

## TIMELINE

# Transport of lipopolysaccharide across the cell envelope: the long road of discovery

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**Abstract** | Intracellular lipid transport is poorly understood. Genetic studies to identify lipid-transport factors are complicated by the essentiality of many lipids, whereas biochemical and cell biology approaches aiming to determine localization and mechanisms of lipid transport are often challenged by the lack of adequate technology. Here, we review the epic history of how different approaches, technological advances and ingenuity contributed to the recent discovery of a multi-protein pathway that transports lipopolysaccharide across the envelope of Gram-negative bacteria.

One hundred and twenty-five years ago, Christian Gram developed a staining method that allows the differentiation of two major classes of bacteria, the Gram positives and the Gram negatives<sup>1</sup>. This classification is widely used by microbiologists and the procedure still remains an important primary diagnostic tool in the clinic. Although it was assumed that the Gram stain reflected differences in the cellular envelope of these bacterial types, it took almost 100 years to understand the basis for the Gram reaction<sup>2,3</sup>. This delay occurred mostly because discovering the composition and structure of the prototypical Gram-positive and Gram-negative cell envelopes depended on the development of new technologies.

Although most progress in determining the composition, structure and function of the different constituents of the bacterial envelope occurred during the 1940s–1970s, many researchers have been fascinated since the 1920s by a glycolipid that is present at the outer membrane (OM) of Gram-negative bacteria. This glycolipid, known as lipopolysaccharide (LPS) or endotoxin, is a potent inducer of the immune system and the causative agent of septic shock<sup>4</sup>. LPS is asymmetrically located at the outer leaflet of the OM<sup>5,6</sup>, and typically the molecule

has three distinct regions: lipid A, a core oligosaccharide and a long polysaccharide called the O-antigen<sup>7</sup> (FIG. 1). Its location at the cell surface and its physicochemical properties make LPS primarily responsible for the barrier quality of the OM, which is crucial for the survival of many Gram-negative bacteria in many environments and the reason why it has been so hard to develop antibiotics against these organisms<sup>8</sup>. What now might seem simple LPS biogenesis facts, such as its chemical composition, cellular localization and site of synthesis, actually took decades to elucidate. As we describe in this Timeline article, answers to these questions required years of study and the application of different approaches (TIMELINE). This great body of work has recently led to the identification of the proteins that are required to transport LPS from its site of synthesis, the inner membrane (IM), to the cell surface. The discovery of this transport pathway poses new questions and raises new technological challenges that will guide future research in the field of bacterial envelope biogenesis.

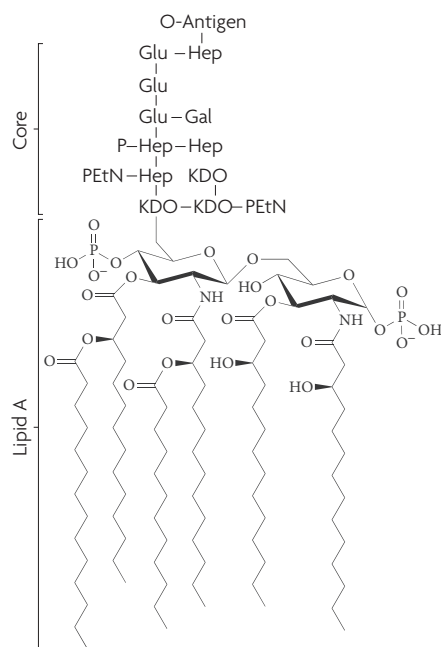
### Uncovering envelope structure

The realization in the 1920s that Gram-negative bacteria produce an endotoxin drove much of the biochemical work

done on the bacterial envelope during the 1920s–1950s. This early work was both facilitated and motivated by the potent immunogenic and toxic properties of LPS. Crude estimates of the chemical composition of the bacterial envelope were emerging but our understanding of its ultrastructure and the location of its constituents could not advance until the development of electron microscopy (EM) and ultracentrifugation. Likewise, although the general composition of LPS was known early on, LPS structure (FIG. 1) was not resolved until nuclear magnetic resonance was used in 1983 to elucidate the structure of lipid A<sup>9–12</sup>.

An excellent review by Glauert and Thornley<sup>13</sup> summarizes early work that probed the physical structure of the bacterial envelope by examining thin sections by EM. Using a variety of staining methods, the cell envelope of Gram-positive bacteria was shown to contain a cytoplasmic membrane surrounded by a thick but uniformly dense layer, which we now know is the cell wall polymer peptidoglycan. By contrast, in Gram-negative bacteria the cytoplasmic membrane was surrounded with what seemed to be a triple-layered structure<sup>14</sup> that in most of these early papers was referred to as the cell wall, a term that can be very confusing to modern readers.

