Novel insights in the lipopolysaccharide transport system of Gram-negative bacteria

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Abstract

The envelope of Gram negative bacteria consists of an inner membrane and an outer membrane, separated by the periplasm. The OM is an asymmetrical bilayer that contains phospholipids on its periplasmic side and LPS on its extracellular side. Although the LPS biosynthetic pathway has long been elucidated, less is known about the transport and assembly of LPS into the OM. Not long ago, the machinery involved in this process has been discovered. Since then, information regarding the machinery involved in LPS transport is accumulating. However, the exact mechanism of action of this machinery remains to be investigated. This review focuses on the most recent developments regarding the LPS transport system, with an emphasis on the components responsible for inserting LPS into the OM. In addition, this review will provide an example of the possible implications of new insights in the LPS transport system for the future, in the form of the development of novel Gram-negative treatment modalities that target the LPS transport system.

Introduction

Gram negative bacteria, as opposed to Gram positive bacteria, are prokaryotes that do not retain violet color in a Gram staining assay developed by Hans Christian Gram in the late 19th century. This type of staining differentiates between bacteria based on the properties of the bacterial envelope, a major line of defense against many potentially harmful compounds and environmental conditions. The envelope of Gram negative bacteria consists of a hydrophilic compartment called the periplasm, sandwiched by an inner (IM) and an outer (OM) membrane (fig. 1). The IM is a symmetric phospholipid bilayer with incorporated proteins that forms the edge of the cytoplasm. These proteins consist of α-helical transmembrane proteins, lipoproteins tethered to the IM facing the periplasm and peripherally attached soluble proteins facing either side of the IM [1]. Three major transport systems are responsible for the insertion of IM proteins into the cytoplasmic membrane and translocation of proteins from the cytoplasm to the periplasm: the secretion (Sec) translocon, the YidC insertase and the twin arginine system [2]. The Sec system is thought to function as the main protein transport mechanism and is responsible for the secretion of various molecules in addition to delivery of unfolded proteins to the periplasm (reviewed by [3]). This compartment contains a peptidoglycan layer providing cell shape and protection against osmotic stress. In addition, the oxidizing environment of the periplasm allows for the formation of disulfide bridges that facilitates correct protein folding, thereby stabilizing essential protein structures [4]. Compounds that cross the periplasm reach the OM, the final barrier of the envelope. The OM is a unique asymmetric bilayer with an outer leaflet composed of lipopolysaccharide (LPS) and an inner leaflet composed of phospholipids. It contains two major classes of proteins: lipoproteins that are generally soluble and tethered to the OM through an N-terminal lipid modification and OM integrated proteins that present β-barrel structures (reviewed by [5]). Assembly and incorporation of these integrated OM proteins (OMPs) into the OM is facilitated by the β-barrel assembly machine (Bam) complex. OMPs function as receptors, porins, channels and other transport machineries that facilitate interaction with the extracellular environment [6].

Components of the bacterial Outer Membrane

The OM forms a highly-selective permeability barrier with properties that differ from the IM mainly due to the presence of LPS. The exact composition of LPS differs between strains, but the classical LPS molecule consists of three separate components: a hydrophobic moiety called Lipid A or endotoxin that anchors LPS to the OM, a non-repeating core oligosaccharide and a distal O-polysaccharide or O-antigen (fig. 1) [7]. The core subunit can be divided in the 'inner core' that consists of 3-deoxy-D-manno-oct-2- ulosonic acid (Kdo) and heptose, and an 'outer core' that consists of galactose, glucose and heptose in addition to a variable number of O-antigen subunits. Several properties of LPS are thought to contribute to the efficiency of the OM as a permeability barrier: 1) low fluidity of the LPS hydrocarbon domain. LPS contains several fully saturated fatty acid side chains that are expected to be in a gel-like state, thereby preventing easy access of potentially harmful chemicals; 2) strong lateral interactions between LPS molecules facilitated by the bridging action of divalent cations; 3) interactions between LPS and OMPs that further complicate access of chemicals [5]. These properties provide Gram-negative bacteria with protection

against many potential dangers including toxic environments, changing temperature, detergents, bile salts, antimicrobial peptides, antibiotics and the hostile host environment encountered during colonization or infection. When encountered by the immune system, LPS presents itself as a potent immune activator with detection levels of most species at picomolar concentrations [5]. Lipid A (or endotoxin) is the epitope for the ancient receptor toll-like receptor 4 (TLR4/MD2). When endotoxin molecules are abundantly present in the host bloodstream during infection, immune responses may precipitate Gram-negative septic shock, a rapidly progressing inflammatory disease with relatively high mortality rates. Unfortunately, treatment of Gram-negative infections to prevent septic shock e.g. by using antibiotics is often complicated by the barrier function of LPS. Therefore, new insights in the pathways involved in LPS homeostasis may provide the basis for novel therapeutics.

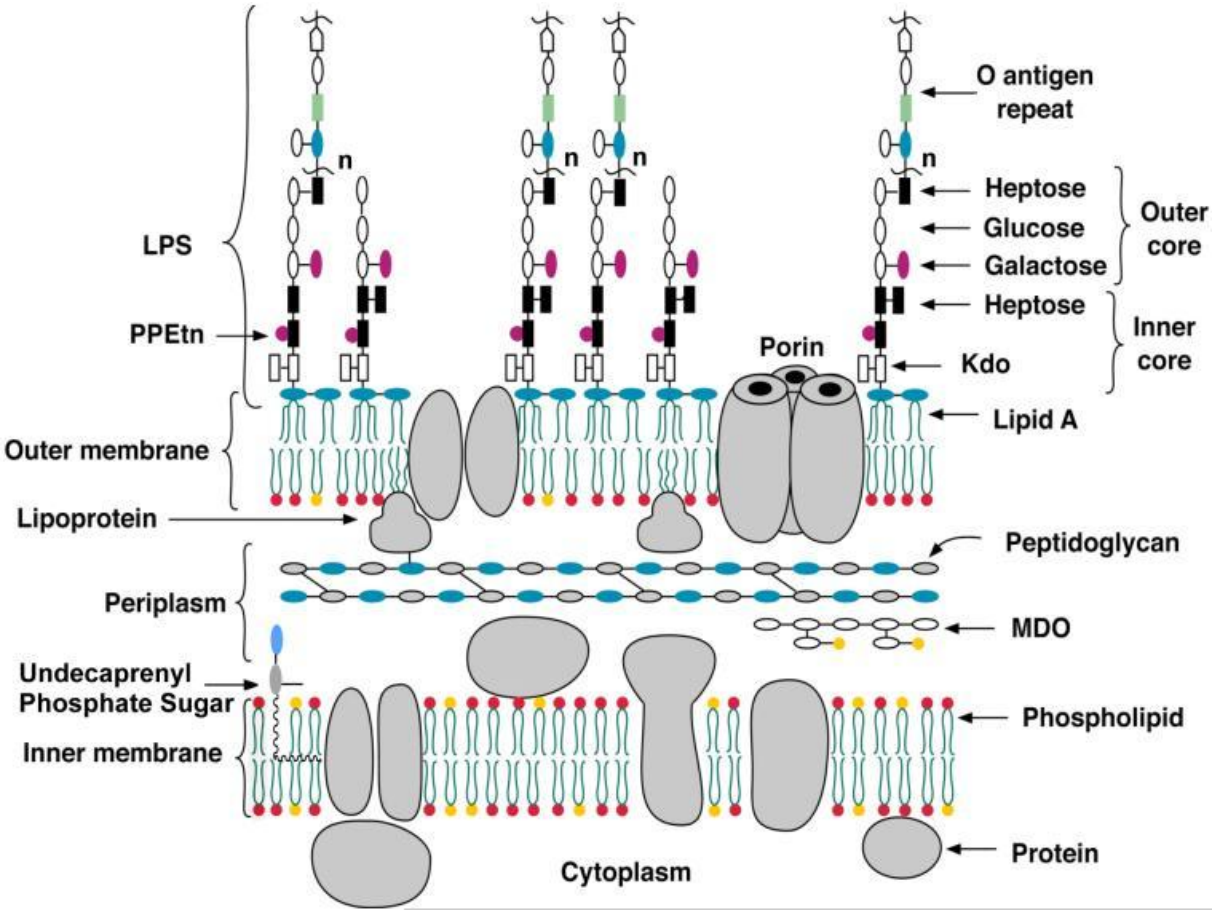


Figure 1. Schematic overview of the *E. coli* K-12 bacterial cell wall. The components of the wall are: 1). A phospholipid inner membrane, 2). An asymmetrical outer membrane with lipopolysaccharide 3). The periplasm that separates the inner from the outer membrane. The figure is adopted from [7]

