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New insights into lipopolysaccharide assembly and export

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Lipopolysaccharide is an important immunomodulatory and structural component found in the outer membrane of Gram-negative bacteria. The biosynthesis of this glycoconjugate proceeds by a highly conserved pathway and, as such, is an attractive target for antibiotic action. We highlight here recent work focused on understanding this pathway with an emphasis on new insights related to chain length control and transport across the inner and outer cell membranes.

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Introduction

Lipopolysaccharide (LPS), a glycoconjugate present in the outer membrane (OM) of Gram-negative bacteria [1], is an important immunomodulatory molecule, contributes significantly to the structural integrity of the OM, and helps shield these organisms from antibiotics and other environmental attacks [2]. Beyond these well-established roles, Rojas *et al.* [3••] have recently shown that LPS, together with membrane proteins, also contributes to cell stiffness. A reduction is OM stiffness is observed when LPS biosynthesis is compromised in turn making the organism more susceptible to mechanical forces, such as turgor pressure. These findings all support the notion that targeting LPS assembly is a viable drug development strategy. Given the rise in drug resistance, effective treatment of infections by these organisms is an unmet medical need [4,5].

Three components comprise LPS (Figure 1): Lipid A, the core oligosaccharide, and the O-antigenic polysaccharide (O-PS). Lipid A is conserved throughout Gram-negative bacteria and is the primary lipid component of the OM outer leaflet. It is synthesized in the cytoplasm by a nine-step enzymatic process (the Raetz pathway) [6] and activates the host innate immune response [7]. The core oligosaccharide moiety, which connects Lipid A to the O-PS, shows greater structural variability across Gram-negative bacteria and is also synthesized in the cytoplasm [8]. After the lipid A–core oligosaccharide is assembled, it is flipped by the ABC transporter MsbA [9] to the periplasm where the O-PS is attached. Recent cryo-EM [10••] and X-ray crystal [11] structures of MsbA reveal that the acyl chains and the 4′-phosphate of lipid A are the structural features needed for LPS flipping.

The O-PS is hyper-variable [12], is an essential virulence determinant [13] and is responsible for resistance to complement-mediated serum killing [14]. The O-PS is synthesized in the cytoplasm by one of three different pathways: the Wzy-dependent pathway [15], the synthase dependent pathway [16], or the ABC transporter-dependent pathway (Figure 2a) [17]. Once the O-PS is synthesized and transported across the inner membrane (IM), it is then ligated to the lipid A–core oligosaccharide by the enzyme, WaaL [18], and transported to the OM by the LPS protein transport (Lpt) machinery (Figure 2b), which is highly conserved in Gram-negative bacteria [19].

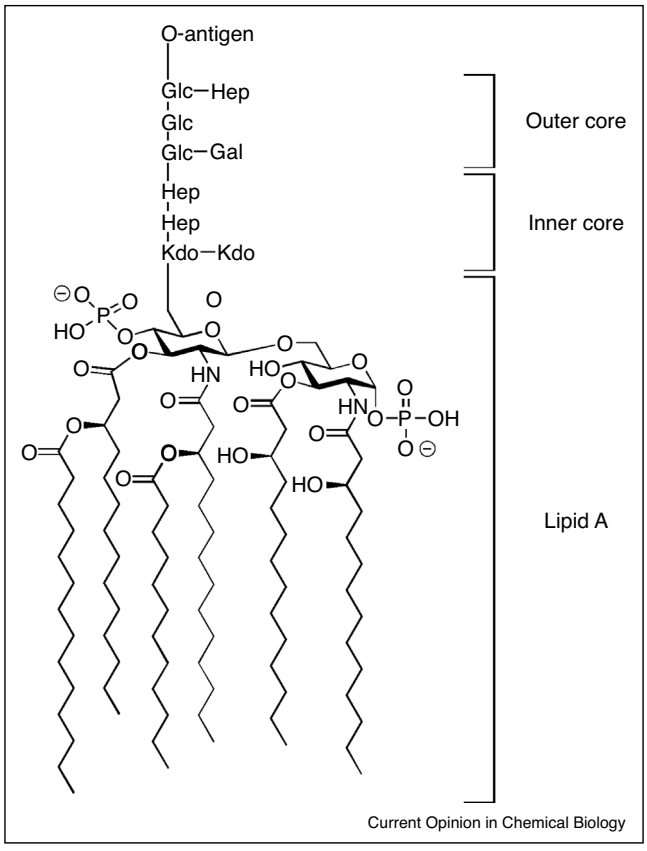
In this perspective, we highlight recent advances in understanding LPS biosynthesis. In particular, the focus is on studies of proteins in the ABC transporter-dependent O-PS biosynthetic pathway and from the Lpt protein machinery complex.