The difficulties encountered in resolving these outer layers probably reflect the fact that the OM is covalently attached to the peptidoglycan. However, as EM techniques improved, a clearer view of the Gram-negative cell envelope emerged. One of the most revealing EM studies was done by Bladen and Mergenhagen<sup>15</sup> with the dental pathogen *Veillonella*. Their micrographs (FIG. 2) showed that the cell envelope contains three distinct layers: the plasma membrane, which confines the cytoplasm, a structure the authors termed a solid membrane and an outermost unit membrane, which they named the outer membrane. The solid membrane was degraded by lysozyme and thus refers to the peptidoglycan layer. They also demonstrated that treatment with phenol-water extracted the OM and



**Figure 1 | Structure of *Escherichia coli* LPS.** The structure of *E. coli* LPS (lipopolysaccharide) with a K-12 core region is shown. The structure of LPS among different species is very diverse<sup>67</sup> and bacteria can further modify the typical LPS structure in response to environmental signals and defects in envelope conditions<sup>37,59,61,68</sup>. EtN, ethanolamine; Gal, D-galactose; Glu, D-glucose; Hep, L-glycero-D-manno-heptose; KDO, 3-deoxy-D-manno-oct-2-ulonic acid; P phosphate.

that this preparation contained LPS. In hindsight, this is a remarkable study not only for the clarity of the EM images but also because it is the first to use the term outer membrane, although it should be pointed out that Murray<sup>16</sup> reported similar

observations for *Escherichia coli* and referred to the outermost layer as the outer unit membrane. Nevertheless, as pointed out by Hiroshi Nikaido<sup>17</sup>, the term outer membrane did not really come into common use until Mary Jane Osborn and colleagues proved the membrane-like nature of this layer by analysing its components<sup>18</sup>.

The lack of electron density between the IM and OM observed in EM micrographs suggested the existence of an aqueous, extracytoplasmic compartment. Evidence that this compartment contains a unique set of proteins came from Leon Heppel and colleagues, who developed methods such as cold osmotic shock and spheroplasting, which release proteins from this compartment without contamination by cytoplasmic proteins<sup>19</sup>. Their work revealed that this extracytoplasmic compartment sequesters hydrolytic enzymes such as ribonuclease and alkaline phosphatase, and contains soluble substrate-binding proteins that are required for the active transport of certain small molecules. The concentration of macromolecules in this compartment is high, and it is, in fact, more viscous than the cytoplasm<sup>20</sup>. This compartment is now called the periplasm, a term apparently coined by Peter Mitchell in 1961, although it did not come into common use until much later. In fact, in the aforementioned 1967 review<sup>19</sup>, Heppel does not use this term. Together, these pioneering works paved the way to many other studies that have provided us with the current detailed picture of the structure and composition of the Gram-negative cell envelope (FIG. 3).

**LPS localization**

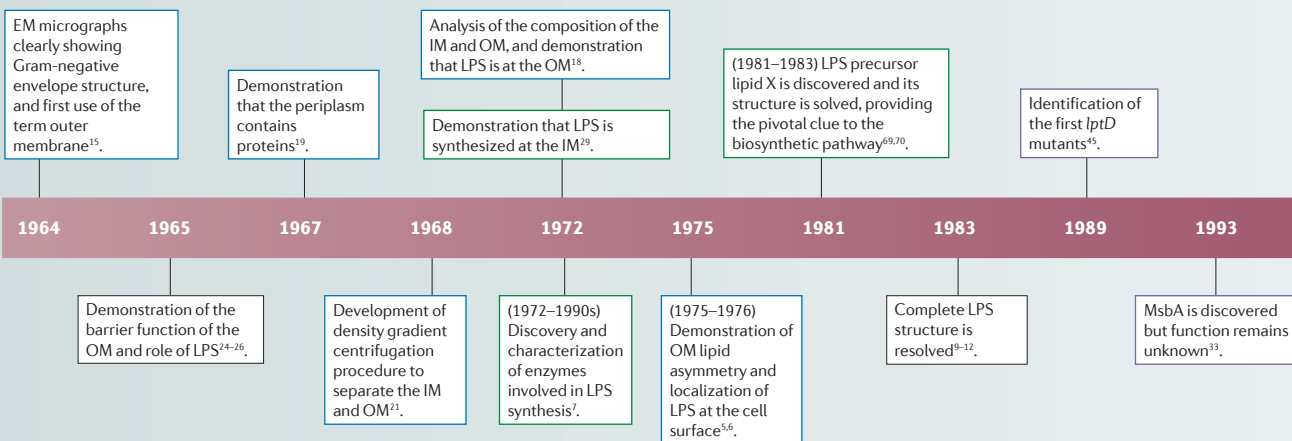
Armed with the knowledge derived from EM of the physical structure of the Gram-negative cell envelope, Miura and Mizushima developed a method to separate the OM from the IM using sucrose density-gradient centrifugation<sup>21</sup>. This technological advance allowed Osborn and collaborators to determine in 1972 the composition of the OM and to conclude that LPS is indeed found in this organelle<sup>18</sup>. Although this method was also crucial for studies (described later) that probed the localization of LPS synthesis and the transport of LPS across the cell envelope, it did not resolve how OM components are arranged.