Lipopolysaccharide biosynthesis in Gram negative bacteria

The chemical structure of LPS and the biosynthetic pathways leading to the formation of LPS have been elucidated in the last two decades (reviewed by [7][8]). The first step in LPS biosynthesis of *Escherichia coli* is the formation of Kdo_2-Lipid A, which is the minimal LPS required for growth in most strains. Kdo_2-Lipid A synthesis is initiated at the cytoplasmic side of the IM by two fatty acylation steps of UDP-N- acetylglucosamine (GlcNAc) (fig. 2) and requires the convergent pathways of Lipid A, Kdo and the oligosaccharide core. Combination

of two diacylated GlcNAc molecules in addition to two phosphorylation steps leads to the formation Lipid IV_A. Two labile CMP-Kdo residues are then incorporated into Lipid IV_A, followed by the addition of two secondary acylation residues to form hexaacylated Kdo_2-Lipid A (fig. 2). The genes involved in these processes are intracellular, constitutively expressed and present in virtually all gram-negative bacteria [7]. Regulation of these first steps in LPS biosynthesis is known to be facilitated by the membrane-bound ATP-dependent metalloprotease FtsH. This protease has been shown to degrade LpxC [9], an enzyme that carries out the first committed step in Lipid A synthesis, in addition to KDO transferase [10], the enzyme responsible for the addition of two KDO residues to lipid IV_A. In this way, FtsH regulates synthesis of the lipid moiety as well as the sugar moiety of LPS, ensuring a balanced synthesis of both LPS components. Recently, a gene called *yciM* was also shown to be involved in the regulation of LPS biosynthesis in *E. Coli* [11]. Inactivation of YicM increased LpxC levels, leading to higher amounts of LPS and even cell death. YicM mediated regulation of LpxC levels was found to be dependent on the presence of functional FtsH, suggesting a regulatory role for YicM towards the LpxC protease FtsH. In another study, the *yciM* homologue *ght* of *N. meningitidis* was implicated in the regulation of LPS biosynthesis [12]. Absence of the IM protein Ght caused a drastic decrease of the total amount of LPS, in sharp contrast to *E. coli*. The observation that LPS could still be detected at the cell surface of *ght* mutants indicated that LPS transport was not impaired. These mutants were overgrown by pseudorevertants that contained normal levels of LPS. Genetic analysis revealed that these pdeudorevertants overexpressed *lpxC*. Therefore, it was suggested that Ght regulates LPS biosynthesis by influencing LpxC activity. These results suggest that both YicM of *E. Coli* and Ght of *N. meningitidis* influence LPS biosynthesis with completely different outcome regarding total LPS levels, underlining that homologues do not necessarily perform the same function across strains.

The remaining residues that form core- Lipid A of LPS with the exception of O-antigen are attached to newly formed Kdo_2-Lipid A by specific glycosyl-transferases (fig. 2). The core-Lipid A with its cytoplasmic orientation is then flipped across the IM to face the periplasm by the ATP-binding cassette (ABC) transporter MsbA (explained below). At the periplasmic side of the IM, O-antigen molecules are attached to core-Lipid A by the WaaL ligase to form mature LPS. Prior to this, cytoplasmically synthesized O-antigen molecules are linked to undecaprenyl phosphate and flipped across the IM to reach the periplasm. The O-antigen is not required for growth in lab environments and is missing in common *E. coli* K12 strains but essential for many other strains under physiological conditions because it provides protection against antibiotics and complement-mediated lysis [13].

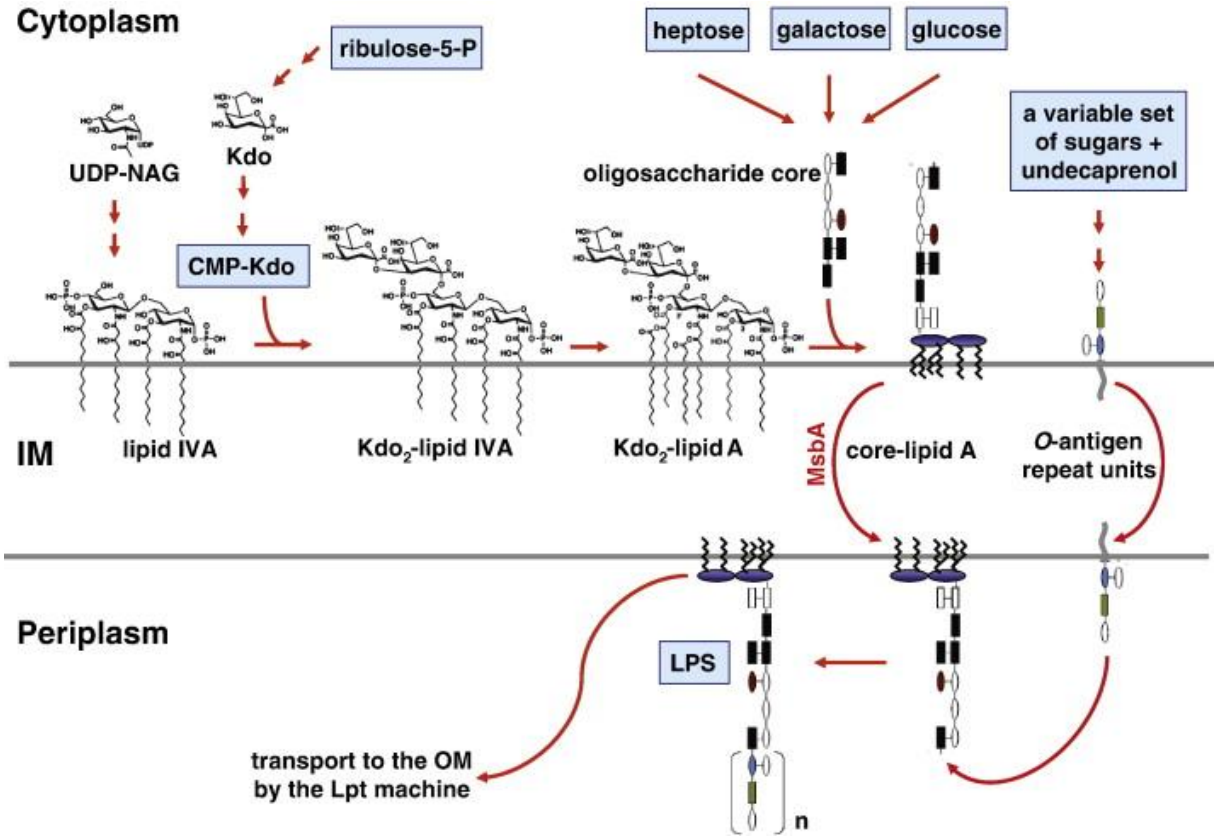


Figure 2. LPS biosynthesis pathway in *E. coli*. Two UDG-NAG molecules form lipid IVA and subsequently form Kdo_2-Lipid A after attachment of Kdo molecules. After two rounds of acylation, the core oligosaccharide is assembled on Kdo_2-Lipid A. Core-Lipid A is proposed to be flipped across the IM by MsbA, where O-antigen units are attached, before transport to the OM. Rectangles and ovals represent sugar moieties as shown in fig. 1. The figure is adopted from [14]

After LPS biosynthesis has been completed, the newly formed molecules are transported across the periplasm where they are incorporated into the OM. One complicating factor for transport of proteins from the IM to the OM is that the periplasm is devoid of ATP or any other high energy carrier and therefore requires endergonic reactions driven by components connected to the cytoplasm through the IM. Compared to protein secretion and trafficking by the Sec system, the machinery involved in transport of LPS is less well understood and has been a topic of investigation. In the last decade, studies using *E. coli* and *Neisseria meningitidis* have brought new insights in this process. This review will summarize the discovery of the machinery involved in LPS transport: the lipopolysaccharide transport (Lpt) system and focus on the most recent findings regarding this system, with an emphasis on the LptDE complex. Additionally, this review will give an example of the possibilities of new insights in the LPS transport system in the form of novel treatment modalities that target this system.