ABC transporter-dependent O-PS biosynthesis

In the ABC transporter-dependent pathway, the O-PS is assembled by glycosyltransferases (GTs) when anchored in the IM cytoplasmic face on a polyprenol pyrophosphate carrier. Once it reaches its full length, the glycan is exported to the periplasm, where it is ligated to the lipid A–core oligosaccharide. Chain length and export of the O-PS are controlled by one of two mechanisms: (1) termination of chain extension by a capping modification [20], or (2) a process by which chain extension and export are coupled [21]. In recent years, key discoveries have focused more on the first mechanism.

In 2015, Hagelueken *et al.* [22••] provided the first structural insights into the capping mechanism, via the crystal structure of the *Escherichia coli* O9a WbdA–WbdD complex (Figure 3a). WbdA is a bifunctional GT responsible for

Figure 1



The general structure of lipopolysaccharide (LPS), based on that present in *E. coli*. Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; Hep, L-*glycero*-D-*manno*-heptose, or D-*glycero*-D-*manno*-heptose; Glc, D-glucose; Gal, D-galactose.

O-PS chain polymerization and WbdD is a bifunctional kinase–methyltransferase that caps the O-PS chain with a methyl phosphate group thus terminating chain extension. The WbdD catalytic domain lies at the end of a coiled-coil region that separates it from the catalytic domain of WbdA by ~200 Å, a distance similar to that calculated for the length of the O-PS based upon the average number of repeating units. This finding suggests that the coiled-coil domain of WbdD acts as a molecular ruler; in support of this hypothesis, varying the length of the coiled-coil domain of WbdD, altered the length of the O-PS.

In a subsequent related study, Williams *et al.* [23•] reported a single protein that assembles the entire O-PS chain of *Raoultella terrigena* LPS (Figure 3b). The protein, WbbB, has three GT domains, with one separated from the other two by a coiled-coil motif. The two adjacent GT domains assemble the O-PS disaccharide repeating unit, while the third is responsible for the capping of the chain with a 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residue. Thus, it appears that coiled-coil molecular rulers are common architectures responsible for chain length control of O-PS, and possibly other polysaccharides.

