a number of amino acid residues is directly visualized in the EM density (Fig. 3c). Mutations of many of these residues generate strong inhibitory effects on bacterial growth, indicating their functional importance for LPS binding and transport[31,32,39]. A cluster of positively charged side chains surround the phosphorylated glucosamines of LPS, and most of these residues are from LptG (Fig. 3f). Thus, although LptF and LptG are structurally homologous, their roles in LPS binding and translocation are not equivalent. This is in contrast with MsbA, where the electrostatic interactions around the glucosamines are contributed symmetrically by two identical subunits[23] (Fig. 2c).

The cryo-EM structure of *E. coli* $LptB_2FGC$[40] (Fig. 3d) and two crystal structures of $LptB_2FGC$ from *Vibrio cholerae* and *Enterobacter cloacae*[41] (Fig. 4e, f) demonstrate a similar architecture of the complex. In these structures, the β-jellyroll domain of LptC is located on top of the β-jellyroll domain of LptF, with essentially no contact with LptG. The concave space in the β-jellyroll domains of LptC and LptF is aligned to form a continuous hydrophobic path, which presumably accommodates the acyl chains of LPS after its extrusion out of the TMDs. Surprisingly, the TM helix of LptC is present between the two TMDs (Fig. 3d, e), mostly interacting with the TM5 of LptF through extensive hydrophobic interactions. The nature of this interaction explains why the length and overall hydrophobicity, but not the exact residues, of the TM helix of LptC are conserved[34]. The presence of an extra TM helix in between two TMDs of an ABC transporter is unprecedented, indicating a novel mechanism for regulation of transporter activity. Indeed, the ATPase activity of $LptB_2FGC$ is substantially lower than that of $LptB_2FG$[36,40,41]. Consistent with the notion that an extra TM helix located between the TMDs would interfere with the conformational changes required for ATP hydrolysis, in the cryo-EM structure of $LptB_2FGC$ trapped by ADP-vanadate, the TM helix of LptC is dissociated from the TMD interface, allowing the inward movement of TMDs and tight dimerization of NBDs[40].

Photocrosslinking experiments demonstrate that LPS enters $LptB_2FGC$ in the region where the TM helix of LptC is sandwiched by the two TMDs, but not on the opposite side of the transporter[41]. In the cryo-EM structure of $LptB_2FGC$, a relatively weak LPS density is observed in the central pocket, and most of the residues that form electrostatic interactions with LPS in $LptB_2FG$ are outside the range for contact (Fig. 3e, f)[40]. This suggests that removal of the TM helix of LptC occurs in concert with the establishment of tight binding to LPS. In the cryo-EM structure of $LptB_2FGC$ trapped with ADP-vanadate, the central pocket is completely collapsed without any substrate inside, thus representing a state after LPS exit. Taken together, the results from cryo-EM analysis suggest a “lock and squeeze” model for

$LptB_2FGC$-driven LPS extraction from the IM (Fig. 3g). In this model, ATP binding or hydrolysis is not needed for LPS entry but required for LPS translocation from the inner cavity to the β- jellyroll domains, and the TM helix of LptC plays a crucial role in sensing the presence of bound LPS to prevent futile ATP hydrolysis. These notions are supported by the results from photocrosslinking experiments that detect the locations of LPS in the Lpt complex in different ATP states[41].

Lpt β-jellyroll bridge

The β-jellyroll domains of LptF and LptG in the cryo-EM structures of *E. coli* $LptB_2FG$ and $LptB_2FGC$ demonstrate two different orientations (Fig. 4c, d), and the structures of $LptB_2FGC$ from three different species also demonstrate different titling angles of the β-jellyroll domains (Fig. 4d–f). Interestingly, in the structure of *V. cholerae* $LptB_2FGC$ with the most upright β-jellyroll domains, the LptF β-jellyroll changes its conformation to close its concave path, likely to block backward flow of LPS[41]. Taken together, both the inter-β-jellyroll interactions and the conformation within each β-jellyroll appear to be highly dynamic.