The LPS transport system of Gram-negative bacteria

MsbA

The first gene shown to be implicated in LPS transport, the ABC transporter MsbA, was originally isolated in a random screening for multicopy suppressors of temperature-sensitive

phenotypes of an *lpxL* (initially identified as *htrB*) mutant [15]. These mutants lack a functional tetraacylated Kdo_2-Lipid A IVA acyl transferase and therefore fail to form pentaacylated Kdo_2-Lipid A. The functional consequence of this is a reduced temperature resistance in addition to alterations in cell morphology, accumulation of phospholipids and of tetraacylated LPS precursor molecules [16]. Expression of *msbA* from a plasmid vector in an *lpxL* mutant suppressed the temperature-sensitive growth by restoring phospholipid composition and inducing transport of tetraacylated LPS precursors to the OM. MsbA therefore appeared to function not by restoring the ability to form pentaacylated Kdo_2-Lipid A, but by facilitating migration of LPS to the OM. In addition, the mutant $msbA_{A270T}$ displayed LPS accumulation on the cytoplasmic side of the IM, indicating that MsbA is involved in LPS transport across the IM [17]. Moreover, it was found that purified MsbA reconstituted into *E. coli* derived proteoliposomes shows flippase activity, indicating that MsbA transfers LPS across the IM by a flippase mechanism. [18]. Together, these results strongly suggest a flippase role for MsbA in the transport of LPS. However, direct flippase activity of MsbA in the bacterial envelope remains to be shown.

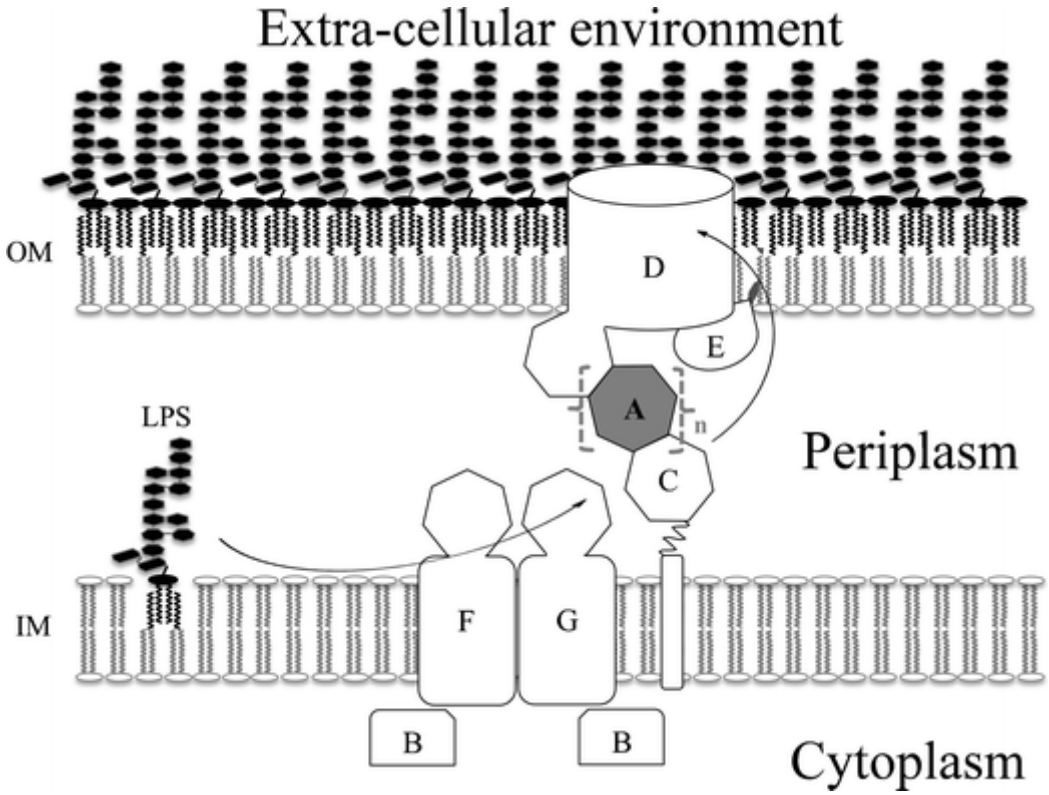


Figure 3. Model for the LPS transport system Lpt. Transport of LPS molecules across the IM to the OM is facilitated by the Lpt system. Details of the mechanism of action of the Lpt machinery are described in the text. The figure is adopted from [19]

The Lpt machinery in LPS transport

Translocation of LPS from the periplasmic side of the IM to the OM is facilitated by the Lpt machinery (fig. 3). The research on the components of the Lpt system can be divided into three parts based on the location of each element involved: LptB, LptC, LptF and LptG in the IM, LptD and LptE in the OM and LptA connecting both parts of the Lpt machinery. LPS first

encounters the Lpt components present in the IM after translocation by MsbA. Together, these components are reported to form the $LptB_2CFG$ complex [20] and are thought to facilitate release of LPS from the IM. As this is an energetically unfavorable process and the periplasm is devoid of ATP or any other high energy carrier, the question arises where the energy to fuel such a step is derived from. LptB, a 26.7 kDa protein facing the cytoplasm where energy carriers are present, was shown to contain an ATP binding cassette (ABC) signature [21]. Therefore, LptB met the requirements to provide the energy required for Lpt-mediated LPS transport. The gene encoding LptB, *lptB*, was originally identified by a genetic screen together with *lptA* and *lptC* (formerly known as *yhbG, yhbN*, and *yrbK*, respectively) [22][23]. Depletion of either LptA, LptB or LptC resulted in a failure to transport LPS to the OM [24]. In addition, mutants lacking these Lpt components were shown to contain LPS species with attached colanic acid residues. As colanic acid is only linked to LPS by the IM protein WaaL ligase, this observation suggested that LPS transport to the OM is impaired in these mutants [25][24]. Together, these results indicated that LptA, LptB and LptC are involved in LPS transport downstream of MsbA.

Recently, LptB mediated ATP hydrolysis has been investigated using the crystal structures of ATP- and ADP-bound LptB [26]. Three residues essential for LptB function were identified: F90, E163 and H195, of which F90 was found to be located in a domain called the Q-loop. The Q-loop is present in a structure that possibly forms a groove and is required for interaction with other members of the $LptB_2CFG$ complex. The groove was shown to undergo conformational changes upon ATP hydrolysis, thereby potentially transferring the energy required for LPS transport from the IM to the periplasm. The side chain of F90 was shown to face the interior of the groove, suggesting that this residue may be involved in the interaction with other members of the Lpt system. These other members, LptF and LptG (formerly known as the 40.4 kDa protein YjgP and the 39.6 kDa protein YjgQ, respectively) are each closely associated with one LptB molecule and may serve to transfer the energy generated by LptB to facilitate LPS transport (fig. 3). Both components were originally identified by a reductionist-based bioinformatics approach, using the small genome of *Blochmannia floridanus* to find novel factors involved in OM biogenesis [27]. It has been shown that LptB levels rapidly decrease without the presence of LptF and LptG, indicating that co-expression of LptF and LptG stabilizes LptB [20]. As noted, LptF and LptG are in a complex with LptC and therefore may also function as a stabilizing factor for LptC. In addition, LptF and LptG may function as the energy integrating component due to their close association with both the energy input LptB and the and the suggested LPS binding element LptC. However, their exact function and possible mechanism of action remains to be elucidated.

The last component of the Lpt machinery present in the IM, LptC, was identified as a 21.6 kDa IM protein with a large domain facing the periplasm and a singular smaller transmembrane domain [24]. *E. coli* expressing a truncated LptC lacking the transmembrane domain are viable [28]. Binding of truncated LptC to the LptBFG complex in these cells is unhindered, suggesting that the essential LptC functions are located in the periplasmic domain. LptC was thought to interact with LptA, an 18.6 kDa protein present in the periplasm that was predicted to form the bridge between the IM and the OM using

bioinformatics [23]. The crystal structures of LptC and LptA revealed large structural similarities in the form of a β-jellyroll fold, even though they do not share significant sequence similarity [29], suggesting that these proteins interact. LptA was reported to assemble into rod-like oligomers of up to at least a pentamer *in vitro* involving disorder-to-order transitions [19]. These oligomers display an LptA concentration-dependent increase in resistance to thermal denaturation, indicating increasing stability upon oligomerization. However, the affinity of LptA for LptC has been suggested to be even stronger than the affinity for LptA oligomerization [30]. These results provide evidence for a model in which LptC interacts with LptA that forms a bridge across the periplasm. Moreover, qualitative *in vitro* assays showed that LptC, in addition to LptA, possesses hydrophobic residues that form a core along the protein, possibly facilitating LPS binding [31]. Later it was found that four residues from LptC and five from LptA directly interact with LPS *in vivo* [32]. Interestingly, LptA is able to displace LPS from LptC *in vitro*, but not vice versa [31], consistent with the current model of LPS transport from the IM to the OM (fig. 3). These data suggest that LPS molecules are transported from LptC to LptA and are subsequently transported across the periplasm by LptA to reach the Lpt components present in the OM.