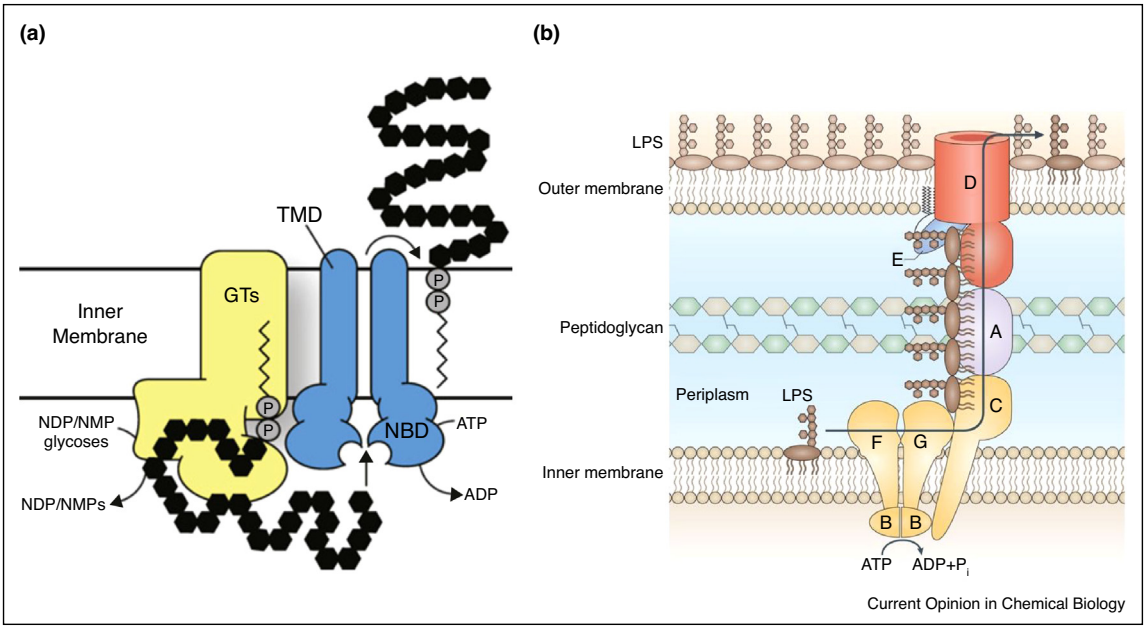
After O-PS polymerization, the glycan is exported to the periplasm by an ABC transporter. When the glycan has a capping moiety, a carbohydrate binding module in the transporter recognizes the O-PS before export. Bi *et al.* [24••] recently reported the first crystal structure of an ABC transporter in an open conformation. The protein investigated, from *Aquifex aeolicus*, is a homolog of the prototypical *E. coli* ABC transporter. The data demonstrate that the transporter channel can accommodate a linear polysaccharide and that 8–10 monosaccharide units can span the IM. The structure also suggests that the polyprenol pyrophosphate is recognized by the gate helix of the transporter. Further studies [25•] revealed that the transporter forms a large, continuous transmembrane channel when it is in a closed, ATP-bound conformation. Molecular dynamics simulations suggest that a lipid gate blocks solvent entrance at the periplasmic opening, preventing water from entering the channel. Taken together, these results support a highly regulated transport system for the export of O-PS. Alignment of the primary sequence of this protein with homologs revealed that gate helices are characteristic features of ABC transporters that accept polyprenol pyrophosphate linked intermediates including those that do not cap O-PS chains before export. These shared structural features imply a similar recognition element for glycan export in both the capping and no-capping mechanisms. There have been few advances in understanding chain length control and export of uncapped O-PS; however, new developments in technology and probes [26] will hopefully shed light on this in the future.

Lpt protein machinery complex

The seven-protein Lpt complex (LptA–G) transports LPS from the IM to the OM (Figure 2b) [19]. $LptB_2FGC$ removes LPS from the IM and starts the transport process to the OM [27]. Within this assembly, $LptB_2FG$ is the ABC transporter; LptC connects $LptB_2FG$ to LptA [28] and coordinates LPS transport (below). In turn, LptA provides a groove along which LPS is pushed across the periplasm [29] before LptDE inserts it into the OM [30,31]. This protein assembly has received significant recent study.

