EcoSal Plus
Cellular and Molecular Biology of
E. coli, Salmonella, and the *Enterobacteriaceae*

DOMAIN 2 CELL ARCHITECTURE AND GROWTH

Function and Biogenesis of Lipopolysaccharides

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ABSTRACT The cell envelope is the first line of defense between a bacterium and the world-at-large. Often, the initial steps that determine the outcome of chemical warfare, bacteriophage infections, and battles with other bacteria or the immune system greatly depend on the structure and composition of the bacterial cell surface. One of the most studied bacterial surface molecules is the glycolipid known as lipopolysaccharide (LPS), which is produced by most Gram-negative bacteria. Much of the initial attention LPS received in the early 1900s was owed to its ability to stimulate the immune system, for which the glycolipid was commonly known as endotoxin. It was later discovered that LPS also creates a permeability barrier at the cell surface and is a main contributor to the innate resistance that Gram-negative bacteria display against many antimicrobials. Not surprisingly, these important properties of LPS have driven a vast and still prolific body of literature for more than a hundred years. LPS research has also led to pioneering studies in bacterial envelope biogenesis and physiology, mostly using *Escherichia coli* and *Salmonella* as model systems. In this review, we will focus on the fundamental knowledge we have gained from studies of the complex structure of the LPS molecule and the biochemical pathways for its synthesis, as well as the transport of LPS across the bacterial envelope and its assembly at the cell surface.

Received: 20 January 2018
Accepted: 01 June 2018
Posted: 01 August 2018
Editor: James M. Slauch, The School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, IL
Citation: EcoSal Plus 2018; doi:10.1128/ecosalplus.ESP-0001-2018.
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doi:10.1128/ecosalplus.ESP-0001-2018

INTRODUCTION

Gram-negative bacteria are characterized by an envelope that contains two membranes: an inner membrane (IM) that surrounds cytoplasmic components, and an outer membrane (OM) that separates the cell from its environment. These two membranes surround an aqueous cellular compartment termed the periplasm, which contains the peptidoglycan cell wall (Fig. 1A) (1). Thus, in Gram-negative bacteria, the OM serves as the first line of defense against environmental threats. Notably, in contrast to many biological membranes, the OM of most Gram-negative bacteria is not a phospholipid bilayer. Instead, it is a highly asymmetric bilayer that contains phospholipids in the inner leaflet and lipopolysaccharide (LPS) molecules in the outer leaflet (1–3). The glycolipid LPS is the focus of this review.

LPS performs several functions in Gram-negative bacteria. The most fundamental function of LPS is to serve as a major structural component of the OM. Perhaps not surprisingly, LPS is an essential component of the cell envelope in most, although interestingly not all, Gram-negative bacteria (4). In addition, LPS molecules transform the OM into an effective permeability barrier against small, hydrophobic molecules that can otherwise cross phospholipid bilayers,

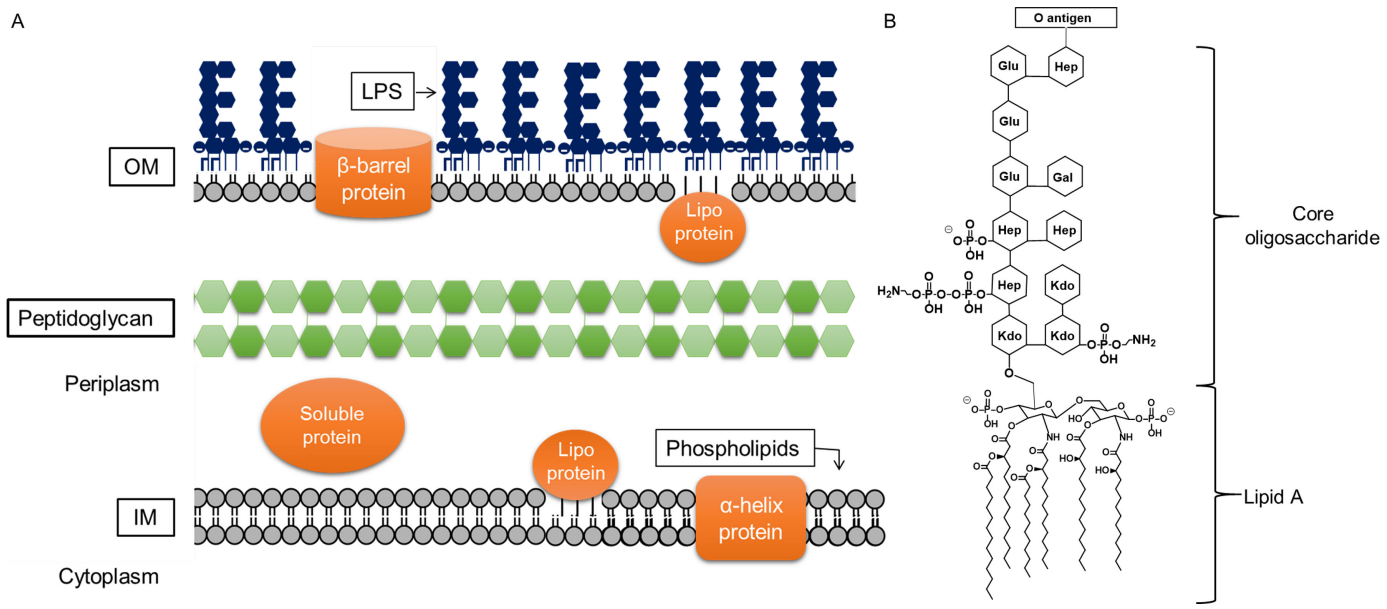

Figure 1 Architecture of the Gram-negative cell envelope. (A) Depiction of the Gram-negative cell envelope and its components. The inner membrane (IM) contains phospholipids, while the outer membrane (OM) contains phospholipids in the inner leaflet and lipopolysaccharide (LPS) in the outer leaflet. (B) Structure of prototypical LPS produced by *E. coli* (shown is the core structure associated with core type K-12).

making Gram-negative bacteria innately resistant to many antimicrobial compounds (5, 6). LPS can also play a crucial role in bacteria-host interactions by modulating responses by the host immune system.

Three main areas of LPS biology are covered in this review. We will first discuss the overall structure of LPS and its function from the bacterial and human point of view. We will then review LPS biosynthesis in *Escherichia coli* and *Salmonella*, and discuss how bacteria can regulate LPS synthesis and modify its chemical structure in response to environmental stressors. Last, in the 1970s, Mary Jane Osborn and collaborators posed a question that has dominated a great part of LPS biogenesis research in the past two decades: since LPS is synthesized in the IM but displayed at the cell surface, how is it transported across the cell envelope (7, 8)? Here, we will summarize the work that has uncovered a novel intermembrane transport system that solves the challenges of shuttling this complex glycolipid across diverse cellular compartments.

STRUCTURE AND FUNCTION OF LPS

Main Features of the Structure of LPS

LPS is a large glycolipid composed of three structural domains: lipid A, the core oligosaccharide, and the O antigen (Fig. 1B) (9). Lipid A, the hydrophobic portion of the molecule, is an acylated β-1′-6-linked glucosamine disaccharide that forms the outer leaflet of the OM (9). In *E. coli* and *Salmonella*, the glucosamines are phosphorylated at the 1 and 4′ positions and acylated at the 2, 3, 2′, and 3′ positions (9). Two additional secondary acyl chains are also typically present in the distal glucosamine so that mature lipid A is mostly hexa-acylated (9). The core oligosaccharide is a nonrepeating oligosaccharide that is linked to the glucosamines of lipid A (9, 10). The core structure usually contains 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) residues, heptoses, and various hexoses, which can be modified with phosphates and other substituents such as phosphoethanolamine (9–12). The O antigen is an extended polysaccharide that is attached to the core oligosaccharide. It is composed of a repeating oligosaccharide made of two to eight sugars (13–15).