3D classification of cryo-EM particle images of $LptB_2FGC$ indicated that the stable attachment of the β-jellyroll domain of LptC on top of LptF is inhibited when the TM helix of LptC is sandwiched between the TMDs; conversely, the interaction between the β-jellyroll domains of LptC and LptF is enhanced when the TM helix of LptC is dissociated from the TMD interface, as observed in the cryo-EM structure of $LptB_2FGC$ trapped by ADP-vanadate[40]. These findings indicate that the stable interaction between the β-jellyroll domains of LptC and LptF occurs preferentially when LPS is bound inside the TMDs and the TM helix of LptC is released from the TMD interface. Thus, the β-jellyroll bridge in the periplasm is unlikely to be static or assembled by default. The structural plasticity and dynamic interactions of the β-jellyroll domains likely contribute to high efficiency of $LptB_2FGC$ in LPS transport, at least in part, by preferentially connecting the LPS-loaded $LptB_2FGC$ to LptA in the periplasm.

Lpt inhibitors

A few compounds have been identified to bind the β-jellyroll domains of Lpt proteins and block LPS transport, including a class of macrocyclic peptidomimetics[42–44], thanatin[45] and IMB-881[46]. Novobiocin, an antibiotic against Gram-positive bacteria by inhibiting DNA gyrase, was co-crystallized with isolated LptB and increases LPS transport instead of inhibiting it[47]. However, when cocrystallized with $LptB_2FGC$, novobicin binds a functionally irrelevant site rather than the interface between LptF/LptG and LptB[41]. Thus, the action mechanism of novobiocin in Gram-negative bacteria has yet to be defined. No small-molecule compounds have been reported to bind the TMDs of $LptB_2FGC$.

Perspectives

A great deal of structural information for the LPS flipping and transport machinery has become available over the past two years. Particularly, single-particle cryo-EM has resolved

LPS bound to MsbA and Lpt proteins for the first time[23,40], and demonstrated its power in studying membrane proteins in native-like bilayer membrane, characterizing structural rearrangement of ABC transporters during their functional cycles, and analyzing the conformational ensemble of flexible domains. Despite the great progress, many important questions related to LPS transport remain to be addressed. How does LPS travel into the central cavities of these transporters? Are there unidentified intermediate conformations? What are the structural details of the transporters during LPS exit? What is the architecture of fully assembled Lpt protein bridge? Future structural studies together with biochemical, functional, and spectroscopic approaches will generate deeper insights and probably more unexpected discoveries.

The development of antibiotics to block LPS transport is still in its infancy. It is worth noting that the conformation of MsbA targeted by the compounds developed by Genentech is different from those in all previously published structures[24], and that the binding pocket for these compounds could not be predicted based on available structures. Like other ABC transporters, MsbA and $LptB_2FGC$ undergo large conformational transition during their functional cycles, and we know very little about which conformations can be effectively targeted by small molecules. Future structural studies of these essential transporters bound to different classes of inhibitors will be crucial for mapping all druggable conformations. Armed with the wealth of knowledge, the search for new and improved antibiotics that target LPS transport will help solve the worldwide antibiotic crisis.

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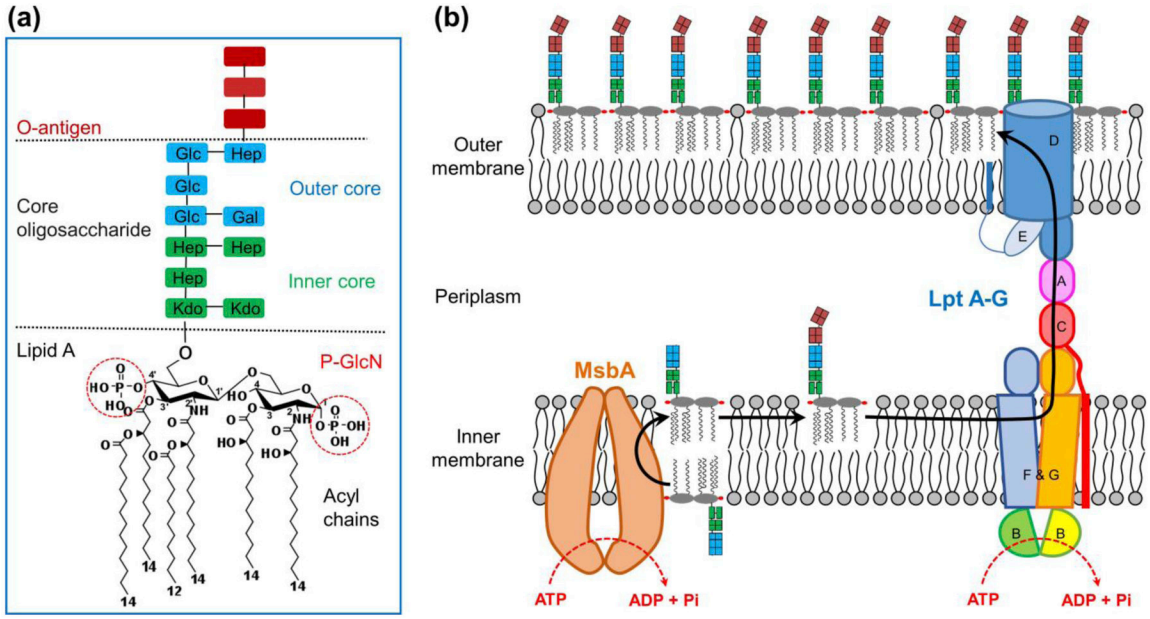

