Lipid A Modification Systems in Gram-Negative Bacteria

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Abstract

The lipid A moiety of lipopolysaccharide forms the outer monolayer of the outer membrane of most gram-negative bacteria. *Escherichia coli* lipid A is synthesized on the cytoplasmic surface of the inner membrane by a conserved pathway of nine constitutive enzymes. Following attachment of the core oligosaccharide, nascent core-lipid A is flipped to the outer surface of the inner membrane by the ABC transporter MsbA, where the O-antigen polymer is attached. Diverse covalent modifications of the lipid A moiety may occur during its transit from the outer surface of the inner membrane to the outer membrane. Lipid A modification enzymes are reporters for lipopolysaccharide trafficking within the bacterial envelope. Modification systems are variable and often regulated by environmental conditions. Although not required for growth, the modification enzymes modulate virulence of some gram-negative pathogens. Heterologous expression of lipid A modification enzymes may enable the development of new vaccines.

Contents

INTRODUCTION

Lipid A (endotoxin), the hydrophobic moiety of lipopolysaccharide (LPS), is a glucosamine-based saccharolipid (1) that makes up the outer monolayer of the outer membranes of most gram-negative bacteria (2–4). The term lipid A is also used to describe the lipid product released by mild-acid hydrolysis of LPS, which selectively cleaves the Kdo-lipid A linkage (2–4). There are approximately 10^6 lipid A residues, 10^7 phospholipids, and 10^5 undecaprenyl phosphate-sugar molecules in an *Escherichia coli* cell (5, 6). With a few exceptions (7, 8), considered further below, the lipid A and Kdo domains of LPS (**Figures 1** and **2**) are required for growth (5, 9, 10). In wild-type strains, additional core and O-antigen sugars are present (**Figure 1**) (2, 3). These complex glycoforms are not needed for growth but protect bacteria from antibiotics and complement-mediated lysis. The core and O-antigen domains are required for virulence and consequently are present in most clinical and environmental isolates (3). The structures and biosynthesis of core and O-antigen sugars are reviewed elsewhere (2, 3). Here, we focus on the biosynthesis of Kdo_2-lipid A and its modification during transport to the outer membrane. The mechanisms of LPS transport are covered in greater detail by Tommassen and coworkers (10a).

The Innate Immune Response to Lipid A

Most gram-negative bacteria synthesize Kdo_2-lipid A molecules resembling those made by *E. coli* (**Figure 2**) (2, 3). The lipid A moiety of LPS is detected by the TLR4/MD2 receptor of the mammalian innate immune system (11–17). Picomolar levels of lipid A induce macrophages to synthesize potent mediators of inflammation, such as TNF-α and IL-1β (18, 19). Lipid A furthermore activates the production of costimulatory molecules required for adaptive immunity (20, 21). With mononuclear and endothelial cells, lipid A stimulates tissue factor production (22, 23). All these events are desirable for clearing local infections. When overproduced systemically during sepsis, however, the inflammation caused by some of these proteins damages small blood vessels and can precipitate gram-negative septic shock (24, 25). LPS, or