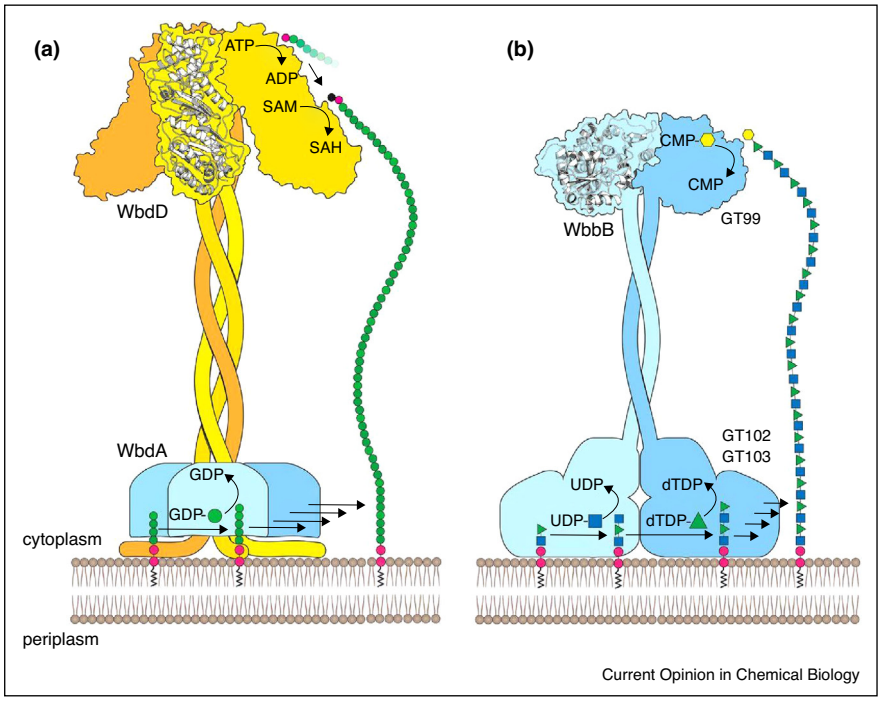
Luo *et al.* [32••] and Dong *et al.* [33••], independently reported crystal structures of $LptB_2FG$ and a mechanism for LPS extraction from the IM. In their model, a gate in $LptB_2FG$ opens upon nucleotide (ATP) binding, resulting in an open conformation that allows LPS access to the central cavity of the complex. Bertani *et al.* [34•] identified key residues within the central cavity that bind LPS by interacting electrostatically with the phosphate esters. These studies suggest a mechanism for LPS extraction by which nucleotide binding alters the conformation of

Figure 2



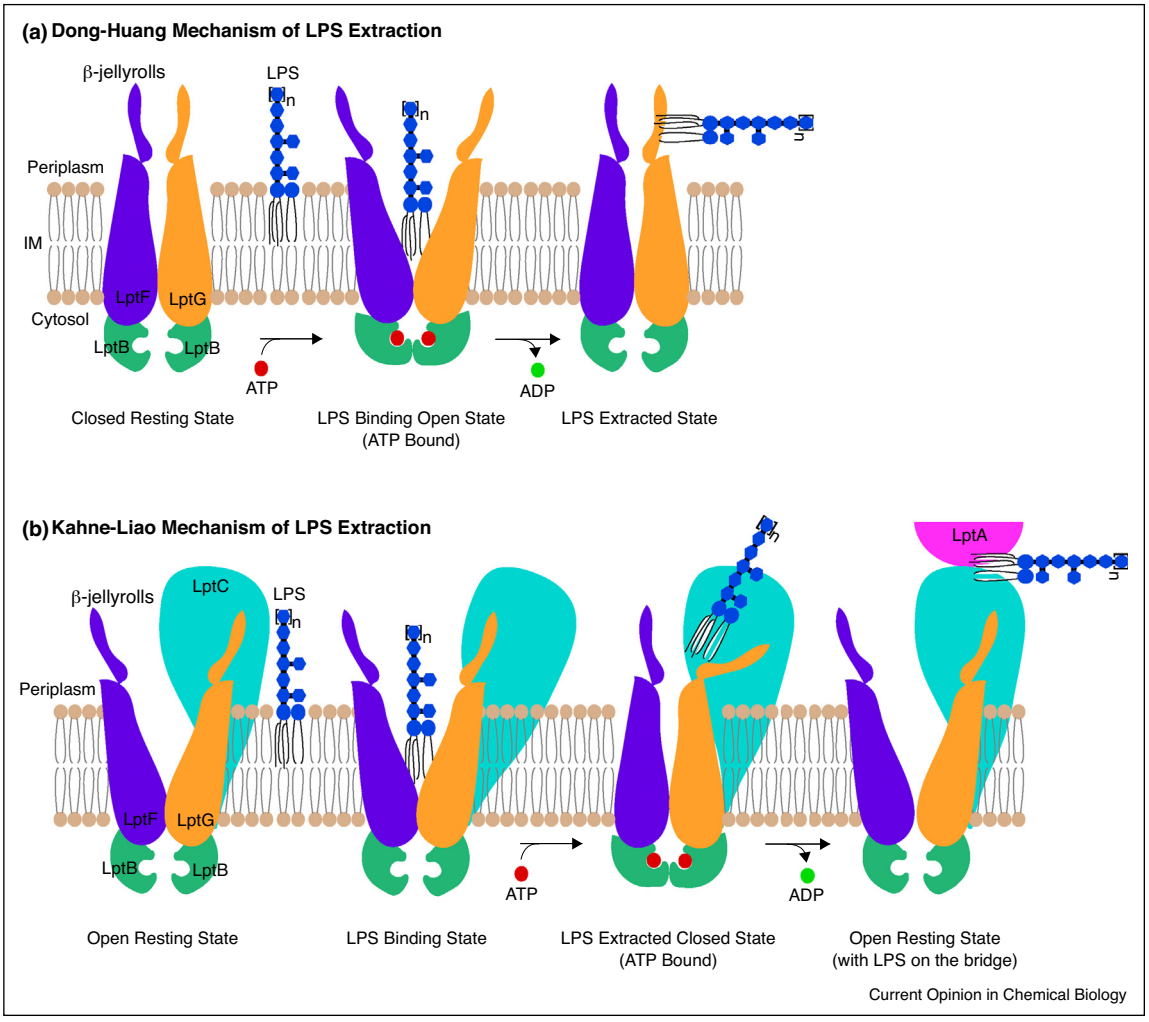
(a) Cartoon representation of the overview of the ABC transporter-dependent O-PS biosynthetic pathway. First, the O-PS is synthesized in the cytoplasm on a lipid carrier, before its recognition and export by an ABC transporter (Adapted with permission from Greenfield LK, Whitfield C: **Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways**. *Carbohydr. Res.* 2012, **356**:12–24).
(b) Lpt protein bridge featuring the 'PEZ model'. After the Lpt proteins carry LPS across the periplasm, LPS completes its journey by being inserted into the outer membrane (Reproduced with permission from Okuda S, Sherman DJ, Silhavy TJ, Ruiz N, Kahne D: **Lipopolysaccharide transport and assembly at the outer membrane: the PEZ model**. *Nat. Rev. Micro.* 2016, **14**:337–345).