Figure 1 |. Lipopolysaccharide structure and transport pathway.

(a) Lipopolysaccharide (LPS) structure in *Escherichia coli*. LPS is composed of three portions: lipid A, core oligosaccharide, and O-antigen. Lipid A has a glucosamine disaccharide with phosphate groups at the 1- and 4′-positions (phospho-N-acetylglucosamine, or P-GlcN), and primary acyl chains at the 2-, 3-, 2′- and 3′-positions. Two secondary acyl chains are attached to the 2′ and 3′ primary acyl chains. The core oligosaccharide is composed of inner and outer saccharides, including 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo), heptose (Hep), glucose (Glu), and galactose (Gal). The O-antigen consists of many repeats of an oligosaccharide unit. **(b)** LPS transport pathway in *E. coli*. Lipid A with core oligosaccharide is synthesized in the cytoplasmic leaflet of the inner membrane (IM). MsbA, a homodimeric ABC transporter, flips the nascent LPS across the IM. The O-antigen is ligated to lipid A-core saccharide on the periplasmic side of the IM. $LptB_2FGC$ extracts the mature LPS out of the IM, and subsequently pushes it across the periplasm through LptA and LptDE. LPS is eventually inserted in the outer leaflet of the outer membrane (OM). MsbA and $LptB_2FGC$ utilize the energy from ATP hydrolysis to power LPS movement.

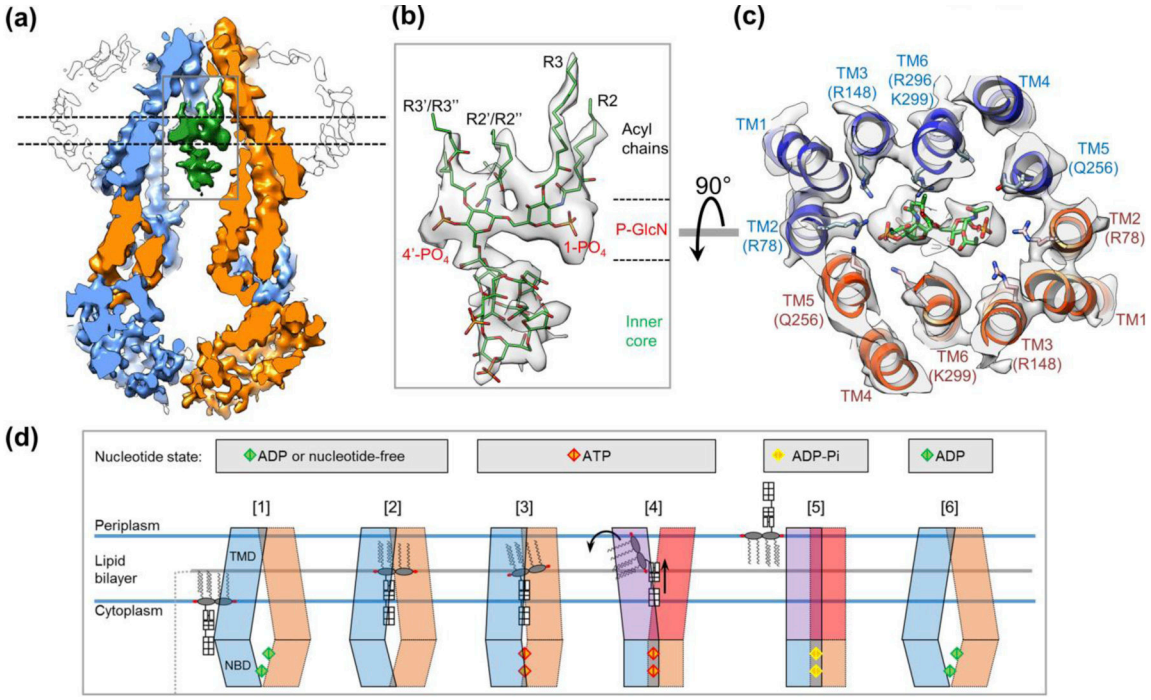

Figure 2 |. Cryo-EM structure of MsbA in nanodiscs.