After transport across the periplasm, LPS reaches the Lpt components present in the OM, LptD and LptE, that are thought to be responsible for LPS incorporation into the OM. The discovery of these components initiated in 1989, when a gene involved in OM permeability was identified by two independent genetic screens and was termed *imp* (for increased membrane permeability) in one study [33] and *ostA* (for organic solvent tolerance) in another [34]. Together, these studies showed that mutations in *lptD* (previously known as *imp/ostA*) alter the permeability of the OM in *E. coli*, leading to increased sensitivity to detergents, dyes and antibiotics. Further studies showed that *lptD* codes for an 87 kDa β-barrel OMP that is essential for envelope biogenesis in *E. coli* [35]. However, it was not until 2004 that *lptD* was shown to be involved in LPS transport, rather than general OM biosynthesis [36]. LptD was found to co-purify with LptE (previously known as rare lipoprotein B) [37]. Depletion of either LptD or LptE generated similar phenotypes e.g. abnormal membrane morphology, altered LPS levels (differs across strains), increased OM density on a sucrose gradient and failure of LPS transport to the OM [37]. Over the years, much information has been gathered on the role of LptD and LptE in the Lpt system.

Latest insights concerning the LptDE complex

After synthesis in the cytoplasm, LptD and LptE pass through the sec translocon that translocates them across the IM. Lipoproteins such as LptE are then released from the IM by LolCDE and delivered to LolA, that facilitates transport across the periplasmic space and delivery to LolB for incorporation into the OM [38]. In *E. coli*, LptD is transported across the periplasmic space by the chaperone SurA that delivers it to the Bam complex responsible for LptD incorporation into the OM [37][39]. Not long ago, LptD and LptE were described to be present in a stable two-protein complex in *E. coli* [40]. Both proteins were shown to migrate together by co-purification of LptD with His-tagged LptE in a high molecular weight complex corresponding to the combined molecular weight of LptDE. The interaction displayed a stoichiometric ratio of 1:1 in a denaturating gel, suggesting a very stable interaction. The stability of the LptDE complex was further emphasized by the notion that trypsin treatment

would produce a truncated trypsin-resistant LptDE complex, whereas trypsin treatment of LptE alone resulted in complete degradation of the protein. In the trypsin treated complex, LptE interacts very strongly with the C-terminal region of LptD. In addition, purified LptE, but not LptD, was suggested to bind LPS *in vitro,* indicating that LptE is responsible for LPS binding [40]. However, the authors of the article that contains this suggestion were unable to verify if purified LptD was properly folded in their experiment. Moreover, LptD but not LptE displays the same β-jellyroll fold as LptA and LptC, which have been shown to bind LPS *in vitro*. Furthermore, the C-terminus of LptA has been shown to interact with the N-terminus of correctly folded LptD, thereby possibly forming an interaction that could transfer LPS to LptD [41]. These observations suggest that LptD is involved in the interaction with LPS, but do not exclude a role for LptE in this process. The exact mechanism of LPS incorporation into the OM by the LptDE complex *in vivo* remains to be investigated.

Initial studies concerning the LptDE complex found that overexpression of LptD is only possible with simultaneous expression of functional LptE [40], suggesting a role for LptE in LptD synthesis or assembly. This notion was later confirmed by generating partial loss-of-function mutations in *lptE* using error-prone polymerase chain reaction [42]. These mutations increased OM permeability by directly affecting the interaction between LptE and LptD. Suppressor mutations were linked to *lptD* and *bamA* by genetic mapping and improved LptD assembly, thereby restoring the OM barrier function. Moreover, Tommassen *et al*. reported that LptE may be the chaperone for LptD and is not directly involved in LPS transport in *N. meningitidis* [43]. In contrast to *E. coli*, *N. meningitidis* can survive without LPS [44] and is therefore considered a valuable strain for the investigation of LPS transport. In agreement with this, it was found that none of the components of the Lpt system is required in this strain. As expected, the absence of most Lpt components, with the exception of LptE, resulted in severe LPS transport defects. The levels of LptD in *lptE* mutants were greatly decreased compared to control and all other *lpt(A-C, F-G)* mutants. In addition, a mutation in LptE generated by error-prone PCR was found that affected the interaction between LptE and LptD in *E. coli* [45]. This mutation was shown to impair LptDE complex stability. A total of 11 suppressor mutations were found that can be divided in two classes based on colony morphology. Using genetic mapping, class I suppressor mutations that improve LptD assembly were linked to *lptD* and class II suppressor mutations to *bamA,* suggesting that LptE and LptD interact during LptD assembly at the OM. Together with the observation that LptE is poorly conserved among LPS-producing bacteria [43], these data indicate a chaperone-like role for LptE in LptD biogenesis.

To gain new insights in the exact function of the LptDE complex, the architecture of LptDE was investigated [46]. Using unnatural amino acid mutagenesis and photocrosslinking *in vivo*, LptD and LptE were shown to interact in multiple interfaces. Seven LptE residues were found to interact with LptD on both edges of the protein, suggesting that LptE resides inside the LptD β-barrel (fig. 4A, encircled residues interact with LptD). In this interaction site, residues T86, F90, F123, and R124 are in a conserved β-sheet and residues M142 and R150 are in a conserved C-terminal α helix. LptE mutants with disturbed interaction sites displayed reduced to severely reduced growth. However, none of the mutations affected LptE protein levels or the stability of the LptDE complex. Conversely, mass spectrometric analysis

showed that LptD contains a ten-residue region spanning from amino acid 529-538 that interacts with LptE. Interestingly, they show that this interaction site is present in the extracellular region of LptD, suggesting that LptE interacts with extracellular LptD. In line with this, it was found that disruption of the extracellular interaction site compromises LptDE biogenesis, underlining the importance of this region for LptDE complex formation. These results strongly suggest that the LptDE complex forms a plug-and-barrel conformation in which LptE resides in the barrel of LptD (fig. 4A & 4B, left picture). Insertion of LPS into the OM by LptDE could be facilitated by diffusion through LptD (fig 4B, right picture). Such movement of hydrophobic compounds through a β-barrel wall has been described in several systems [47], indicating that such a mechanism is possible. However, future research is required to determine if this is the actual mechanism of action for LptDE-mediated LPS incorporation into the OM.