The overall structure of LPS is conserved, but there are many variations that can occur at the species and strain level (9–12, 16, 17). Similarly, the lipid A structure is conserved at the species level; however, as described below, it can undergo regulated modifications in response to environmental conditions (12, 18–20). The core oligosaccharides vary among species and even between some strains of one species (9–12). However, the most diverse component of LPS is the O antigen (13, 17). Not

only can the structure and composition of the O antigen differ within a species at the strain level, but, in addition, some Gram-negative bacteria do not synthesize this component of LPS (13, 21). In such cases, molecules composed of only lipid A and the core oligosaccharide are typically referred to as lipooligosaccharides, or LOS (9). Classically, LOS has been referred to as "rough" LPS, as opposed to "smooth" LPS, which includes the O antigen (9).

The Function of LPS

While the structure of LPS (or LOS) may vary among bacteria, in all cases, this glycolipid populates much of the cell surface and establishes a permeability barrier that protects the cell from the entry of toxic molecules such as antibiotics and bile salts (5, 22). Additionally, because LPS is the primary bacterial component encountered by the host immune system, LPS often plays a major role in bacterial pathogenicity (20, 23).

The barrier function of LPS stems in part from its strong amphipathic nature. As in other lipid bilayers, the acyl portion of lipid A provides hydrophobic character that inhibits the passage of hydrophilic molecules through the OM. However, in contrast to other bilayers, the core oligosaccharide and O antigen additionally provide extensive hydrophilic character to LPS that makes the OM particularly impermeable to hydrophobic compounds as well (5, 22). The effectiveness of the barrier posed by LPS is also heavily reliant on the ability of LPS to pack densely within the outer leaflet of the OM. This dense packing is mediated in part by hydrophobicity-driven association of the acyl chains of lipid A. Because lipid A molecules typically bear a large number (that is, 4 to 7) of saturated fatty acid moieties, the extensive interactions between these acyl chains result in low fluidity within the membrane bilayer (5). However, packing of LPS is complicated by the presence of negatively charged phosphate groups throughout its structure. Most salient and conserved are the phosphates of the 1 and 4′ positions of the glucosamines in lipid A, which lie at the exterior surface of the OM, but phosphates can also be found in the core oligosaccharide (9, 12). To prevent repulsion between these negatively charged phosphates, divalent cations such as Mg^{2+} intercalate between LPS molecules, forming polyionic interactions that greatly enhance LPS packing and, consequently, promote the barrier function of the OM (5, 22).

Because LPS decorates the surface of many bacterial pathogens, the host immune system has evolved to respond dramatically to its presence, making LPS a PAMP, or pathogen-associated molecular pattern (20, 23, 24). In fact, this response can be so dramatic as to prove toxic to the host. For this reason, LPS has been classically termed "endotoxin" in reference to the cell-associated (endo) toxicity observed for many Gram-negative organisms. Understandably, the immune system has evolved to respond primarily to the most conserved feature of LPS, the lipid A structure, for which host toll-like receptor 4 (TLR4) is the primary receptor (20, 23, 24). However, as mentioned above, there is considerable diversity in LPS structures, even within lipid A. A consequence of this diversity is that different LPS structures have varying ability to trigger the host immune response (20). Thus, while the classical, hexacylated, bisphosphorylated lipid A molecule produced by *E. coli* and *Salmonella* is highly immunogenic, other forms of lipid A are less so. Some forms of lipid A not only elicit no response themselves, but inhibit the host response to more immunogenic varieties (20, 25–27). In fact, production of less immunogenic lipid A is a strategy used by certain pathogens to evade the host immune response. For example, *Yersinia pestis*, the causative agent of the bubonic plague, modulates the acylation of its lipid A at mammalian body temperature to produce less immunogenic lipid A (28). Alternatively, some organisms evade the host immune response by masking the more conserved aspects of their LPS with a highly variable O antigen (23). Although the O antigen induces the production of antibodies, the length of the O-antigen chain prevents the antibody-mediated deposition of complement at the bacterial cell surface (13, 29, 30). Consequently, the O-antigen structure protects bacteria from lysis by complement. Possession of an O antigen has also been shown to contribute to pathogen evasion of phagocytosis by immune cells (13, 31).

We should also mention that the combination of the antigenicity and great structural diversity of O antigens has been exploited in the clinic to identify and classify pathogens. This application relies on the fact that the immune system produces specific antibodies that recognize one type of O antigen. Collections of O-antigen-specific antisera have been classically utilized to categorize Gram-negative organisms by serotype, that is, antigenicity in serological testing, of their O antigen (17).

LPS SYNTHESIS PATHWAY

Kdo_2-Lipid A Biosynthesis: the Raetz Pathway

Lipid A was first identified as the lipid component that could be released from the rest of the LPS molecules by mild-acid hydrolysis (32–34). Historically, this degradation product was marked as one of the three structural components of LPS. However, it is worth noting that cells synthesize lipid A together with the Kdo moieties of the core oligosaccharide using a biosynthetic pathway that is the most conserved aspect of LPS synthesis (9). This pathway has been extensively characterized in *E. coli* and *Salmonella* (9, 35) and is referred to as the Raetz pathway because much of the research describing it was led by Christian Raetz and his team (Fig. 2).

The process of lipid A synthesis begins in the cytoplasm with the precursor molecule *N*-acetylglucosamine linked to a nucleotide carrier (UDP-GlcNAc). This UDP-GlcNAc precursor is initially acylated by the enzyme LpxA to yield UDP-3-*O*-(acyl)-GlcNAc (36–38). LpxA is selective for the 14-carbon acyl group β-hydroxymyristate carried by the acyl carrier protein (ACP) (37). This selectivity is based on the LpxA active site functioning as a hydrocarbon ruler that most readily incorporates 14-carbon substrates (39, 40). The acylation of UDP-GlcNAc is unfavorable, however, and thus the first committed step of lipid A synthesis is the second reaction in the pathway, which is the irreversible deacetylation of UDP-3-*O*-(acyl)-GlcNAc to UDP-3-*O*-(acyl)-GlcN by the Zn^{2+}-dependent metalloenzyme LpxC (41–43). Because LpxC catalyzes the first committed step in the synthesis of LPS, much of the regulation of this pathway, which will be discussed below, appears to center around this enzyme. Following the action of LpxC, UDP-3-*O*-(acyl)-GlcN is subsequently acylated a second time by LpxD to yield UDP-2,3-diacylglucosamine (44, 45). Like its earlier homologous counterpart LpxA, LpxD is selective for β-hydroxymyristate-ACP as a donor (44). In fact, it has been suggested that LpxD could, to some extent, be capable of substituting for LpxA in the first acylation step. However, since both enzymes are essential (44, 46, 47), it appears any cross-specificity between LpxA and LpxD is insufficient to support growth. After the second