Indeed, demonstration that LPS is localized at the outer leaflet of the OM was accomplished in 1975 by Muhlradt and Golecki<sup>5</sup> using immuno-EM. A year later, the study by Kamio and Nikaido showing that the outer leaflet of the OM of *Salmonella enterica* serovar Typhimurium is devoid of its most abundant phospholipid, phosphatidylethanolamine<sup>6</sup>, further confirmed the lipid asymmetry of the OM. These findings, together with the research described in the following section, were the foundation for our understanding of the barrier quality of the OM.

**Function of LPS**

Like most lipid bilayers, the OM is impermeant to large hydrophilic molecules, but it also has the unusual property of preventing the rapid diffusion of small hydrophobic molecules<sup>22</sup>. This is why Gram-negative bacteria are generally more resistant to

**Timeline | Landmarks in lipopolysaccharide biogenesis**



Envelope composition and structure: blue boxes, LPS synthesis: green boxes, LPS transport: purple boxes. EM, electron microscopy; IM, inner membrane; LPS, lipopolysaccharide; Lpt, lipopolysaccharide transport; OM, outer membrane.

hydrophobic antibiotics and detergents than their Gram-positive counterparts. Of course, the OM still allows the exchange of solutes with its environment. Small hydrophilic molecules of up to approximately 600 Da diffuse through nonspecific OM protein channels called porins, and certain large molecules such as vitamins and iron chelates can pass through specialized channels.

Probing the barrier function of the OM became possible when in 1958 MacGregor and Elliker realized that ethylenediaminetetraacetic acid (EDTA) increases the sensitivity of *Pseudomonas aeruginosa* to quaternary amines<sup>23</sup>. In the 1960s–1970s, Loretta Leive demonstrated that this was because EDTA promotes the entry of several compounds into Gram-negative cells<sup>24,25</sup>. However, understanding how this compound increases permeability was not possible until biochemical and genetic studies proved, respectively, that EDTA releases LPS from the cell surface<sup>26</sup> and that certain mutants producing truncated forms of LPS (known as rough mutants) are more permeable than their wild-type counterparts<sup>27,28</sup>.

Most recent physical studies on the barrier quality of the OM have been conducted in enteric bacteria and are discussed in detail by Nikaido<sup>22</sup>. They have revealed that the main feature responsible for the impermeability of the OM is the low fluidity conferred by the high degree of packing of LPS molecules into the outer leaflet. This is accomplished by the following properties of LPS. LPS has a higher number of fatty-acid chains per molecule

than phospholipids, which results in a higher number of hydrophobic interactions between neighbouring molecules. In the Enterobacteriaceae, lateral interactions between LPS molecules are also facilitated by hydrogen bonds and by cations ( $Mg^{2+}$  and  $Ca^{2+}$ ) that neutralize the negative charges of the phosphate groups present in lipid A. Furthermore, in the LPS of enteric bacteria, the fatty-acid chains in lipid A are saturated, which allows tighter packing. Consequently, altering either the structure or the cellular location of LPS compromises the barrier function of the OM. We now understand that the aforementioned EDTA treatments chelate the cations that are intercalated between LPS molecules, and this results in the repulsion and release of LPS molecules into the medium. Somehow, LPS release causes the translocation of phospholipids to the outer leaflet, which increases the permeability of the OM as it now contains phospholipid bilayers.

#### Where is LPS synthesized?

Separation of the IM and OM using sucrose density gradient ultracentrifugation allowed Osborn and collaborators to prove not only that LPS is located at the OM, but that LPS is synthesized in the IM and that translocation of LPS to the OM is unidirectional<sup>18,29</sup>. Those studies were seminal in the field of LPS biogenesis as they began sketching the pathway of LPS transport across the cell envelope.