(a) Slice view of the cryo-EM map of MsbA with bound LPS[23]. The two MsbA subunits and LPS are colored in blue, orange and green. Nanodisc is shown as outline. Within the inner cavity of MsbA, LPS is elevated relative to the cytoplasmic membrane leaflet. **(b)** Close-up view of LPS as highlighted by the rectangle in **a**. Cryo-EM map (grey) is superimposed with the model of LPS (green). **(c)** Cross-section of the cryo-EM map of MsbA superimposed with the associated model is viewed from the periplasm in perpendicular to the membrane plane, at the level of the phosphorylated glucosamine (P-GlcN) as indicated by dashed lines in **a**. **(d)** Proposed 'trap and flip' model for LPS flipping by MsbA. Proposed nucleotide states are indicated at top. 1) LPS in the cytoplasmic leaflet of the IM enter the central cavity of MsbA in its inward-facing open conformation. 2) LPS achieves stable binding through electrostatic and hydrophobic interactions, which restrict the opening of MsbA and align two NBDs for ATP binding. 3) ATP binding induces conformational changes of TMDs to abolish the LPS-MsbA interactions. 4) The outward opening of TMDs exposes the hydrophobic acyl chains of LPS to the aqueous periplasm, and the incompatibility causes the rotation of acyl chains into the periplasmic leaflet of the IM and also upward movement of the core oligosaccharides. 5) After LPS is flipped out, the TM helices move inward and collapse the central cavity, and the ATP is hydrolyzed. 6) MsbA returns to the inward-facing open conformation for the next round LPS flipping.

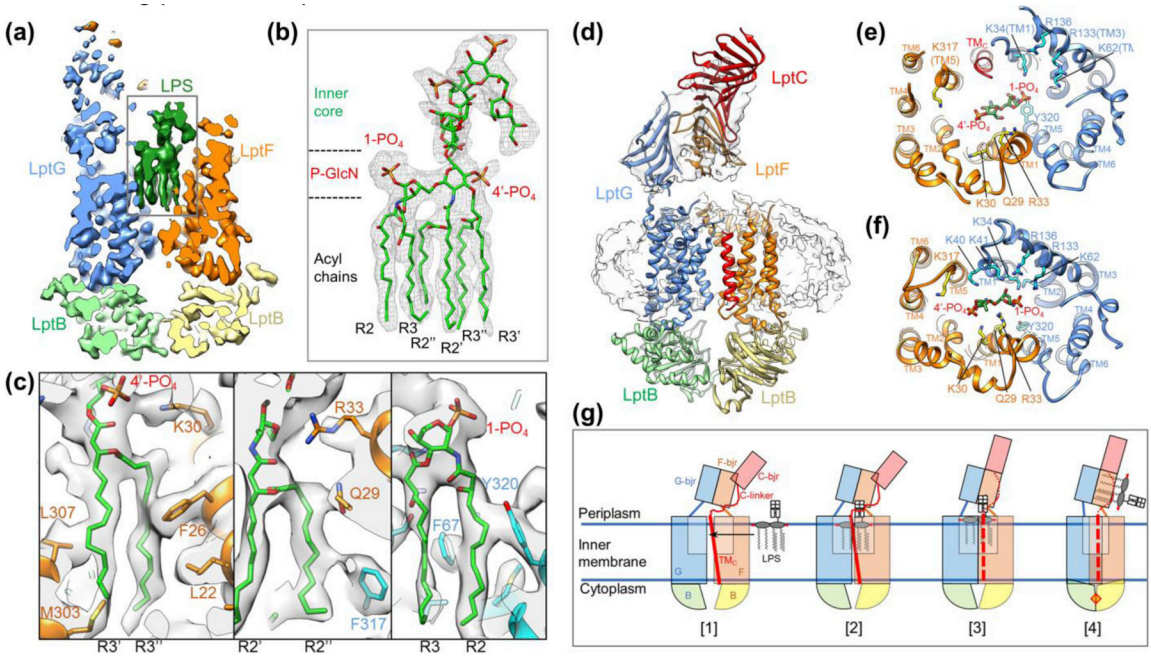

Figure 3 |. Cryo-EM structures of $LptB_2FG$ and $LptB_2FGC$ in nanodiscs.