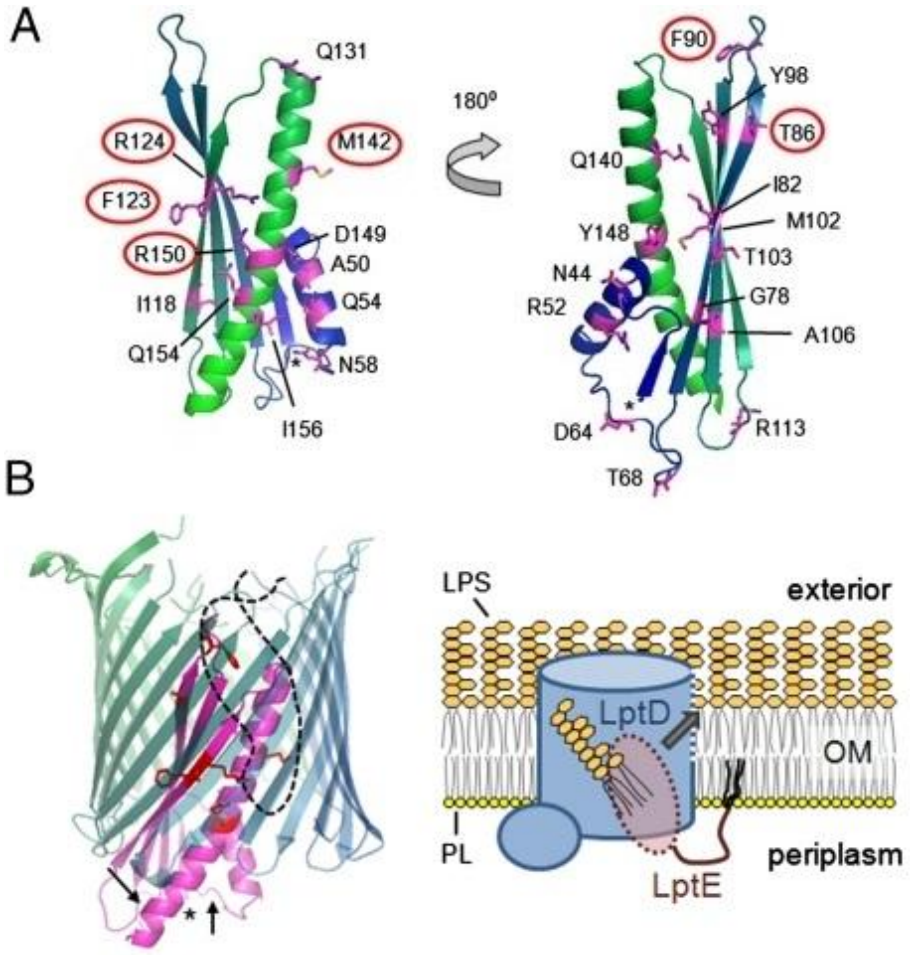
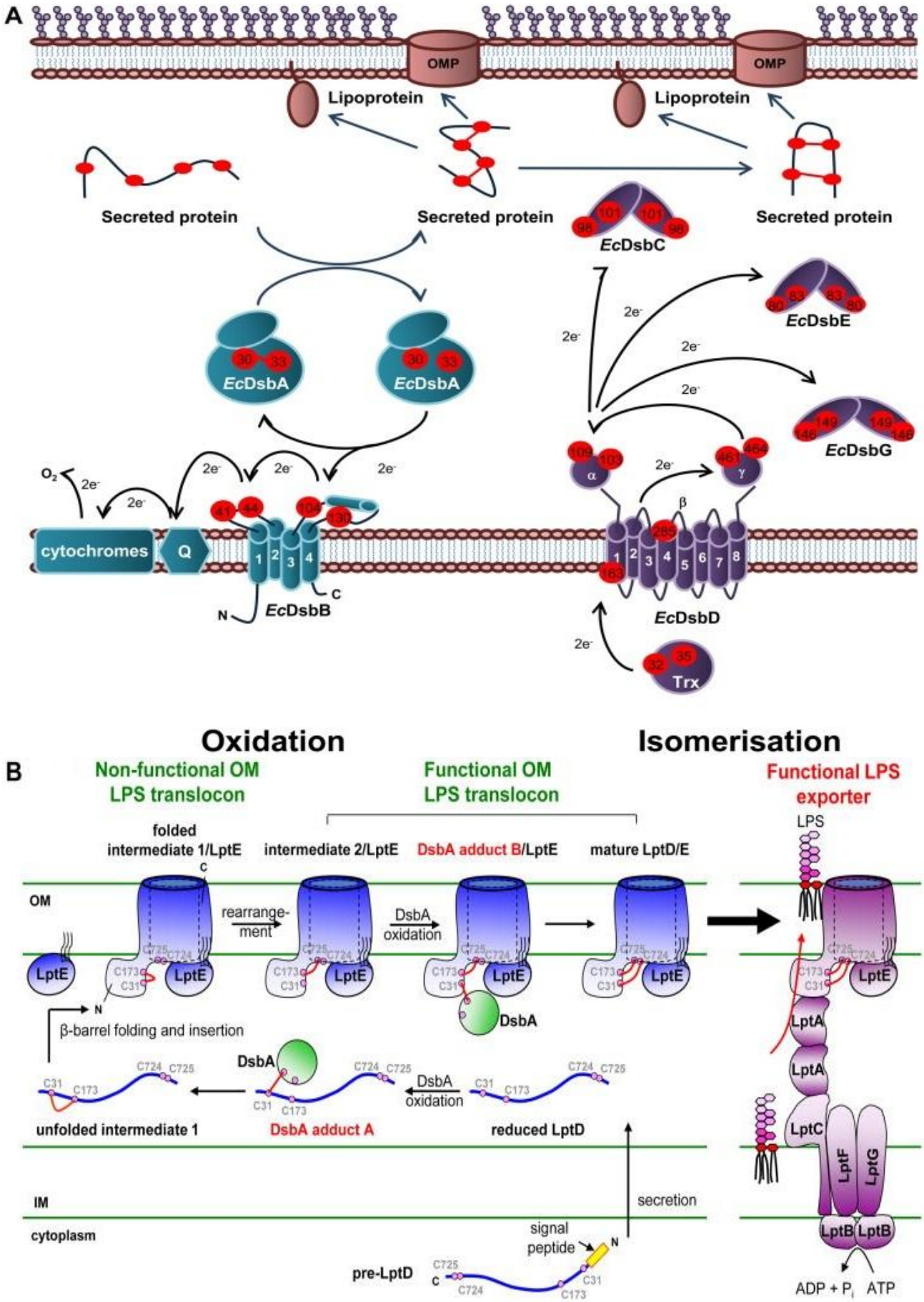


Figure 4. (A) Overview of the identified LptE residues that interact with LptD. Residues involved in LptD/E interactions are encircled in red. The asterisk denotes the N-terminus of the structure, which represents residue I36 of the full-length E. coli LptE. (B) Proposed models for the structure and function of LptDE. The left structure depicts the LptDE interaction in which a model LptE structure is shown in magenta and the sites interacting with LptD are shown as red sticks. The asterisk denotes the N-terminus of the LptE model structure. The LptD β-barrel is shown in blue-green. The arrows denote the approximate location of trypsin cleavage sites. The right picture shows a proposed model for the LptDE mechanism of LPS insertion into the OM. Figure adapted from [46]

In another study, a mutation in *lptE* was found that increases OM permeability of *E. coli* to erythromycin even in presence of wild-type LptE [48]. Grabowicz *et al*. showed that this dominant negative mutation does not affect LptD assembly or LPS transport, indicating that LptE performs a function apart from acting as a chaperone for LptD. Suppressor mutants restored OM permeability as a direct consequence of reduced LptD levels in the OM. Thus, the observed increased permeability caused by a mutation in *lptE* is counteracted by reducing the amount of LptD in the OM. Taken together, these data provide further evidence for the proposed plug-and-barrel conformation of LptDE by showing that functional LptE is required to maintain the OM barrier function, probably by forming a selective plug inside the LptD β-barrel.

As previously noted, LptE is reported to perform a chaperone-like role in LptD biogenesis. However, periplasmic oxidoreductases have also been implicated in LPS biogenesis of *E. coli* by ensuring correct folding of LptD [39]. Disulfide bond (Dsb) proteins are oxidoreductases that catalyze disulfide bond formation, covalent attachments between two cysteine residues, in OMPs and secreted proteins as they migrate through the periplasm to the OM. Dsb proteins facilitate correct disulfide bridge formation by two independent but parallel pathways: the oxidation and the isomerization pathways (reviewed by [49]). Of these two, the oxidation pathway is primarily responsible for correct disulfide bridge formation, whereas the main function of the isomerization pathway is implicated in disulfide bridge rearrangement to their correct location within the protein. The main protein in the oxidation pathway is DsbA, the primary electron acceptor interacting with its substrates to catalyze disulfide bridge formation. DsbA is kept in an oxidized state by DsbB, which in turn is oxidized by passing electrons through quinones of the electron transport pathway in the IM (fig. 5A). Oxidized DsbA contains a disulfide bond between cysteines of the active site that is donated to an unfolded protein substrate as a biomolecular nucleophilic substitution reaction. The main player in the isomerization pathway is DsbC, that is kept in a reduced active form by the IM protein DsbD, which in turn is reduced by thioredoxin and NADPH. It must be noted that the oxidizing and isomerization pathways described here belongs to *E. coli*. Both pathways have also been elucidated in *N. meningitidis*, and function in a similar manner to the *E. coli* pathways with minor differences (reviewed by [49]).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LptB
IM
cytoplasm
secretion
signal peptide
pre-LptD
ADP + Pi
ATP

Figure 5. Folding of LptD (A) Oxidation and isomerisation pathways in periplasmic protein folding in *E. coli.* EcDsbA (Ec in this sense stands for *E. coli*) promotes disulfide formation and is kept in an oxidized state by EcDsbB in the IM, which in turn donates electrons to cytochromes via ubiquinone (Q). Oxidoreductase EcDsbC is responsible for shuffling of incorrect disulfide bonds and is kept in a reduced state by EcDsbB in the IM in addition to EcDsbE and EcDsbG. The oxidase pathway is depicted in turquoise and the isomerisation pathway is depicted in purple. Cysteines are denoted by a red circle with a number representing the position of the cysteine in the protein sequence. The figure is adopted from [49]. (B) A proposed model of the LptD oxidative folding pathway. The experimentally observed intermediate LptD species are depicted as identified after translocation of LptD secretion across the IM. Details of the model are described in the text. The figure is adopted from [50]