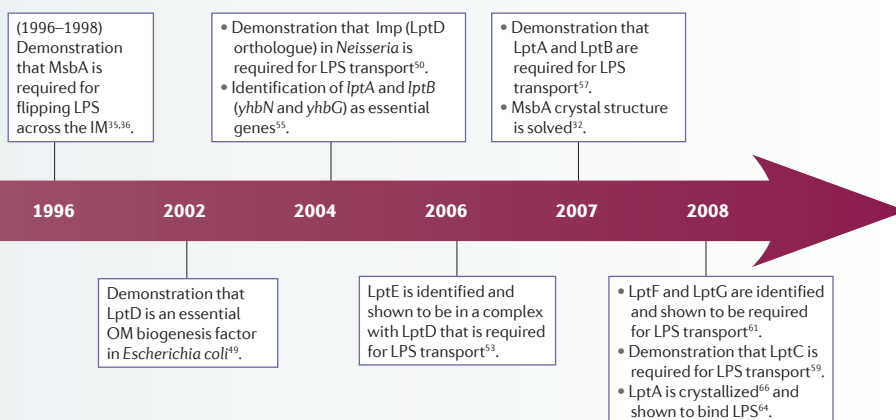
During the next decades, the biosynthetic pathway of LPS (often referred to as the Raetz pathway), its participating

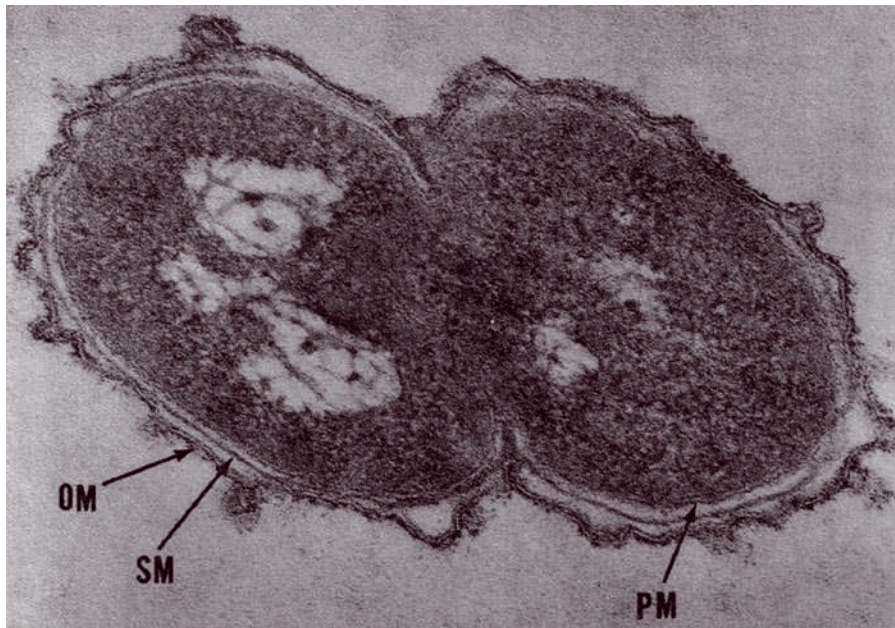
enzymes and their location were mainly elucidated by Christian Raetz and collaborators (reviewed in REF. 7). From those studies we know that all components of LPS (lipid A, core and O-antigen) are synthesized at the inner leaflet of the IM where lipid A and core are ligated to form what is known as rough LPS. Rough LPS and the O-antigen are independently flipped across the IM and ligated at the outer leaflet of the IM. Notably, *E. coli* K-12 strains only synthesize rough LPS because they carry a mutation in the O-antigen biosynthetic pathway<sup>30</sup>.

Once we knew that LPS is synthesized at the IM, it became clear that LPS molecules are first translocated across the IM, then transported across the periplasm, and ultimately delivered to the cell surface. At the same time, many questions emerged. Are specific proteins required for LPS transport? Is LPS transport to the OM coupled to the transport of OM proteins? Are the controversial zones of contact between the IM and the OM bridges that mediated LPS transport across the cell envelope? What is the source of energy for LPS assembly at the OM, an environment devoid of ATP? We now know the answer to some of these questions. For example, Rothfield and Pearlman-Kothencz determined early on that inhibition of protein synthesis did not prevent either LPS synthesis or transport to the OM for hours<sup>31</sup>, implying that OM proteins and LPS were not transported to and assembled at the OM as a unit. Most importantly, employing a variety of genetic, biochemical and bioinformatic approaches, a number of laboratories have recently discovered the proteins that transport LPS across the cell envelope, and a model for LPS biogenesis has emerged.