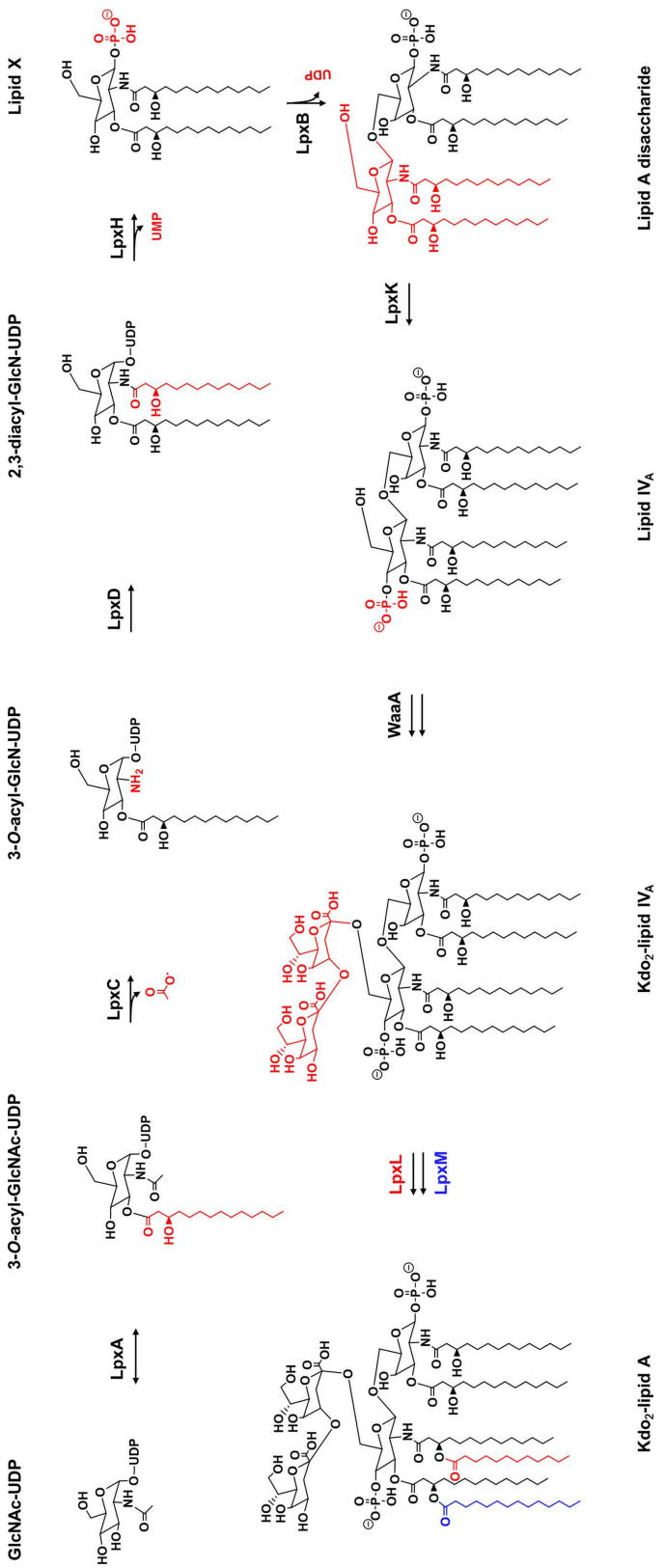

Figure 2 Lipid A biosynthesis pathway. Modifications to the preceding structure made by each enzyme in the pathway are marked in red, with the exception of the last step, where the modifications made by LpxL and LpxM are colored in red and blue, respectively. Donor molecules are not shown. At low temperatures, LpxP acts instead of LpxL to add a C16:1 palmitoleoyl group instead of a lauroyl group.

acylation by LpxD, LpxH removes the sugar nucleotide carrier from UDP-2,3-diacylglucosamine to generate 2,3-diacylglucosamine-1-phosphate, otherwise known as lipid X (48–50). Lipid X is subsequently added by LpxB to a molecule of UDP-2,3-diacylglucosamine (the product of the LpxD reaction) through a β-1′-6 linkage that releases the UDP nucleotide carrier. The resulting product is a tetraacylated glucosamine disaccharide that is inserted in the inner leaflet of the IM and is sometimes referred to as lipid A disaccharide (51, 52). Following this condensation step, lipid A disaccharide is phosphorylated at the 4′ position by the kinase LpxK, becoming the bisphosphorylated lipid IV_A (53, 54). As noted above, while not strictly part of lipid A synthesis, the next step is the addition of two Kdo sugar groups of the core oligosaccharide to lipid IV_A (55–57). This step is mediated by the enzyme WaaA, previously known as KdtA, which sequentially adds Kdo groups to lipid IV_A from activated Kdo (CMP-Kdo) (56, 57). Finally, two additional acylation events catalyzed by the LpxL and LpxM acyltransferases occur in sequence (58–60). LpxL adds a lauroyl group to the hydroxyl of the 2′-hydroxymyristoyl group and, subsequently, LpxM transfers a myristoyl group to the hydroxyl of the 3′-hydroxymyristoyl group (58–60). Like their earlier counterparts LpxA and LpxD, LpxL and LpxM only utilize substrates carried by ACP (58–60). LpxM functions best after the lauroyl group has already been added by LpxL, but it is capable of functioning to some extent in the absence of LpxL activity (60). After the sequential action of LpxL and LpxM, mature, hexacylated lipid A, which also contains the first two Kdo residues of the inner core, is ready to serve as an acceptor for the sugar groups composing the core oligosaccharide.

An important note is the fact that, under normal laboratory growth conditions, about one-third of LPS molecules are modified by LpxT, which adds a second phosphate group to the 1-phosphate of lipid A, utilizing the pyrophosphate-undecaprenol (Und-PP) as the donor (discussed below in "Modifications of Lipid A" and shown in Fig. 6) (61, 62). This reaction is not part of the Raetz pathway and occurs after the core-lipid A molecule is flipped across the IM. In addition, as discussed below, LpxT activity is subject to regulation by environmental conditions.

Biosynthesis of the Core Oligosaccharide

The core oligosaccharide can be subdivided into an inner core, which is proximal to lipid A, and an outer core, which becomes the attachment site for the O antigen (9). The inner core is generally well conserved and is composed of Kdo and L-*glycero*-D-*manno*-heptose (heptose) groups (9, 63, 64). The outer core constituents are less conserved, and will vary depending on the type of core oligosaccharide, but in general consist of a series of hexoses (9, 63, 64). For the sake of simplicity, we will focus on the synthesis pathway for the K-12 core type from *E. coli* (Fig. 3), although various types of core oligosaccharide structures of *E. coli* and *Salmonella* can be found in Fig. 4 (9, 11).

The first step of core oligosaccharide synthesis is the sequential addition by the WaaA enzyme of the first two Kdo groups to the glucosamines of lipid A, which, as discussed above, occurs before the final acylation steps that conclude lipid A synthesis (Fig. 2) (56–60). Next,

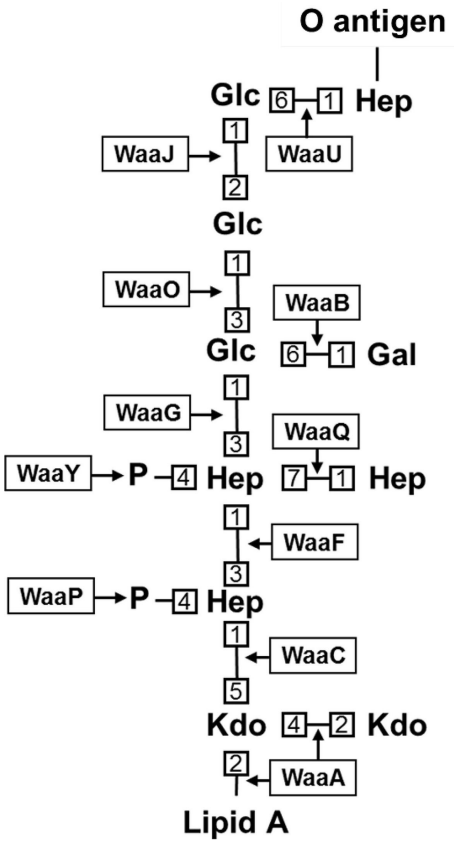

Figure 3 Structure and biosynthetic enzymes of the *E. coli* K-12 core oligosaccharide. Numbers represent bond positions between sugars. Note that nonstoichiometric modifications are not shown. All linkages are α-anomeric unless preceded by the β symbol, which specifies the β-anomeric state. Enzyme names are boxed, with arrows indicating the linkages they catalyze. It is worth noting that, while the O-antigen ligation site is indicated, *E. coli* K-12 does not typically produce O antigen because of an ancestral mutation that inactivates its synthesis.