(a) Slice view of the cryo-EM map of $LptB_2FG$ with bound LPS[40]. The Lpt proteins and LPS are colored differently. **(b)** Close-up view of LPS as highlighted by the rectangle in **a**. The cryo-EM density is of sufficient quality to directly visualize all six acyl chains, phosphorylated glucosamines (P-GlcN), and inner core sugars. **(c)** Zoomed-in views of LPS binding pocket. **(d)** Cryo-EM map and associated model of $LptB_2FGC$. The TM helix of LptC (red) binds between the two TMDs formed by LptF and LptG. The β-jellyroll domain of LptC stacks on top of the β-jellyroll domain of LptF (orange), forming a continuous groove path. **(e, f)** Views from the periplasm of the LPS binding pocket for $LptB_2FGC$ (**e**) and $LptB_2FG$ (**f**). The presence of the LptC TM helix causes the TM helices of LptF and LptG to spread apart (**e**). In the absence of LptC the two halves of the transporter move closer together, providing for a tight electrostatic lock on the bound LPS (**f**). **(g)** Proposed 'lock and squeeze' model for LPS extraction by $LptB_2FGC$. 1) LPS traverses laterally from the outer leaflet of the IM into the central cavity of $LptB_2FGC$. 2) Binding of LPS in the central cavity induces the closure of TMDs to lock the bound LPS, and promotes the release of the LptC TM helix. 3) The LptC TM helix (red dashed line) is dissociated from $LptB_2FG$, which facilitates the attachment of the β-jellyroll domain of LptC onto LptF β-jellyroll domain. 4) ATP binding induces tight dimerization of LptB subunits, and causes collapse of the LPS binding pocket to squeeze out the bound LPS.

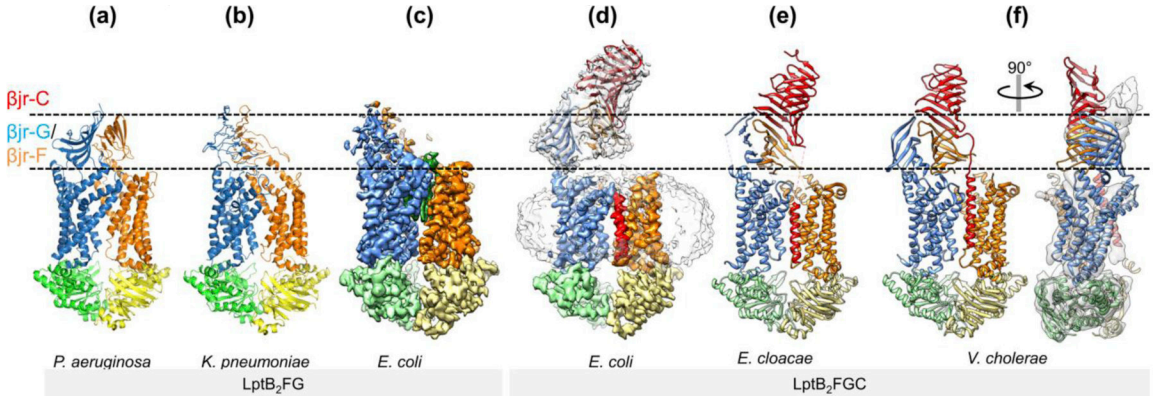

Figure 4 |. Dynamic β-jellyroll domains.

Surface views of the crystal (**a**, **b**, **e**, **f**) and cryo-EM structures (**c**, **d**) of $LptB_2FG$[31,32] and $LptB_2FGC$[40,41] show conformational plasticity of the periplasmic β-jellyroll domains. The regions corresponding to the β-jellyroll domains of LptF/LptG and LptC are delineated by dashed lines. In **f**, the crystal structure of $LptB_2FGC$ from *V. cholerae* is superimposed onto the cryo-EM map of *E. coli* $LptB_2FGC$ (grey as in **d**), demonstrating the different orientations of the β-jellyroll domains in these two structures.