#### Current model for LPS biogenesis

For clarity, we first summarize the current model for LPS biogenesis (FIG. 4). This model was based on the fact that every envelope compartment has at least one factor required for LPS transport and from the genetic, biochemical and bioinformatic studies described later. After its synthesis, rough LPS is translocated across the IM by the ABC (ATP-binding cassette) transporter MsbA. The O-antigen is independently translocated across the IM, usually by the Wzx flippase as an undecaprenol-phosphate-linked molecule; however, in some *E. coli* strains, the Wzm–Wzt ABC transporter translocates undecaprenol-linked O-antigen. The WaaL ligase then attaches the O-antigen to rough LPS at





**Figure 2 | Electron micrograph of *Veillonella* illustrating the ultrastructure of the Gram-negative cell envelope.** Bladen and Mergenhagen published this micrograph in 1964 (REF. 15) and they labelled the different structures as the outer membrane (OM), the solid membrane (SM), which is known nowadays as the peptidoglycan, and the plasma membrane (PM), which we refer to as the inner or cytoplasmic membrane. Figure is reproduced, with permission, from REF. 15 © (1964) American Society for Microbiology.

the periplasmic face of the IM<sup>7</sup>. Next, LPS (or rough LPS in the case of *E. coli* K-12 strains) interacts with the Lpt (LPS transport) pathway in which LPS is first extracted from the IM by an ABC transporter composed of LptB, LptF and LptG, and probably the bitopic membrane protein LptC. For LPS to cross the periplasm and be assembled at the OM, the periplasmic protein LptA, the OM lipoprotein LptE and the  $\beta$ -barrel OM protein LptD are required. As we discuss later, although we think that every essential Lpt factor has been identified, we still lack a fundamental understanding of the mechanism of LPS transport.

### Flipping LPS across the IM

The ABC transporter MsbA translocates rough LPS across the IM. Perhaps because it was identified early and because it belongs to the ABC transporter superfamily, MsbA has been the subject of many biophysical and protein biochemical studies during the past decade; as a result, MsbA is the best understood protein involved in LPS transport. We now have four x-ray structures of MsbA trapped in different conformations!<sup>32</sup> However, it all started with a simple genetic selection.

Karow and Georgopoulos identified *msbA* in 1993 as a multicopy suppressor of the temperature sensitivity conferred by

*htrB* null alleles<sup>33</sup>. Primary sequence and genetic analyses revealed that MsbA was probably an essential ABC transporter in *E. coli* but lack of knowledge on the function of HtrB (later renamed LpxL) obscured the identity of the transported substrate. Indeed, the demonstration in 1996 by Raetz and collaborators that LpxL is a lipid A laurate acetyltransferase<sup>34</sup> led Polissi and Georgopoulos<sup>35</sup> to show that same year that LPS transport to the OM is defective in an *htrB* mutant under non-permissive conditions. More importantly, after demonstrating that when present in multiple copies, *msbA* alleviates this defect in *htrB* mutants and that loss of MsbA causes accumulation of LPS species at the IM, they proposed that MsbA was a translocator of either LPS or one of its precursors.

In an effort to demonstrate a role for MsbA in the transport of LPS across the IM, Raetz and collaborators made an important contribution by developing a biochemical assay to probe LPS transport system *in vivo*<sup>36</sup>. In this assay, lipid A and its phosphorylated precursors are radiolabelled and these species are then extracted and analysed by thin-layer chromatography. Using this assay in combination with sucrose-gradient fractionations to separate the IM and OM, Zhou *et al.*<sup>36</sup> showed that under non-permissive conditions,

*htrB* mutants accumulate tetra-acylated lipid A (lipid IV<sub>A</sub>) species at the IM and that overexpressing MsbA does not correct this defect by favouring the maturation of LPS, but rather suppresses it by increasing the transport of the immature lipid IV<sub>A</sub> species to the OM. They also demonstrated that cells depleted of MsbA accumulate LPS species containing mature hexa-acylated lipid A at the IM.

Proof that this accumulation of the hexa-acylated lipid A occurs at the inner leaflet of the IM was obtained by exploiting the fact that LPS undergoes chemical modification at different locations in the cell<sup>37</sup>. Specifically, Doerrler *et al.*<sup>38</sup> showed that in cells deficient in MsbA function, the LPS that accumulates at the IM is not accessible to modifications by ArnT and EptA/PmrC, two enzymes that catalyse, respectively, the transfer of aminoarabinose and phosphoethanolamine to lipid A at the periplasmic side of the IM<sup>37,39–41</sup>. In the next section we describe a similar strategy used to determine whether LPS reaches the cell surface.

Therefore, genetic and biochemical studies *in vivo* agree that MsbA translocates LPS across the IM. Crystal structure analyses reveal that MsbA forms a dimer in which each monomer provides six transmembrane (TM) helices and half of a cytoplasmic nucleotide-binding domain<sup>32</sup>. The dimer hydrolyses ATP and undergoes large conformational changes thought to be required for transport. Although reconstitution of the flippase activity of MsbA awaits, *in vitro* biochemical studies have shown that hexa-acylated lipid A species and lipophilic drugs stimulate the ATPase activity of MsbA<sup>42,43</sup>. In fact, the Sharom laboratory has recently shown that the MsbA dimer contains two high-affinity substrate-binding sites capable of binding lipid A and amphipathic drugs<sup>44</sup> and has suggested that MsbA may function as both a lipid flippase and a multidrug transporter<sup>43,44</sup>.