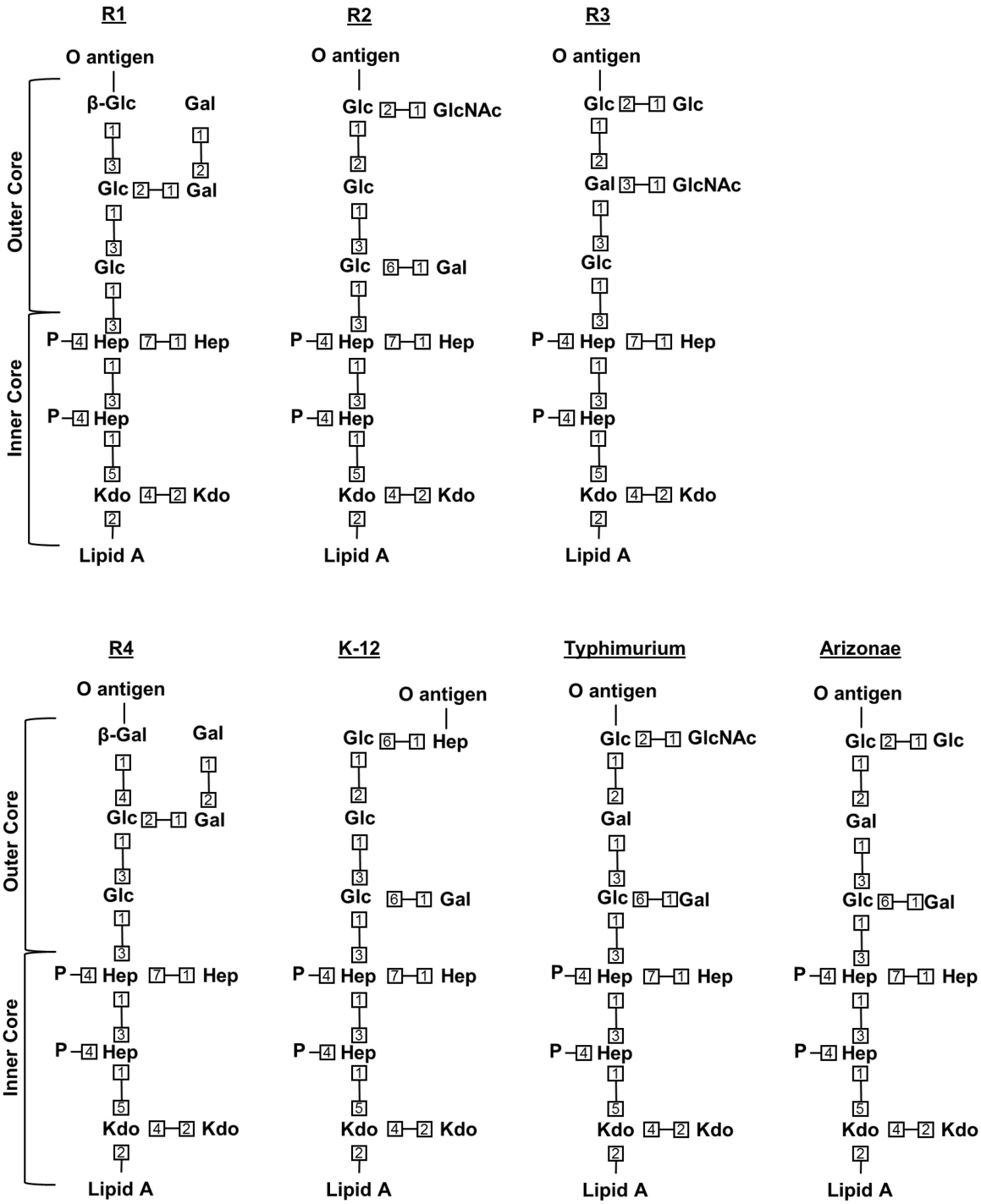

Figure 4 Structure of various core oligosaccharides. Shown are the known core types in *E. coli* (R1 to R4, K-12) and *S. enterica* serovar Typhimurium and *S. enterica* serovar Arizonae IIIA. Numbers represent bond positions between sugars. Note that nonstoichiometric modifications are not shown. All linkages are α-anomeric unless preceded by the β symbol, which specifies the β-anomeric state.

following completion of lipid A synthesis by LpxL and LpxM, the inner core is extended with two heptose residues by the sequential action of WaaC and WaaF (11, 65). ADP-L-*glycero*-D-*manno*-heptose generally serves as the donor substrate for these inner core glycosylation reactions (65). After the addition of the heptoses by WaaC and WaaF, the final three steps that complete inner core synthesis must be catalyzed in order by the enzymes WaaP, WaaQ, and WaaY, respectively (66). WaaP is a kinase that phosphorylates the first heptose

of the inner core, which was added by WaaC (66, 67). WaaQ then transfers an additional heptose to the second heptose of the inner core, which was added by WaaF. This third heptose added by WaaQ is then phosphorylated by the WaaY kinase (66). Interestingly, while loss of inner core phosphorylation inhibits outer core extension, the loss of the enzymes that extend the outer core also inhibits inner core phosphorylation, implying complexities in the synthesis pathway that have not been fully explored (68).

Synthesis of the outer core begins with the addition of a glucose group to the second heptose, not only in the K-12 core type, but in all *E. coli* and *Salmonella* LPS structures (11, 64). This addition is mediated by WaaG (and its homologs), which utilizes UDP-glucose as its donor substrate (11, 64). The glucose added by WaaG is acted on by the glycosyltransferases WaaO and WaaB, which independently add a glucose and a galactose group, respectively, from UDP-bound donors (11, 64). Additionally, it has been shown that WaaO activity is dependent on divalent cations (64). Next, the penultimate glucose residue is added by the enzyme WaaJ (alternatively known as WaaR), whose activity depends on that of WaaB (11, 69). The final step of core synthesis, the addition of a heptose group to the penultimate glucose, is mediated by the WaaU glycosyltransferase (10, 11). This final heptose group serves as the acceptor of the O antigen after this core-lipid A precursor is translocated to the outer leaflet of the IM.

Biosynthesis of the O Antigen

As might be expected for the outermost, and therefore most exposed, component of LPS, O-antigen structures are highly diverse, with roughly 200 different serogroups identified to date in *E. coli* alone (9, 17). Because of the great diversity of O antigens, the following discussion will focus on the more conserved aspects of O-antigen biosynthesis and its subsequent ligation to the core-lipid A molecule. We should also note that *E. coli* K-12 strains, which are often used in research, do not produce O antigen as the result of an ancestral mutation that inactivates its synthesis in that lineage (21).

The O antigen consists of a variable number of repeating oligosaccharide units, and as such can vary in size from molecule to molecule quite dramatically (9, 70). Additionally, rather than being synthesized directly on the core-lipid A molecule, the O antigen is fully synthesized independently from the rest of the LPS molecule. The O antigen is first built stepwise on a lipid carrier molecule, undecaprenyl phosphate (Und-P), and is then transferred to the core oligosaccharide of the nascent LPS molecule in the periplasmic face of the IM (71, 72). Despite the polymorphic nature of O antigens as a whole, the first step in their synthesis is well conserved, and consists of the transfer of a sugar monophosphate to the carrier molecule Und-P at the inner leaflet of the IM. The resulting sugar-pyrophosphate-undecaprenol (sugar-Und-PP) serves as an acceptor for additional glycosylation reactions (13). Aside from this conserved feature, the routes taken to complete the O antigen vary among different organisms and even strains, but generally fall into three categories: the so-called Wzy-dependent pathway, named for the polymerase that founded the group; the ABC-dependent pathway, which, as the name suggests, relies on an ATP-binding cassette (ABC) transporter to translocate the completed O antigen across the IM; and the synthase-dependent pathway, which is poorly characterized and has only been identified in a single species of *Salmonella* (9, 13). A summary of these different routes can be found in Fig. 5.

The Wzy-dependent pathway entails the synthesis of single O units on Und-P, requiring initiation for each O-antigen subunit, and the subsequent flipping of these Und-P-linked O units to the periplasmic face of the IM by the Wzx flippase (9, 13, 73, 74). These O units are then polymerized on a single Und-P carrier molecule through the activity of Wzy. Polymer length is controlled by a partner protein, Wzz, and exhibits a modal distribution of polymer sizes (9, 13, 75–77). Recent structural studies using cryoelectron microscopy have proposed a new model for how Wzz controls polymer length through a synergistic interaction with Wzy (78). Specifically, this model proposes that association between Wzz and Wzy serves to trigger polymerization, with polymer length being controlled by both a molecular ruler mechanism based on Wzz's polysaccharide-binding capacity, and a molecular stopwatch mechanism based on the time of association between Wzz and Wzy. Finally, the polymerized O antigen is ligated to the core-lipid A acceptor at the outer leaflet of the IM by the ligase WaaL, and the Und-PP carrier is recycled (9). It is worth noting that this biosynthetic strategy of generating O antigen by initiating the synthesis of each subunit on an Und-P molecule places substantial demand on the Und-P carrier pool (9). Indeed, interrupting O-antigen biosynthesis at certain steps in this pathway can lead to the sequestration of Und-P by O-antigen precursors, which causes severe