Figure 3



Cartoon representation of the O-PS chain elongation and termination mechanisms from *E. coli* O9a **(a)** and *R. terrigena* **(b)** (Reproduced with permission from Williams DM, Ovchinnikova OG, Koizumi A, Mainprize IL, Kimber MS, Lowary TL, Whitfield C: **Single polysaccharide assembly protein that integrates polymerization, termination, and chain-length quality control**. *Proc. Natl. Acad. Sci. U.S.A.* 2017, **114**: E1215–E1223).

Figure 4



Mechanistic proposals for the extraction of LPS from the inner membrane.
(a) Mechanism proposed by Dong and co-workers and Huang and co-workers. First, ATP binds, which causes a conformational change to LptFG (LPS Binding Open State). Then, LPS enters the cavity, and after ADP is released, LPS is extracted into the Lpt protein complex (Original artwork adapted from: Luo Q, Yang X, Yu S, Shi H, Wang K, Le Xiao, Zhu G, Sun C, Li T, Li D, et al.: **Structural basis for lipopolysaccharide extraction by ABC transporter LptB$_2$FG**. *Nat. Struct. Mol. Biol.* 2017, **24**:469–474).
(b) Mechanism proposed by Kahne and co-workers and Liao and co-workers. LPS first binds in the cavity of LptFG, pushing LptC away from the cavity (LPS Binding State). Then upon ATP binding, the cavity of LptFG is forced inward, pushing LPS through the β-jellyroll gate of LptG and onto LptC (LPS Extracted Closed State). Once LPS is on the Lpt protein bridge, the gate spontaneously closes, causing the unidirectional movement of the glycan.

LptB$_2$FG, which then binds LPS and initiates its transport to the OM (Figure 4a).