### From the IM to the surface

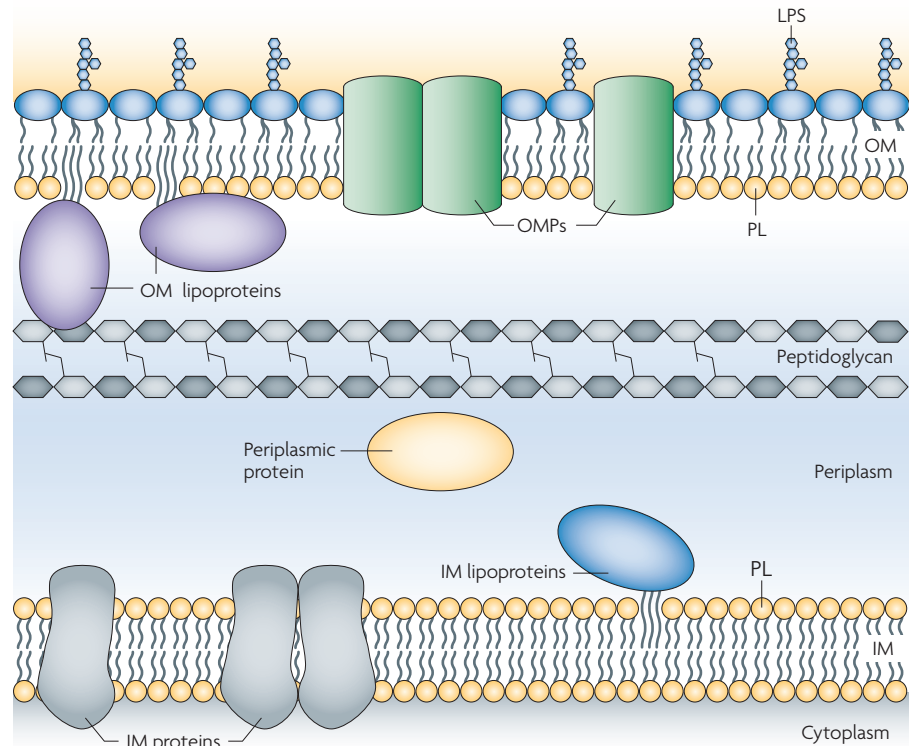
The Lpt pathway transports LPS from the IM to the cell surface (FIG. 4). Most of the Lpt factors have been identified in the past 3 years but the first *lpt* mutations were isolated in *lptD* (formerly known as *imp* or *ostA*) in 1989. Benson and collaborators designed a genetic selection for mutants which allowed maltodextrins to pass across their OM in the absence of the maltodextrin-specific channel LamB<sup>45</sup>. The *lptD* mutations increased membrane

permeability not only to maltodextrins but also to many hydrophobic and hydrophilic antibiotics, implying that the overall barrier function of the OM was compromised.

Although those early studies suggested the essential nature of *lptD*, it took more than a decade to uncover the role of LptD in OM biogenesis. New attention to *lptD* was sparked by two findings. First, *lptD* is located immediately upstream of *surA*, a gene that encodes a key periplasmic chaperone involved in the assembly of OM proteins<sup>46</sup>. Second, both *lptD* and *surA* are in an operon regulated by  $\sigma^E$  envelope stress response<sup>47</sup>. The  $\sigma^E$  response monitors the status of the cell envelope and, when activated, increases the production of envelope-biogenesis factors<sup>48</sup>. With this information in hand, Braun and Silhavy showed that LptD is an essential OM protein required for the biogenesis of the *E. coli* cell envelope<sup>49</sup>. Proof of its role in LPS assembly at the surface came later from work in *Neisseria meningitidis* by Tommassen and collaborators<sup>50</sup>, who showed that in *N. meningitidis* mutants that lack the LptD orthologue, LPS does not reach the cell surface as it is not accessible to either cleavage by extracellular neuraminidase or modification by the OM enzyme PagL. Notably, in *N. meningitidis* neither LptD nor LPS are essential<sup>50,51</sup>; likewise, LptD is also not essential in *Helicobacter pylori*<sup>52</sup>.

To identify additional Lpt factors, Kahne and collaborators took a biochemical approach. Using affinity purification of an epitope-tagged LptD, they discovered that in *E. coli* LptD is in a complex with the essential OM lipoprotein LptE (formerly known as RlpB)<sup>53</sup>. They also showed LPS molecules synthesized after depletion of either LptD or LptE are not accessible to modification by PagP, an OM enzyme that palmitoylates LPS at the outer leaflet of the OM<sup>37,54</sup>. Therefore, both LptD and LptE are required for LPS transport to the cell surface in *E. coli*.