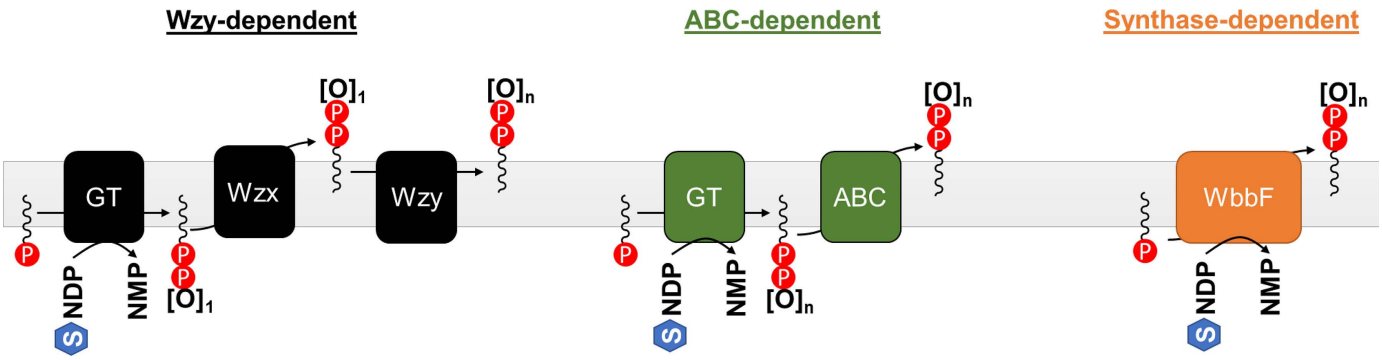

Figure 5 Summary of different O-antigen synthesis pathways. GT stands for glycosyltransferase, and for the purposes of illustration represents all GTs required to generate the O antigen. [O] represents a repeating unit of the O antigen, while the subscript represents the number of repeats present (n being an arbitrary integer). Individual sugar units are represented by "S" inside a hexagon, and are shown bound to an arbitrary nucleotide carrier NDP. The lipid carrier is Und-P.

growth defects because Und-P also functions in the synthesis of several envelope glycopolymers, including the essential peptidoglycan cell wall (79, 80).

In contrast to the Wzx-dependent pathway, the ABC-dependent pathway only requires a single initiation event per molecule of polymerized O antigen and performs the entirety of its polymerization in the cytoplasm (9, 13). Glycosyltransferases first polymerize the completed O antigen on a single Und-P carrier molecule in the inner leaflet of the IM utilizing nucleotide-activated sugar donors. The completely polymerized O-antigen-Und-PP molecule is subsequently flipped to the periplasmic face of the IM by an ABC transporter, where the O-antigen portion is then added to the core-lipid A molecule by the WaaL ligase (13, 81).

The synthase-dependent pathway is peculiar to *Salmonella enterica* serovar Borreze (rfbO:54) (13, 82). The mechanistic details for this mechanism of O-antigen synthesis are unclear, but the titular synthase (WbbF) of the pathway is presumed to simultaneously polymerize and translocate the O antigen across the IM (13, 82). As in the other two biosynthetic pathways, the resulting O-antigen-Und-PP molecule is then used as a donor by the WaaL ligase, which transfers the O-antigen polymer to the outer core oligosaccharide of nascent LPS molecules and releases the lipid carrier, which is then recycled.

REGULATION OF LPS BIOSYNTHESIS

The pathways for the biosynthesis of phospholipids and LPS share a common precursor, β-hydroxymyristate-ACP, the substrate of the FabZ and LpxA enzymes, respectively. As a result, proper balance in lipid biosynthesis, and thereby balanced growth of the IM and OM, requires regulation of LPS synthesis. As stated earlier, LpxC mediates the first committed step in LPS synthesis (9). Consequently, this step becomes a logical control point for the pathway, and indeed, much of our understanding of the regulation of LPS synthesis centers around LpxC. It was noted as early as 1996 that LpxC activity was upregulated when the early steps of LPS synthesis were inhibited, while many other enzymes of the pathway were nonresponsive (83). This response was later shown to be due to regulation of LpxC protein levels by the essential, AAA^+ metalloprotease FtsH (for a recent review regarding FtsH, the reader is directed to reference 84) (85). Interestingly, FtsH has also been shown to degrade WaaA, which adds Kdo groups to lipid IV_A (86). Proteolysis of LpxC by FtsH is dependent on a sequence present at the carboxyl terminus of LpxC, and is controlled by the cellular growth state and levels of the alarmone (p)ppGpp. Specifically, LpxC is stable during fast growth but degraded by FtsH during slow growth; this relationship is inverted with the loss of (p)ppGpp synthesis (87, 88). The precise signal(s) that directly controls FtsH proteolysis of LpxC has not been elucidated, although feedback through lipid A disaccharide has been proposed as a candidate (46). Nonetheless, a protein that regulates the FtsH-dependent proteolysis of LpxC has also been discovered. The bitopic IM protein LapB (formerly YciM) functions as a negative regulator of LpxC levels in an FtsH-dependent manner (89–91). LapB has also been reported to copurify with, in addition to FtsH and LPS, several LPS synthesis and transport proteins, including WaaC and the entirety of the LPS transport (Lpt) complex (described below). These copurification results imply that LapB may serve a larger role in coordinating disparate aspects of LPS biogenesis. How-

ever, the precise nature and mechanism of that role and the relevance of some of those interactions are not clear (91). Furthermore, additional proteolytic control of LpxC by an unknown protease has also been proposed (92). Clearly, more research is needed to understand regulation of LPS synthesis.

MODIFICATION OF LPS STRUCTURE

To better adapt to their varying environments, Gram-negative bacteria will often deviate from the LPS biosynthesis pathways described above. The most frequent modifications include changes in the number and type of acyl chains, as well as the number of phosphates in lipid A; the addition of covalent modifications to lipid A, generally at the 1- and 4′-phosphates, and the core oligosaccharide; and the conversion of the type of O antigen whenever the genomic locus responsible for O-antigen synthesis is exchanged through horizontal gene transfer (18–20). Additionally, due to the incorporation of non-stoichiometric modifications, LPS synthesized by a single strain is not entirely uniform under any growth condition. The most relevant modifications in *E. coli* and *Salmonella* are discussed in detail below, and those in the lipid A and core oligosaccharide regions are summarized in Figs. 6 and 7.

Modifications of Lipid A

Bacteria adapt to different temperatures by modulating the fluidity of their membranes through alterations of the type of acyl chains of their membrane lipids (93). LPS is no exception. Under low-temperature conditions (~12°C), *E. coli* will express an alternate acyltransferase homologous to LpxL, termed LpxP, that adds a 16-carbon palmitoyl group in place of the 12-carbon lauroyl group normally added by LpxL (18, 94, 95). Presumably, this modification serves to offset the decrease in membrane fluidity resultant from lower temperatures (18). Like its counterpart LpxL, LpxP requires ACP-bound substrates for activity (94).

However, most of the changes to lipid A structure occur in response to the amount of cations and positively charged antimicrobials in the environment. A number of enzymes that catalyze those modifications are under control of the PhoQP two-component regulatory system (96, 97). In *Salmonella*, the PhoQP system is primarily implicated in responding to low levels of divalent cations, namely Mg^{2+}, the presence of cationic antimicrobial peptides (CAMPs), and host interactions (97–99). When the kinase PhoQ is activated by these signals, it phosphorylates its cognate response regulator PhoP. Once phosphorylated, PhoP activates the transcription of several