In related work, Li *et al.* [35••] solved high-resolution cryo-EM structures of the LptB$_2$FGC complex with and without LPS bound. LPS binds strongly to the LptB$_2$FG cavity; however, when the transmembrane helix of LptC is present, the binding weakens. Structures with ADP–vanadate trapped in the nucleotide binding domain show dimerization of the LptB subunits, which, in turn, push the transmembrane domains of LptFG towards each other, constricting the cavity. No bound LPS was observed in these vanadate-trapped structures, implying that they represent conformations after LPS expulsion from the cavity. Using X-ray crystallography, Owens *et al.* [36••] reported two structures of the LptB$_2$FGC complex and also observed that the LptC transmembrane helix is inserted in the LptFG cavity. When this helix is removed, LPS transport to LptA is reduced. Studies revealed that LPS bound tightly in the cavity of wild-type LptB$_2$FGC complexes. Further experiments showed that LPS accumulates in a β-jellyroll domain of LptF and LptC,

suggesting LPS enters the cavity independent of ATP, and ATP powers transport through the channel. Disulfide introduction into the β-jellyroll of LptF provided a non-functional complex *in vivo*, but LPS transport returned upon disulfide reduction *in vitro*. ATP hydrolysis was seen, regardless of the opening state of the gate, suggesting ATP drives LPS movement in the cavity, and that LPS pushes the gate open upon arrival.

The Li *et al.* [35••] and Owens *et al.* [36••] studies suggest a mechanism of LPS extraction from the IM that differs greatly from that proposed by Luo *et al.* [32••] and Dong *et al.* [33••] (Figure 4b). First, LPS binds to the cavity, causing a conformational change that pushes LptC away from it. ATP then binds to and dimerizes the LptB subunits, which forces the transmembrane domains of LptF and LptG inward, pushing LPS forward to LptC and LptA. LPS is then forced through a β-jellyroll gate of LptF onto LptC, after which the gate spontaneously closes, forcing unidirectional movement of the glycan to the OM.

LptC is also thought to facilitate the transfer of the LPS to LptA, which then shuttles LPS across the periplasm. Laguri *et al.* [37] showed that the LPS lipid moiety interacts strongly with hydrophobic cavities of both LptC and LptA. Around the same time, Schultz *et al.* [38] characterized the binding of LPS to LptA and established the location of LPS binding to LptA, that they bind in a 1:1 ratio, and that the N-terminus of the protein, which interacts with LptD [39], unfolds in the presence of the glycan. These studies also provided evidence implying that when LptA cannot form oligomers, it has drastically lower affinity for LPS. It was proposed that the protein oligomerizes to cross the periplasm, and that the unfolding of the N-terminus facilitates LPS transfer to LptD. Schultz and Klug [40] later showed that LptC also binds LPS in a 1:1 ratio with similar binding constants to those observed with LPS–LptA binding.

Sherman *et al.* [41••] later provided direct evidence that LptA forms a bridge spanning the periplasm. Their studies did not support the oligomerization of LptA as a prerequisite for transfer and they hypothesized that one or two LptA proteins would be sufficient to bridge the gap between LptC and LptD. Subsequent studies using a fluorescent probe that detects LPS [42•] demonstrated [43••] that a OM translocon of LptDE communicates with the $LptB_2FGC$ complex on the IM to stop LPS transport. LPS cannot be inserted into the OM beyond a certain threshold, based on the structure of the translocon and its affinity for LPS. Moreover, an inability to insert LPS into the OM stops ATP hydrolysis at the IM. This long-range communication regulates LPS transport and prevents the cell from wasting resources by starting the transfer of the LPS to the OM and expending ATP. These results suggest that the Lpt protein complex shuttles LPS to the OM in a highly regulated manner that efficiently uses ATP, the cellular power source.

Conclusions

LPS is a critical component of the OM in Gram-negative bacteria and given its attractiveness as a target for drug action, the pathways by which the molecule is assembled have received significant attention. Recent advances in structural biology and probe synthesis have provided new insights into how chain length is controlled and how the molecule is transported from the cytosol, across them IM and then on to the OM. Given space considerations, it was not possible to highlight here molecules that inhibit the action of many of these proteins; however, a number of such compounds are now available [11,44–48]. Further refinement of the details of these processes, and optimization of the lead molecules identified to date are expected to provide new approaches for treating infections caused by Gram-negative bacteria.

Conflict of interest statement

Nothing declared.

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