The remaining Lpt factors were identified by different approaches. Polissi and collaborators found *lptA* and *lptB* (formerly known as *yhbN* and *yhbG*, respectively) in a genetic screen searching for essential genes<sup>55</sup>. Clues that these genes could be involved in LPS biogenesis first came from the realization that they are part of a highly conserved locus that includes genes encoding enzymes involved in the synthesis of LPS core<sup>56</sup>. This led Polissi and collaborators to demonstrate using sucrose density-gradient centrifugation that depletion of

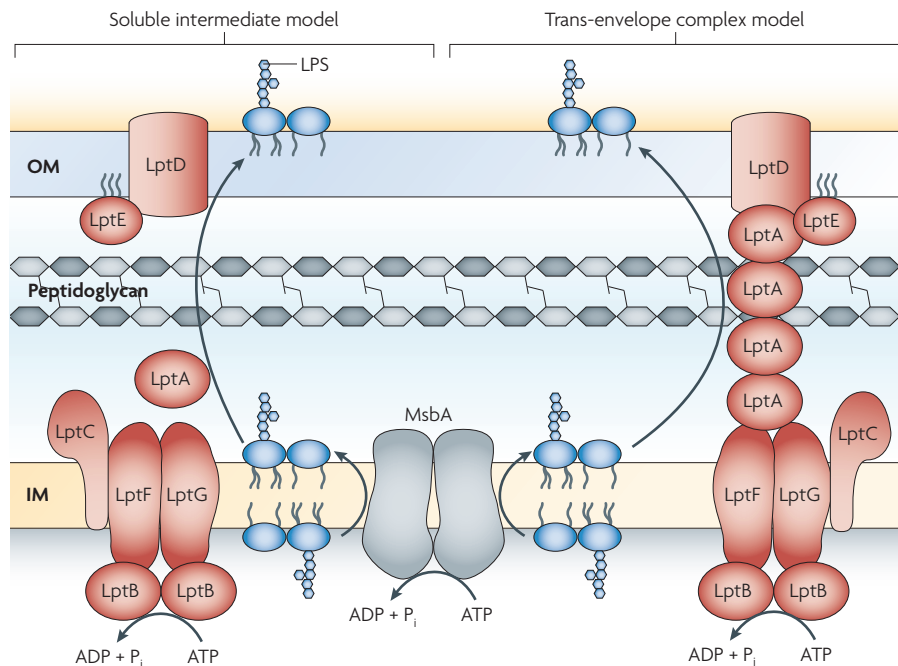


**Figure 3 | Structure of the Gram-negative cell envelope.** In Gram-negative bacteria, the cytoplasm is surrounded by the inner membrane (IM), that is, a phospholipid (PL) bilayer that also contains proteins. There are two types of proteins in the IM: integral IM proteins, which span the membrane through  $\alpha$ -helical transmembrane domains, and IM lipoproteins, which are anchored to the outer leaflet through a lipid moiety. The periplasm is the aqueous compartment bounded by the IM and the outer membrane (OM); it contains soluble proteins and the peptidoglycan layer. The OM is anchored to the rest of the cell via proteins that are covalently attached to the peptidoglycan. The OM is asymmetric, as it contains phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. In addition, the OM contains two types of proteins, integral OM proteins (OMPs) and lipoproteins.

either LptA or LptB halts the transport of LPS across the cell envelope, causing the accumulation of LPS at the IM<sup>57</sup>. Because sequence analysis predicts that LptB is an IM-associated<sup>58</sup> cytoplasmic protein with a nucleotide-binding domain typical of ABC transporters and LptA is a periplasmic protein, they proposed the following model. LptB hydrolyzes ATP to provide energy to an ABC transporter that extracts LPS from the IM, whereas LptA facilitates LPS transport across the periplasm<sup>57</sup>. ABC transporters require an IM component, therefore, they also suggested that the IM protein encoded by the essential gene *yrbK*<sup>56</sup> might have this role as the 3' end of *yrbK* overlaps with the 5' end of *lptA*. Such chromosomal arrangement is usually indicative of genes specifying related functions.

Since then, Polissi and collaborators have proved that YrbK (renamed LptC) is required for the extraction of LPS from the IM<sup>59</sup>. However, LptC is a bitopic protein and typically the integral components of

ABC transporters are composed of either one IM protein with 12 TM domains or two with 6 TM domains each<sup>60</sup>. Therefore, it was likely that we were missing at least one more Lpt factor. Indeed, using a reductionist bioinformatic approach designed to identify OM-biogenesis factors, Ruiz and collaborators recently found the 6-TM-domain IM proteins LptF and LptG (formerly known as YjgP and YjgQ)<sup>61</sup>. Their approach took advantage of the high degree of conservation of OM-biogenesis factors among Gram-negative bacteria, including endosymbionts. They reasoned that even though endosymbionts have lost many genes during their adaptation to intracellular life, these intracellular bacteria must still possess those encoding factors essential for OM biogenesis. Thereby, they identified LptF and LptG as the missing components of the ABC transporter, which together with LptB, and possibly LptC, extracts LPS from the outer leaflet of the IM so that it