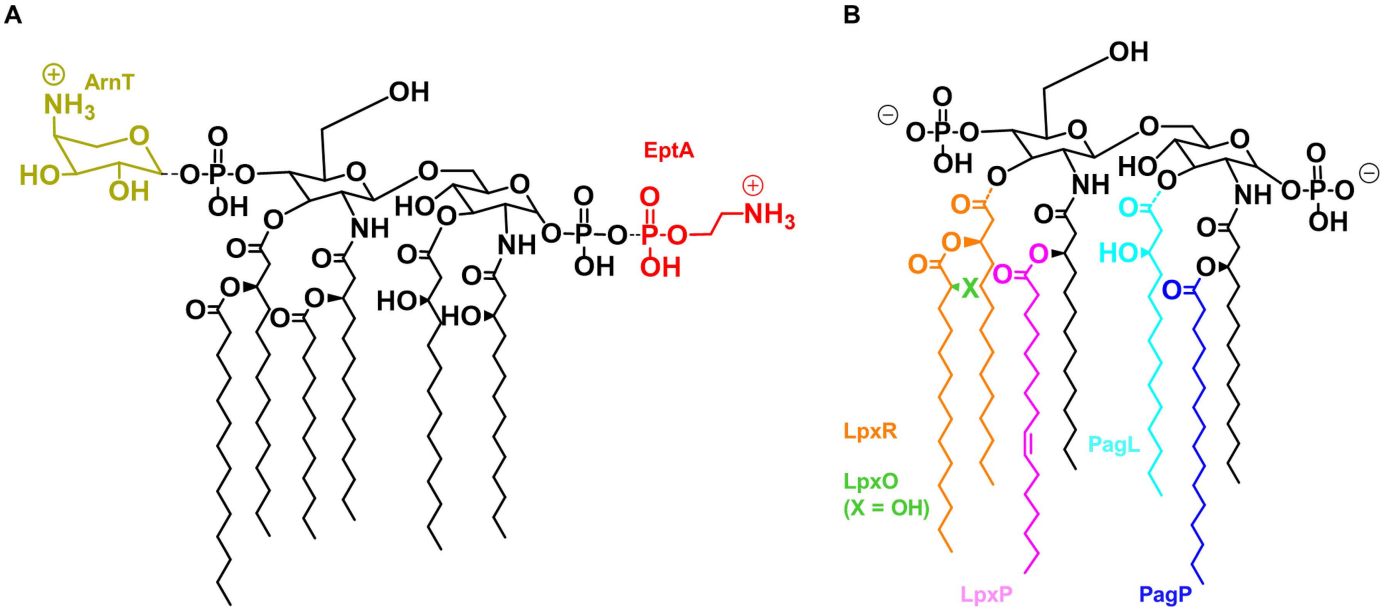

Figure 6 Modifications to the structure of lipid A. Shown are modifications made to lipid A described in the text, alongside the enzymes that mediate them in corresponding colors. (A) Modifications made to the glucosamine phosphates. While shown in their preferred positions, it is possible for either phosphate to be modified with either substituent. (B) Modifications made to the acyl groups. The X indicated for LpxO is a hydroxyl (-OH) when LpxO is active, and a hydrogen (-H) when it is not. When acyl chains are removed, the cleaved bond is shown as a dotted line. It is important to note that LpxP is part of the conserved Raetz pathway but only active at low temperatures, at which LpxP substitutes for LpxL to add a C16:1 palmitoleoyl group instead of a lauroyl group.

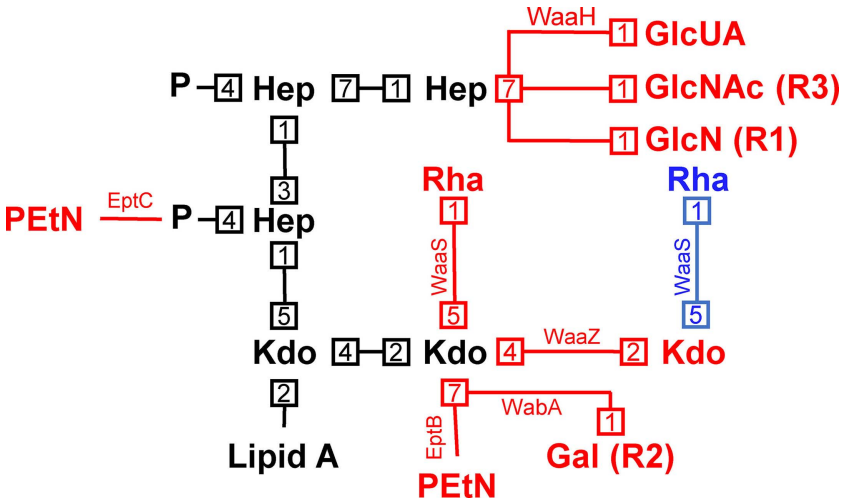

Figure 7 Modifications to the structure of the core oligosaccharide. Shown is the conserved inner core oligosaccharide (and its linkage to lipid A) in black, with potential modifications being indicated in red (although the alternate rhamnose modification by WaaS when the second Kdo is modified with PEtN by EptB is shown in blue). Numbers represent bond positions between sugars. Enzymes mediating modifications are next to each linkage, and, when not associated with *E. coli* K-12, core type associations are listed in parentheses beside the modification.

genes encoding LPS-modifying enzymes. Among them, PagP is an enzyme that resides in the OM and transfers a palmitoyl group to LPS, resulting in heptacylated LPS species that are implicated in CAMP resistance (18, 100, 101). In this transfer, PagP uses the 16-carbon acyl chains of phospholipids that are mislocalized to the outer leaflet of the OM as donors (18, 100, 101). Additionally controlled by PhoQP are LpxR and PagL (the latter is absent in *E. coli*), which also reside in the OM and modify the acyl chain content in lipid A. LpxR and PagL, respectively, mediate the removal of the 3′-acyl groups and the 3-hydroxymyristoyl group, which modulate the immunogenicity of lipid A (102, 103).

The PhoQP system also controls modification to the glucosamine disaccharide portion of lipid A, albeit indirectly through another two-component regulatory system. These modifications are directly regulated by the PmrBA (alternatively BasSR for *E. coli*) two-component system. The PmrBA system is positively regulated by PhoQP through the adapter protein PmrD, which protects the phosphorylated response regulator PmrA (BasR) from dephosphorylation (104–107). In addition, the sensor kinase PmrB (BasS) directly responds to acidic pH and elevated concentrations of certain metals such as Fe^{3+}, Al^{3+}, and Zn^{2+} (12, 107–109). When phosphorylated, PmrA upregulates the covalent modification of lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (PEtN) (18). L-Ara4N modification is mediated by the enzyme ArnT, which transfers L-Ara4N from Und-P to lipid A primarily at the 4′-phosphate, although it can also act on the 1-phosphate (110, 111). PEtN modification is mediated by the enzyme EptA, which transfers PEtN from phosphatidylethanolamine to lipid A primarily at the 1-phosphate, although it can also act on the 4′-phosphate (112, 113). Activation of PmrBA also inhibits modification of lipid A by LpxT, the kinase that adds a second phosphate group to the 1-phosphate from Und-PP to ~33% of LPS molecules under standard laboratory growth conditions (61, 62). Both the addition of positively charged moieties to lipid A by ArnT and EptA and the loss of the negatively charged modification catalyzed by LpxT are associated with increased resistance to CAMPs, presumably due to the masking of negative charges (i.e., phosphates) in LPS to which CAMPs bind (62, 107).

In addition, *Salmonella* can alter lipid A through the action of LpxO, which is absent in *E. coli*. LpxO is not regulated by PhoQP and mediates the oxygen-dependent hydroxylation of the secondary 3′-acyl group immediately after the carboxyl group in the cytoplasm (114, 115).

It is worth noting that, while these are the primary well-characterized modifications to lipid A performed by *Salmonella* and *E. coli* species, other species use similar strategies with alternate modifying groups. For example, certain isolates of *Vibrio cholerae* modify the acyl chains of their lipid A with amino acid moieties (116, 117). Additionally, some *Bordetella* isolates modify the phosphates of their lipid A with glucosamine (118,

119). Both of these modifications promote CAMP resistance, much like many of their *Salmonella* and *E. coli* counterparts.

Modifications to the Core Oligosaccharide

Given that the outer core is relatively variable, well-characterized modifications tend to be confined to the inner core region. Modifications to the core oligosaccharide include the addition of various sugar groups as well as other moieties, such as PEtN. What types of modifications occur will also depend to some extent on the core type. The best characterized modifications will be discussed in detail below, and are also summarized in Fig. 7.