LptD contains four cysteine residues: Cys_{31}, Cys_{173}, Cys_{724}, and Cys_{725} that are shown to form intramolecular disulfide bonds in *E. coli* and are essential for its function [51]. LptD mutants in which three of the cysteines have been replaced by serine are not viable. In contrast, LptD mutants in which two cysteines are replaced by serine are viable but only when disulfide bonds between Cys_{173}- Cys_{725} or Cys_{31}- Cys_{724} are allowed to form. This observation indicates that nonconsecutive disulfide bond formation is required for LptD function. In addition, it was shown that *dsbA* mutants display defects in LptD oxidation, but still contain a small fully oxidized LptD population. This indicates that DsbA is important in, but not required for the oxidation of LptD. Furthermore, absence of the isomerase DsbC did not alter the levels or oxidation of LptD, suggesting that DsbC is not involved in LptD biogenesis. Absence of LptE was shown to produce SDS-resistant LptD aggregates that are sensitive to reducing agents. Thus, LptE is required for proper oxidation of LptD. In a follow up study, intermediate LptD products were identified by introducing LptE as the limiting agent in LptD biogenesis [50]. In such a setting, a non-functional LptD intermediate accumulated that migrated comparably to the $LptD_{CCSS}$ mutant in which consecutive disulfide bond formation between Cys_{31} and Cys_{173} was allowed. Subsequent pulse-chase experiments showed that this accumulating LptD species is an intermediate along the oxidative-folding pathway of LptD *in vivo*. In addition, it was suggested that LptD β-barrel formation occurs before disulfide rearrangement of the found intermediate product. Furthermore, the role of DsbA in the oxidation of LptD was investigated using an LptD mutant that forms kinetically stable mixed-disulfide intermediates with substrate proteins. Two stable LptD species were found that contained DsbA and were found to correspond to reduced LptD with DsbA bound to Cys_{31} (annotated as adduct A) and as LptD containing a disulfide bond between Cys_{173}-Cys_{725} in addition to DsbA bound to Cys_{31} (annotated as adduct B, fig. 5B). Together, these data form the basis for a model of the LptD oxidative-folding pathway (fig. 5B). In this model, reduced and unfolded LptD is oxidized by DsbA that introduces disulfide bond formation between Cys_{31} and Cys_{173}. After this, LptD is folded into a β-barrel and incorporated into the OM, assisted by LptE. A disulfide rearrangement followed by another round of DsbA-mediated oxidation ensures the formation of mature, properly oxidized LptDE (fig. 5B).

Recently, the periplasmic chaperone Skp that has long been implicated in the assembly of OMPs, was reported to be involved in the assembly of LptD [52]. Schwalm *et al*. found that the simultaneous loss of Skp and FkpA, a protein that has been shown to act as a chaperone for nonnative or mutant proteins, caused reduced levels of functional LptD. They showed

that the reduced LptD levels could not be attributed to defects in DsbA mediated disulfide bridge formation or reduced amounts of other chaperones. Furthermore, overexpression of SurA, the main periplasmic chaperone involved in OMP assembly, in a *skp fkpA* double mutant background or vice versa did not restore LptD levels, showing that the effect is not caused by overloading SurA with substrate. These results demonstrate that Skp and FkpA are involved in the assembly of LptD. In another recent paper, a similar function to Skp was found for the protein BepA, a putative periplasmic metalloprotease regulated by the σ^E stress response [53]. It was previously shown that BepA disruption increases sensitivity for multiple drugs, indicating a role for BepA in OM integrity. BepA was found to alter the biogenesis of LptD by promoting intramolecular disulfide bond rearrangement. A fast migrating intermediate product of LptD containing nonnative disulfide bonds accumulated in absence of BepA. This effect was suppressed upon overexpression of a wild-type, but not of a proteolytically inactive BepA. These data suggest that BepA is required for correct disulfide bond rearrangement in addition to degradation of incorrectly folded LptD.

Conclusion and perspectives

Recent discoveries have led to new insights concerning the Lpt transport system of LPS in Gram-negative bacteria. Although the mechanism of action of all individual Lpt system components remains to be fully understood, a model for LPS transport from the IM to the OM can be derived based on current data. The route of LPS transport begins with flipping across the IM by MsbA after synthesis in the cytoplasm. At the periplasmic side of the IM, LPS most likely localizes to LptC that has been suggested to bind LPS (fig.3). The energy required for LPS release from the IM may be provided by ATP hydrolysis of LptB that induces conformational changes in this protein [26]. These conformational changes may then be passed on to LptF and/or LptG and finally to LptC that releases LPS from the IM. The periplasm-spanning LptA is a likely candidate to accept LPS from LptC and facilitate transport across the periplasm, as LptA has been shown to interact with LptC in the IM and with LptD in the OM [41], forming a bridge between the IM and OM components of the Lpt system. Moreover, LptA is able to displace LPS from LptC, but not vice versa [31], strongly suggesting that LptA is the LPS transport component downstream of LptC. Further movement of LPS across the periplasm may also be mediated by LptA, but remains to be investigated. After crossing the periplasm LPS encounters the stable LptDE complex present in the OM. As the C-terminus of LptA has been shown to interact with the N-terminus of LptD [41], it seems likely that LPS first encounters the N-terminus of LptD before moving through the LptD β-barrel and incorporation into the OM. In *E. coli*, LptE has been shown to reside inside the LptD β-barrel where it possibly functions as a plug that is important for maintaining OM impermeability to harmful compounds [46][48]. It seems plausible that LptE has to be removed from the LptD β-barrel in order for LPS to pass through the barrel to the OM. As LptE has been shown to specifically bind LPS *in vitro* [40] and forms an interaction with a predicted extracellular loop of LptD [46], it may be possible that LptE assists in LPS movement through the LptD β-barrel when LptE falls back into the barrel to plug LptD (fig. 4B). Direct insertion of LPS into the predicted extracellular loop of LptD would imply that LPS moves laterally through LptD into the outer leaflet of the OM. Such movement of hydrophobic structures through the lumen of a β-barrel to the OM bilayer has been

described in several systems [reviewed in 47]. In line with this, LPS is a substrate of PagP and is thought to access the active site of PagP via a lateral opening in its β-barrel [54]. The openings in the β-barrels through which hydrophobic substrates are described to migrate are delimited by proline residues that interrupt hydrogen bond formation between adjacent β-strands. Interestingly, two pairs of conserved proline residues have been found by an alignment of LptD between 20 different species in adjacent β-strands [46], suggesting that lateral movement of LPS through the LptD β-barrel into the outer leaflet of the OM may occur. Taken together, these data provide indications for a model for LPS transport from the IM to the OM. Notably, this review has described recent developments regarding LptDE complex assembly. Although incomplete, this has led to a model for LptD folding (fig 5B) in which the exact function of LptE, Skp, FkpA and BepA remain to be clarified. Further research is required before the complete mechanism-of-action of the Lpt system and folding pathways of LptDE can be determined.

The search for novel antibiotics becomes increasingly important as bacterial strains become more resistant to antimicrobial compounds. Recently, LptD has been shown to be a target of novel peptidomimetic antibiotics in *Pseudomonas aeruginosa*. Peptidomimetic antibiotics based on the antimicrobial peptide protegrin I were synthesized and optimized against Gram-negative *Pseudomonas* spp [55]. One potent peptidomimetic antibiotic was shown to target LptD and displayed potent antimicrobial activity in a mouse septicemia infection model. This family of novel peptidomimetic antibiotics was later shown to produce an enhanced membrane composition resembling alterations caused by depletion of LptD, that disrupt OM integrity [56]. Using LPS modifications as markers that can be used to distinguish between the IM and the OM, it was found that antibiotic treated wild-type cells displayed the same LPS transport defects as *lptD* mutants. A follow-up study reported that β-hairpin structures were essential for antimicrobial activity of peptidomimetic antibiotics that target LptD, possibly by inhibiting transport of LPS through the LptD β-barrel [57]. Together, these studies show that β-hairpin peptidomimetic antibiotics function by targeting LptD and provide a basis for the development for potent novel antimicrobial compounds that target the LPS transport system in Gram-negative bacteria. In addition, the thermodynamic and kinetic parameters of the interaction between LptC and a fluorescent lipo-oligosaccharide (fLOS) *in vitro* were recently reported [58]. fLOS binding to LptC was shown to be mostly irreversible, suggesting that the compound may lead to the development of novel antibiotics targeting LPS transport.