**Figure 4 | Current models for LPS transport across the cell envelope.** After being synthesized, rough lipopolysaccharide (LPS) molecules (that is, those containing only lipid A and core) are flipped across the inner membrane (IM) by the ABC transporter MsbA. Strains that produce the O-antigen translocate it independently across the IM and then ligate it to the rough LPS by the WaaL ligase (step not shown). LPS is then extracted from the IM by LptC and the LptF, LptG, LptB ABC transporter. In the soluble-intermediate model, LptA serves as a soluble periplasmic chaperone that transports LPS from the IM to the LptD and LptE assembly site on the outer membrane (OM). In the trans-envelope complex model, the Lpt factors constitute a multiprotein complex that spans the cell envelope. Because LptA crystallizes as a fibre in the presence of LPS, it is possible that it serves as a bridge between the OM and IM components. Note that the only physical interaction between Lpt factors that has been conclusively demonstrated to date is that between LptD and LptE<sup>53</sup>.

can be transported to the OM. This bioinformatic analysis also suggests that all essential Lpt factors of *E. coli* have been identified.

**Lpt pathway order**

In *E. coli*, every cellular compartment contains proteins required for LPS transport. Phenotypes exhibited by cells depleted of MsbA are distinct from those observed on depletion of Lpt factors: LPS is not flipped across the IM in the absence of MsbA but it is in the absence of Lpt factors. However, disruption of the pathway at any step after MsbA leads to indistinguishable phenotypes: in all cases, LPS accumulates in the outer leaflet of the IM<sup>59,61,62</sup>. Therefore, we cannot conduct epistasis analysis to determine exact pathway order. We can only rely on the information about the cellular location of these proteins to predict temporal order in the pathway as follows: MsbA followed by a complex of LptB, LptC, LptF and LptG, followed by LptA and finally by a complex of LptD and LptE.

**Future directions**

The central question of LPS biogenesis at the moment is how, mechanistically, LPS is transported across the aqueous periplasmic compartment and inserted in functional form in its correct spatial location, that is the outer leaflet of the OM. In a very real sense, the mechanism by which the Lpt proteins are transported is a stereochemical problem of the highest order. As it involves proteins in two different membranes that are separated by an aqueous compartment, it is not even clear what kinds of tools will be needed to study this problem.

LPS cannot traverse the aqueous periplasm by diffusion owing to its amphiphilic nature. Therefore, LPS is somehow assisted across the periplasm and through the peptidoglycan layer. Two general models have been proposed for how LPS crosses the periplasm to reach the OM (FIG. 4)<sup>46</sup>. One model posits that LPS is bound by a soluble periplasmic chaperone protein that diffuses across the periplasmic

space to a docking site at the inner leaflet of the OM. Specifically, according to this model, LPS transport would resemble OM lipoprotein transport in which a soluble chaperone, LolA, binds lipoproteins after they have been extracted from the IM by the ABC transporter composed of LolC, LolD and LolE and carries them to an assembly site, LolB, in the OM<sup>63</sup>. In fact, LptA has recently been shown to bind LPS *in vitro*, an observation that the authors suggest is consistent with the chaperone model<sup>64</sup>. An alternative model posits that there is some sort of proteinaceous bridge that physically connects the IM and OM and allows for direct efflux of LPS to the cell surface. It has been demonstrated that spheroplasts, which are effectively drained of periplasmic contents, are still able to transport newly synthesized LPS to the outer leaflet of the OM, implying that all components required for LPS transport remain stably associated with the spheroplasts<sup>65</sup>. In addition, the recently solved crystal structure of LptA suggests that the protein can form fibrils composed of LptA molecules oriented in a head-to-tail fashion, and thus could physically bridge the gap between the two membranes<sup>66</sup>. It is important to note, however, that none of the evidence presented for one model can rule out the other model entirely. More biochemical experiments need to be done to discern between these two transport models.

Even though we still do not understand how the Lpt system functions, we have clearly made great advances in understanding LPS assembly in the recent years. We hope that the scientific history presented here illustrates how this endeavour was only possible because of the development of new technologies and the use of different approaches throughout decades. We foresee that the same level of creativity and diversity of scientific approaches will be needed to explore and elucidate the molecular mechanisms of the multi-component, inter-membrane Lpt transport system.

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**FURTHER INFORMATION**

Thomas J. Silhavy's homepage: <http://www.molbio1.princeton.edu/labs/silhavy/index.html>

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