Starting from the lipid A-proximal sugars and working up the sugar chain of the core, the second Kdo residue can be modified with additional Kdo, PEtN, rhamnose, or galactose groups (11, 12). Kdo addition is mediated by the WaaZ transferase. Modification of the second Kdo with PEtN is mediated by EptB and confers resistance to polymyxin B and high levels of Ca^{2+} (12, 120–122). Rhamnose addition is mediated by WaaS, and can occur to either the second Kdo or the third Kdo added by WaaZ, but both activities are dependent on prior addition of the third Kdo (12, 123, 124). When PEtN is present on the second Kdo group, WaaS can only act on the third Kdo (added by WaaZ), whereas it otherwise acts on the second Kdo (12, 124). At least in *E. coli* K-12, these modifications are associated with induction of the envelope stress response effector σ^E (for a review on the function and induction of σ^E, the reader is directed to reference 125), as well as with the truncation of the outer core caused by downregulation of WaaJ (WaaR), which leads to the loss of the terminal glucose and heptose groups (12, 124). In addition, EptB is negatively regulated by PhoQP, but induced by high levels of Ca^{2+} (12, 120, 121). Last, addition of galactose to the second Kdo group is mediated by WabA, which is absent in *E. coli* K-12 and was instead characterized in *E. coli* with the R2 core type (11).

The first heptose of the inner core may be modified with PEtN, specifically at the phosphate added by WaaP, which confers resistance to CAMPs (126). This addition is mediated by EptC, which is induced by PhoBR, a two-component system responsive to phosphate-limiting conditions (126–128). The third heptose of the inner core (added by WaaQ) may be modified with several different glucose derivatives, including GlcNAc, GlcN, and glucuronic acid (GlcUA) (11, 126). The former two modifications were characterized in *E. coli* with the R3 and R1 core types, respectively, whereas the latter is found in *E. coli* K-12 (11, 126). Modification of the third heptose with GlcUA is mediated by the enzyme WaaH, which like EptC, is induced by the PhoBR system (12, 126). Consistent with its response to phosphate-limiting conditions, the GlcUA addition mediated by WaaH proceeds more efficiently in the absence of the phosphate group added by WaaY (12, 126).

O-Antigen Modification

While modifications to basic O-antigen structures have been reported, they will not be discussed in detail here due to the sheer breadth of O-antigen synthesis (13, 129). More striking than minor additions or replacements of individual sugars is that the O antigen may be replaced entirely. In some cases, a serotype of O antigen is replaced by another through the genetic exchange of part or the entire biosynthetic locus resulting from horizontal gene transfer (16, 130). In addition, the O antigen can be replaced altogether by a different type of polysaccharide. Specifically, in *E. coli* K-12, it has been shown that induction of capsular colanic acid synthesis results in replacement of the O antigen in LPS with colanic acid repeats in a WaaL-dependent manner (131, 132). A similar phenomenon has been observed with the enterobacterial common antigen (ECA) (133, 134). Curiously, a missense mutation in *waaL* that broadens the substrate specificity of the ligase led to the discovery that this WaaL variant can modify the LPS core with the peptidoglycan-building block by utilizing lipid II as a donor as well (135).

LPS TRANSPORT

Overview

In 1972, Mary Jane Osborn and collaborators published seminal studies demonstrating that LPS is synthesized at the IM and, therefore, must be transported across the envelope to the OM. Furthermore, the authors demonstrated that this intermembrane transport is unidirectional (7, 8). That body of work opened a new area of research that eventually led to the discovery of the factors required for LPS transport during the 1990s and 2000s. We now know that, as described in the sections above, the core-lipid A and Und-P-linked O-antigen components of the LPS structure are independently synthesized

at the cytoplasmic face of the IM. Using different transporters, these two subunits are separately flipped to the periplasmic leaflet of the IM. Once there, the O antigen can be ligated onto the core oligosaccharide moiety by the WaaL ligase. The resulting newly synthesized LPS molecules must then be extracted from the IM, cross the aqueous periplasm, and traverse the OM to ultimately be assembled at the cell surface. We describe our current knowledge of these steps in LPS transport in the next sections.

Crossing the IM: MsbA

Transport of the core-lipid A molecule from the inner leaflet to the outer leaflet of the IM is mediated by a homodimer of MsbA, a flippase that belongs to the ABC transporter superfamily (Fig. 8) (33, 136–138). MsbA was first characterized as a multicopy suppressor of the loss of LpxL (previously known as HtrB) activity, and was named accordingly (multicopy suppressor of *htrB* A) (136). It would not be until years later that the flippase activity of MsbA was demonstrated, both by the accumulation of LPS in the cytoplasmic side of the IM upon MsbA depletion and the *in vitro* reconstitution of functional MsbA (139, 140). Recent structural studies of MsbA have put forth a model in which LPS directly enters a largely hydrophobic cavity within the MsbA homodimer (141, 142). Interestingly, this cavity is localized in the outer leaflet of the IM, indicating that LPS traverses the IM before being flipped (141, 142). Interactions between this cavity and the lipid A moiety of LPS, together with ATP hydrolysis by MsbA, cause conformational changes in MsbA that lead to the exposure of this cavity to the aqueous periplasmic environment to drive LPS into the outer leaflet of the IM in concert with the closing of the cavity (141). These studies suggest that interaction between positively charged residues within the cavity and the phosphates of lipid A serve as recognition determinants for LPS by MsbA (141, 142). Moreover, the placement of these interactions has been proposed to favor accommodation of shorter acyl chains within the cavity, which may serve to discriminate against flipping of phospholipids (142). It has also been revealed that MsbA's substrate specificity likely drives the requirement for Kdo_2-lipid A as the minimal LPS structure. Flipping of LPS by MsbA is most efficient when the glycolipid contains the late-stage acyl chains, which are added by LpxL and LpxM after the addition of the first two Kdo residues (143–145). The requirement for Kdo_2-lipid A as the minimal LPS structure is thus primarily a reflection of the preference of MsbA for hexacylated substrates (142).

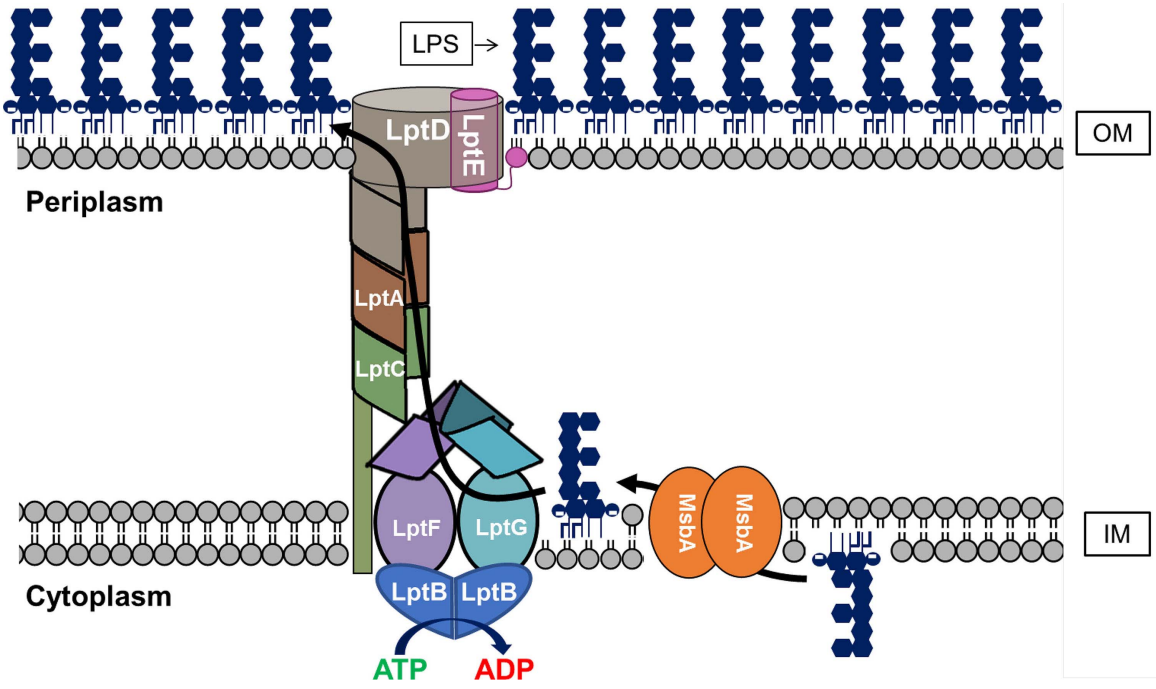

Figure 8 Transport of LPS across the cell envelope. Shown are representations of MsbA, which mediates the transport of core-lipid A across the IM, and the Lpt complex ($LptB_2FGCADE$), which mediates LPS extraction from the IM and its transport through the periplasm and OM. As described in the text, the O antigen can be synthesized on Und-P and transported across the IM by different pathways (Fig. 5). If made, the O antigen is ligated to core-lipid A in the periplasmic leaflet of the IM by WaaL (not shown).