The development of novel antibiotics are an excellent example of the beneficial consequences of new insights in the LPS transport system, thereby underlining the importance of research on this topic. Therefore, future studies are required to unravel the exact mechanism of LPS transport from the IM to the outer leaflet of the OM that may lead to novel treatment moieties that target the Lpt system.

References

[1] J. Luirink, Z. Yu, S. Wagner and J.-W. de Gier, “Biogenesis of inner membrane proteins in *Escherichia coli*.,” *Biochim. Biophys. Acta*, vol. 1817, no. 6, pp. 965–76, Jun. 2012.

[2] R. Kudva, K. Denks, P. Kuhn, A. Vogt, M. Müller and H.-G. Koch, “Protein translocation across the inner membrane of Gram-negative bacteria: the Sec and Tat dependent protein transport pathways.,” *Res. Microbiol.*, vol. 164, no. 6, pp. 505–34, Apr. 2013.

[3] A. J. M. Driessen and N. Nouwen, “Protein translocation across the bacterial cytoplasmic membrane.,” *Annu. Rev. Biochem.*, vol. 77, pp. 643–67, Jan. 2008.

[4] M. Merdanovic, T. Clausen, M. Kaiser, R. Huber and M. Ehrmann, “Protein quality control in the bacterial periplasm.,” *Annu. Rev. Microbiol.*, vol. 65, pp. 149–68, Jan. 2011.

[5] H. Nikaido, “Molecular Basis of Bacterial Outer Membrane Permeability Revisited,” vol. 67, no. 4, pp. 593–656, Dec. 2003.

[6] S. K. Aoki, J. C. Malinverni, K. Jacoby, B. Thomas, R. Pamma, B. N. Trinh, S. Remers, J. Webb, B. a Braaten, T. J. Silhavy and D. a Low, “Contact-dependent growth inhibition requires the essential outer membrane protein BamA (YaeT) as the receptor and the inner membrane transport protein AcrB.,” *Mol. Microbiol.*, vol. 70, no. 2, pp. 323–40, Oct. 2008.

[7] C. R. H. Raetz and C. Whitfield, “Lipopolysaccharide endotoxins.,” *Annu. Rev. Biochem.*, vol. 71, pp. 635–700, Jan. 2002.

[8] C. R. H. Raetz, C. M. Reynolds, M. S. Trent and R. E. Bishop, “Lipid A modification systems in gram-negative bacteria.,” *Annu. Rev. Biochem.*, vol. 76, pp. 295–329, Jan. 2007.

[9] T. Ogura, K. Inoue, T. Tatsuta, T. Suzaki, K. Karata, K. Young, L. H. Su, C. a Fierke, J. E. Jackman, C. R. Raetz, J. Coleman, T. Tomoyasu and H. Matsuzawa, “Balanced biosynthesis of major membrane components through regulated degradation of the committed enzyme of lipid A biosynthesis by the AAA protease FtsH (HflB) in *Escherichia coli*.,” *Mol. Microbiol.*, vol. 31, no. 3, pp. 833–44, Feb. 1999.

[10] C. Katz and E. Z. Ron, “Dual role of FtsH in regulating lipopolysaccharide biosynthesis in *Escherichia coli*.,” *J. Bacteriol.*, vol. 190, no. 21, pp. 7117–22, Nov. 2008.

[11] S. Mahalakshmi, M. R. Sunayana, L. SaiSree and M. Reddy, “yciM is an essential gene required for regulation of lipopolysaccharide synthesis in *Escherichia coli*.,” *Mol. Microbiol.*, vol. 91, no. 1, pp. 145–57, Jan. 2014.

[12] F. Putker, A. Grutsch, J. Tommassen and M. P. Bos, “Ght protein of *Neisseria meningitidis* is involved in the regulation of lipopolysaccharide biosynthesis.,” *J. Bacteriol.*, vol. 196, no. 4, pp. 780–9, Feb. 2014.

[13] M. S. Trent, C. M. Stead, A. X. Tran and J. V Hankins, “Diversity of endotoxin and its impact on pathogenesis.,” *J. Endotoxin Res.*, vol. 12, no. 4, pp. 205–23, Jan. 2006.

[14] P. Sperandeo, G. Dehò and A. Polissi, “The lipopolysaccharide transport system of Gram-negative bacteria.,” *Biochim. Biophys. Acta*, vol. 1791, no. 7, pp. 594–602, Jul. 2009.

[15] M. Karow and C. Georgopoulos, “The essential Escherichia coii msbA gene , a multicopy suppressor of null mutations in the htrB gene , is related to the universally conserved family of ATP-dependent translocators,” vol. 7, pp. 69–79, Sep. 1993.

[16] M. Karow, O. Fayet and C. Georgopoulos, “The lethal phenotype caused by null mutations in the *Escherichia coli* htrB gene is suppressed by mutations in the accBC operon, encoding two subunits of acetyl coenzyme A carboxylase.,” *J. Bacteriol.*, vol. 174, no. 22, pp. 7407–18, Nov. 1992.

[17] W. T. Doerrler, H. S. Gibbons and C. R. H. Raetz, “MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*.,” *J. Biol. Chem.*, vol. 279, no. 43, pp. 45102–9, Oct. 2004.

[18] P. D. W. Eckford and F. J. Sharom, “The reconstituted *Escherichia coli* MsbA protein displays lipid flippase activity.,” *Biochem. J.*, vol. 429, no. 1, pp. 195–203, Jul. 2010.

[19] C. Santambrogio, P. Sperandeo, R. Villa, F. Sobott, A. Polissi and R. Grandori, “LptA assembles into rod-like oligomers involving disorder-to-order transitions.,” *J. Am. Soc. Mass Spectrom.*, vol. 24, no. 10, pp. 1593–602, Oct. 2013.

[20] S. Narita and H. Tokuda, “Biochemical characterization of an ABC transporter LptBFGC complex required for the outer membrane sorting of lipopolysaccharides.,” *FEBS Lett.*, vol. 583, no. 13, pp. 2160–4, Jul. 2009.

[21] F. Stenberg, P. Chovanec, S. L. Maslen, C. V Robinson, L. L. Ilag, G. von Heijne and D. O. Daley, “Protein complexes of the *Escherichia coli* cell envelope.,” *J. Biol. Chem.*, vol. 280, no. 41, pp. 34409–19, Oct. 2005.

[22] S. Serina, F. Nozza, G. Nicastro, F. Faggioni, H. Mottl, G. Dehò and A. Polissi, “Scanning the *Escherichia coli* chromosome by random transposon mutagenesis and multiple phenotypic screening.,” *Res. Microbiol.*, vol. 155, no. 8, pp. 692–701, Oct. 2004.

[23] P. Sperandeo, C. Pozzi, G. Dehò and A. Polissi, “Non-essential KDO biosynthesis and new essential cell envelope biogenesis genes in the *Escherichia coli* yrbG-yhbG locus.,” *Res. Microbiol.*, vol. 157, no. 6, pp. 547–58, Feb. 2006.

[24] P. Sperandeo, F. K. Lau, A. Carpentieri, C. De Castro, A. Molinaro, G. Dehò, T. J. Silhavy and A. Polissi, “Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*.,” *J. Bacteriol.*, vol. 190, no. 13, pp. 4460–9, Jul. 2008.

[25] C. R. H. Raetz and C. Whitfield, “Lipopolysaccharide endotoxins.,” *Annu. Rev. Biochem.*, vol. 71, pp. 635–700, Jan. 2002.

[26] D. J. Sherman, M. B. Lazarus, L. Murphy, C. Liu, S. Walker, N. Ruiz and D. Kahne, “Decoupling catalytic activity from biological function of the ATPase that powers lipopolysaccharide transport.,” *Proc. Natl. Acad. Sci. U. S. A.*, Mar. 2014.

[27] N. Ruiz, L. S. Gronenberg, D. Kahne and T. J. Silhavy, “Identification of two inner-membrane proteins required for the transport of lipopolysaccharide to the outer membrane of *Escherichia coli*.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 105, no. 14, pp. 5537–42, Apr. 2008.