From the IM to the OM: the Lpt System

After its translocation across the IM by MsbA, and possibly undergoing the addition of the O antigen and other chemical modifications (e.g., by the addition of L-Ara4N and PEtN) at the outer leaflet of the IM, LPS must be transported across the periplasm and OM. This transport necessitates the extraction of LPS from the IM, the sheltering of its acyl moieties as it traverses the aqueous periplasm, and, finally, its translocation across the OM to arrive at its final destination in the outer leaflet of the OM. All these processes are mediated by a protein complex, termed the Lpt complex (Fig. 8) (1). The Lpt complex is composed of seven different proteins that span all compartments of the cell: LptB in the cytoplasm; LptF, LptG, and LptC in the IM; LptA in the periplasm; and LptD and LptE in the OM (1, 146). A dimer of LptB proteins together with LptF and LptG form the ABC transporter $LptB_2FG$. LptB constitutes the nucleotide-binding domains (NBDs) that bind and hydrolyze cytoplasmic ATP to power the transport of LPS from the IM to the OM (147, 148). Recent structural studies have revealed that LptF and LptG, the transmembrane domains of the $LptB_2FG$ ABC transporter, form a cavity that is predicted to accommodate LPS during its extraction from the IM (149–151). Whether or not the bitopic IM protein LptC is involved in the extraction process is still somewhat unclear, although it is worth noting that its single transmembrane domain is dispensable for function (152). While the precise mechanism of extraction of LPS from the IM remains to be elucidated, it is known that, eventually, LPS makes its way to the periplasmic domain of LptC (146, 148). The periplasmic domain of LptC consists of a series of antiparallel β-strands arranged to form a β-jellyroll domain that contains a hydrophobic groove that is thought to shelter the acyl chains of LPS during its transit across the periplasm (153, 154). LptA and the periplasmic domain of LptD, LptF, and LptG possess similar β-jellyroll folds. Moreover, the β-jellyroll domains of LptC, LptA, and LptD have been shown to associate in a head-to-tail manner to extend the hydrophobic groove across the periplasm (154–156). These domains in LptCAD are thus thought to form a bridge extending across the periplasm through which LPS can travel to reach the OM. In support of this model, LPS has been covalently cross-linked to each individual member of this bridge, and the *in vitro* reconstitution of LPS transport requires the formation of a bridge composed of the Lpt periplasmic components (148, 157, 158). It is worth noting that, in addition to its ability to associate with LptC and LptD at either end of the bridge, LptA can also form homo-oligomeric complexes (154, 159). As such, it is possible that more than one LptA protein may be incorporated into the Lpt complex. However, the ability of an LptA variant deficient in homo-oligomerization to complement the deletion of the native *lptA* gene in *E. coli* may suggest that one subunit of LptA per complex is sufficient under normal growth conditions (160). Nevertheless, a recent study has demonstrated that, in *Salmonella*, the width of the periplasm (i.e., the distance between the IM and OM) can be modulated by altering the length of Lpp, the OM lipoprotein that covalently tethers the OM to the peptidoglycan layer (161). It is therefore tempting to suggest that the number of LptA proteins per Lpt bridge might change depending on the width of the periplasm.

On arrival at the OM, LPS must be specifically inserted into the outer leaflet, a process mediated by LptD and LptE (146). The membrane-associated portion of LptD is a large, crenellated β-barrel, in which hydrogen bonding between strands is disrupted along the first and last β-strands, creating a crenellation, or small gap (146, 156). LptE is a lipoprotein that resides in the lumen of the barrel portion of LptD (162). Together, LptD and LptE are proposed to form the OM translocon responsible for the insertion of LPS into the outer leaflet of the OM. Based on structural studies, it has been proposed that when LPS arrives at the periplasmic β-jellyroll domain of LptD, its acyl chains are directly deposited into the hydrophobic environment of the OM through a gap that exists between this periplasmic domain and the β-barrel domain of LptD; the crenellation of LptD may then provide for a means by which the barrel might open enough to allow lateral passage of the hydrophilic portion of LPS through the lumen of LptD and out into the OM (156). The role of LptE is unclear, but its high affinity for LPS suggests that it is more than simply a plug for the LptD barrel (163, 164). It has also been demonstrated that LptE plays a critical role in the proper assembly of LptD in the OM (162, 165).

Together, these data have led to the so-called "PEZ Model" of Lpt-mediated LPS transport (146). This model likens LPS to the originally Austrian PEZ candies, and the Lpt transport machinery to the mechanical PEZ candy dispenser invented by Oskar Uxa. Such a dispenser works by way of a spring-loaded platform at the base of the dispenser, which pushes a stack of PEZ candies up through the central channel of the dispenser, so that a candy is always present at the exit of the dispenser and

ready to be taken and consumed by the user. Likewise, this model predicts that LPS travels as a stream of molecules, and that the driving force of LPS transport by the Lpt machinery is derived from the $LptB_2FG$ transporter at the base of the periplasmic channel formed by LptCAD. Accordingly, the $LptB_2FG$ transporter acts much like the spring-loaded platform of a PEZ dispenser by providing constant pressure to the base of the traveling LPS stream by constantly loading new LPS molecules into the channel. Thus, in this model, the channel formed by LptCAD is largely passive in transport, simply providing a compatible route by which LPS molecules might traverse the aqueous periplasm prior to their assembly at the cell surface through the action of the LptDE translocon, which, like the top of the PEZ dispenser, opens up to deliver its cargo. Importantly, the recent *in vitro* reconstitution of LPS transport supports the PEZ model for LPS transport (146, 158). This great technical achievement has demonstrated that transport of LPS can occur in an ATP-dependent manner between IM-like (i.e., containing $LptB_2FGC$) and OM-like (containing LptDE) proteoliposomes only when they are physically connected by soluble LptA.

CONCLUDING REMARKS

Although much is known about the complex structure, synthesis, regulation, and transport of LPS, fundamental questions remain unanswered. In particular, the mechanism of transport for this complex glycolipid, and the regulation of LPS biosynthesis, as well, are highly active fields of investigation. Additionally, as the means for culturing different organisms and characterizing LPS modifications become more sophisticated and available, the various strategies used by Gram-negative bacteria to adapt their cell surfaces to their environment through nonstoichiometric modification of LPS are coming under heavy scrutiny.

In addition, the rather curious fact that LPS is essential in most, but not all, Gram-negative bacteria has recently received considerable attention. Efforts to elucidate why that is have primarily centered around those organisms in which LPS is not essential, which include *Neisseria meningitidis, Acinetobacter baumannii*, and *Moraxella catarrhalis* (35, 166–168). The best studied of these cases is that of *A. baumannii*, for which LPS essentiality has been linked to the activity of the peptidoglycan cell wall polymerase PBP1A (169). However, the precise mechanisms underlying the essentiality of LPS for *A. baumannii*, as well as other organisms, remain to be clarified. For a more in-depth review of the topic, the reader is directed to reference 170.

LPS biogenesis has also received attention as a potential drug target to treat infections caused by Gram-negative bacteria. Inhibitors of LPS biogenesis could kill organisms in which the glycolipid is essential, but they could also render all LPS producers highly permeable to other antibiotics. Several compounds that inhibit LPS biosynthesis and transport have been developed, and some are undergoing clinical trials, but no compound has been approved yet for clinical use (171–174). We can only hope that enhancing our understanding of LPS biogenesis will, in turn, bolster our ability to produce clinically relevant inhibitors, as well as facilitate our understanding of bacterial lifestyles as a whole.

ACKNOWLEDGMENTS

The authors would like to thank other members of the Ruiz laboratory (Sujeet Kumar, Emily Lundstedt, Rebecca Davis, and Brent Simpson) for assistance in editing this manuscript. This work was supported by the National Institute of General Medical Sciences of the National Institutes of Health under award number R01 GM100951 (to N.R.).

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