[28] R. Villa, A. M. Martorana, S. Okuda, L. J. Gourlay, M. Nardini, P. Sperandeo, G. Dehò, M. Bolognesi, D. Kahne and A. Polissi, “The *Escherichia coli* Lpt transenvelope protein complex for lipopolysaccharide export is assembled via conserved structurally homologous domains.,” *J. Bacteriol.*, vol. 195, no. 5, pp. 1100–8, Mar. 2013.

[29] M. D. L. Suits, P. Sperandeo, G. Dehò, A. Polissi and Z. Jia, “Novel structure of the conserved gram-negative lipopolysaccharide transport protein A and mutagenesis analysis.,” *J. Mol. Biol.*, vol. 380, no. 3, pp. 476–88, Jul. 2008.

[30] K. M. Schultz, J. B. Feix and C. S. Klug, “Disruption of LptA oligomerization and affinity of the LptA-LptC interaction.,” *Protein Sci.*, vol. 22, no. 11, pp. 1639–45, Nov. 2013.

[31] A. X. Tran, C. Dong and C. Whitfield, “Structure and functional analysis of LptC, a conserved membrane protein involved in the lipopolysaccharide export pathway in *Escherichia coli*.,” *J. Biol. Chem.*, vol. 285, no. 43, pp. 33529–39, Oct. 2010.

[32] S. Okuda, E. Freinkman and D. Kahne, “Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*.,” *Science*, vol. 338, no. 6111, pp. 1214–7, Nov. 2012.

[33] B. A. Sampson, R. Misra and S. A. Benson, “Identification and Characterization of a New Gene of *Escherichia coli* K-12 Involved in Outer Membrane Permeability.,” no. 1972, Mar. 1989.

[34] R. Aono, T. Negishi, K. Aibe, A. Inoue and K. Horikoshi, “Mapping of organic solvent tolerance gene *ostA* in *Escherichia coli* K-12., ” no. 58, vol. 7, Oct. 1993.

[35] M. Braun and T. J. Silhavy, “Imp/OstA is required for cell envelope biogenesis in *Escherichia coli*.,” *Mol. Microbiol.*, vol. 45, no. 5, pp. 1289–302, Sep. 2002.

[36] M. P. Bos, B. Tefsen, J. Geurtsen and J. Tommassen, “Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 101, no. 25, pp. 9417–22, Jun. 2004.

[37] T. Wu, A. C. Mccandlish, L. S. Gronenberg, S. Chng, T. J. Silhavy and D. Kahne, “Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*,” Jun. 2006.

[38] H. Tokuda, “Biogenesis of Outer Membranes in Gram-Negative Bacteria,” *Biosci. Biotechnol. Biochem.*, vol. 73, no. 3, pp. 465–473, Mar. 2009.

[39] K. Denoncin, D. Vertommen, E. Paek and J.-F. Collet, “The protein-disulfide isomerase DsbC cooperates with SurA and DsbA in the assembly of the essential β-barrel protein LptD.,” *J. Biol. Chem.*, vol. 285, no. 38, pp. 29425–33, Sep. 2010.

[40] S.-S. Chng, N. Ruiz, G. Chimalakonda, T. J. Silhavy and D. Kahne, “Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 12, pp. 5363–8, Mar. 2010.

[41] E. Freinkman, S. Okuda, N. Ruiz and D. Kahne, “Regulated assembly of the transenvelope protein complex required for lipopolysaccharide export.,” *Biochemistry*, vol. 51, no. 24, pp. 4800–6, Jun. 2012.

[42] R. Haarmann, M. Ibrahim, M. Stevanovic, R. Bredemeier and E. Schleiff, “The properties of the outer membrane localized Lipid A transporter LptD.,” *J. Phys. Condens. Matter*, vol. 22, no. 45, p. 454124, Nov. 2010.

[43] M. P. Bos and J. Tommassen, “The LptD chaperone LptE is not directly involved in lipopolysaccharide transport in *Neisseria meningitidis*.,” *J. Biol. Chem.*, vol. 286, no. 33, pp. 28688–96, Aug. 2011.

[44] L. Steeghs, R. den Hartog, a den Boer, B. Zomer, P. Roholl and P. van der Ley, “*Meningitis* bacterium is viable without endotoxin.,” *Nature*, vol. 392, no. 6675, pp. 449–50, Apr. 1998.

[45] G. Chimalakonda, N. Ruiz, S.-S. Chng, R. a Garner, D. Kahne and T. J. Silhavy, “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. U. S. A.*, vol. 108, no. 6, pp. 2492–7, Feb. 2011.

[46] E. Freinkman, S. Chng and D. Kahne, “The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel,” Dec. 2010.

[47] B. van den Berg, “Going forward laterally: transmembrane passage of hydrophobic molecules through protein channel walls.,” *Chembiochem*, vol. 11, no. 10, pp. 1339–43, Jul. 2010.

[48] M. Grabowicz, J. Yeh and T. J. Silhavy, “Dominant negative lptE mutation that supports a role for LptE as a plug in the LptD barrel.,” *J. Bacteriol.*, vol. 195, no. 6, pp. 1327–34, Mar. 2013.

[49] S. Piek and C. M. Kahler, “A comparison of the endotoxin biosynthesis and protein oxidation pathways in the biogenesis of the outer membrane of *Escherichia coli* and *Neisseria meningitidis*.,” *Front. Cell. Infect. Microbiol.*, vol. 2, no. December, p. 162, Jan. 2012.

[50] S.-S. Chng, M. Xue, R. a Garner, H. Kadokura, D. Boyd, J. Beckwith and D. Kahne, “Disulfide rearrangement triggered by translocon assembly controls lipopolysaccharide export.,” *Science*, vol. 337, no. 6102, pp. 1665–8, Sep. 2012.

[51] N. Ruiz, S.-S. Chng, A. Hiniker, D. Kahne and T. J. Silhavy, “Nonconsecutive disulfide bond formation in an essential integral outer membrane protein.,” *Proc. Natl. Acad. Sci. U. S. A.*, vol. 107, no. 27, pp. 12245–50, Jul. 2010.

[52] J. Schwalm, T. F. Mahoney, G. R. Soltes and T. J. Silhavy, “Role for Skp in LptD assembly in *Escherichia coli*.,” *J. Bacteriol.*, vol. 195, no. 16, pp. 3734–42, Aug. 2013.

[53] S. Narita, C. Masui, T. Suzuki, N. Dohmae and Y. Akiyama, “Protease homolog BepA (YfgC) promotes assembly and degradation of β -barrel membrane proteins in *Escherichia coli*.,” Aug. 2013.

[54] R. E. Bishop, “The lipid A palmitoyltransferase PagP: molecular mechanisms and role in bacterial pathogenesis.,” *Mol. Microbiol.*, vol. 57, no. 4, pp. 900–12, Aug. 2005.

[55] N. Srinivas, P. Jetter, B. J. Ueberbacher, M. Werneburg, K. Zerbe, J. Steinmann, B. Van der Meijden, F. Bernardini, a. Lederer, R. L. a. Dias, P. E. Misson, H. Henze, J. Zumbrunn, F. O. Gombert, D. Obrecht, P. Hunziker, S. Schauer, U. Ziegler, a. Kach, L. Eberl, K. Riedel, S. J. DeMarco and J. a. Robinson, "Peptidomimetic Antibiotics Target Outer-Membrane Biogenesis in Pseudomonas aeruginosa.," *Science (80-.).*, vol. 327, no. 5968, pp. 1010–1013, Feb. 2010.

[56] M. Werneburg, K. Zerbe, M. Juhas, L. Bigler, U. Stalder, A. Kaech, U. Ziegler, D. Obrecht, L. Eberl and J. a Robinson, "Inhibition of lipopolysaccharide transport to the outer membrane in Pseudomonas aeruginosa by peptidomimetic antibiotics.," *Chembiochem*, vol. 13, no. 12, pp. 1767–75, Aug. 2012.

[57] J. Schmidt, K. Patora-Komisarska, K. Moehle, D. Obrecht and J. a Robinson, "Structural studies of β-hairpin peptidomimetic antibiotics that target LptD in Pseudomonas sp.," *Bioorg. Med. Chem.*, vol. 21, no. 18, pp. 5806–10, Sep. 2013.

[58] S. E. Sestito, P. Sperandeo, C. Santambrogio, C. Ciaramelli, V. Calabrese, G. E. Rovati, L. Zambelloni, R. Grandori, A. Polissi and F. Peri, "Functional Characterization of *E. coli* LptC: Interaction with LPS and a Synthetic Ligand.," *ChemBioChem*, vol. 15, no. 5, pp. 734–742, Mar. 2014.