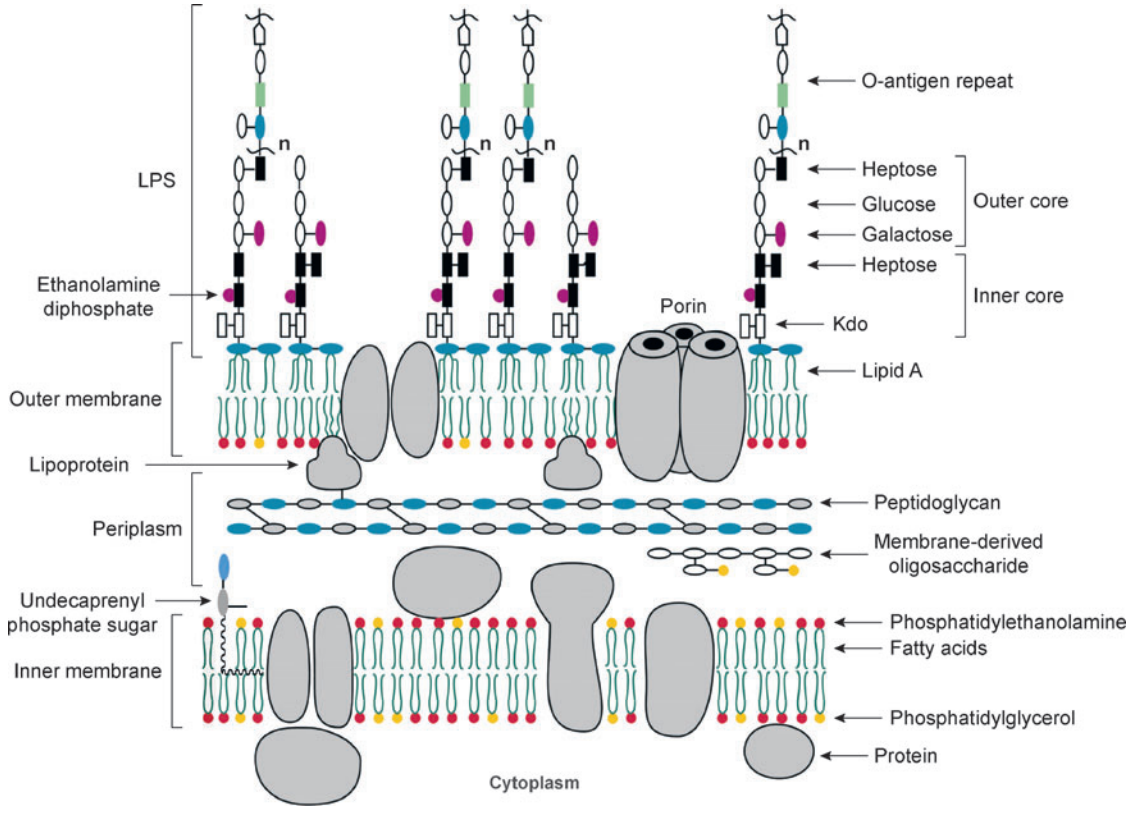

Figure 1

Schematic structure of the *E. coli* K-12 cell envelope. The structure and biosynthesis of LPS (2, 3), peptidoglycan (186), membrane-derived oligosaccharides (239, 240), lipoproteins (241), and phospholipids (242, 243) are reviewed elsewhere. Strains of *E. coli* K-12 normally do not make O-antigen ($n \sim 1 - 50$), unless a mutation in the O-antigen operon is corrected (244). The Kdo_2-lipid A substructure of LPS (the topic of this review) usually represents the minimal substructure required for growth of gram-negative bacteria. Exceptions include some spirochetes and strains of *Sphinogmonas*, in which the Kdo_2-lipid A biosynthesis genes are absent, and *Neisseria meningitidis* in which *lpxA* knockouts lacking LPS are viable (142). If the ABC transporter MsbA (the inner membrane flippase for LPS) is overexpressed, *E. coli* can grow without Kdo (8). These strains still make the tetra-acylated precursor lipid IV_A, which is nevertheless required for growth (8). The phospholipids represent phosphatidylethanolamine (*red*) and phosphatidylglycerol (*yellow*). Abbreviations: Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LPS, lipopolysaccharide; heptose, L-*glycero*-D-*manno*-heptose.

even synthetic *E. coli* lipid A by itself, causes a similar pathology when injected into animals (26–28), supporting its proposed role in sepsis. The characteristic structural features of *E. coli* lipid A (**Figure 2**), especially its two phosphate and acyloxyacyl groups, are needed to trigger full TLR4/MD2 activation in human cells (26). However, partial activation of TLR4/MD2 by certain lipid A substructures and analogues results in the production of an altered cytokine profile that retains the beneficial adjuvant effects of endotoxin but minimizes animal toxicity (29–31). Some lipid A analogues (usually containing fewer acyl chains) are potent TLR4/MD2 antagonists (16, 32–35), with potential utility as human therapeutics (36). A crystal structure of TLR4/MD2 with a bound lipid A molecule or lipid A analogue is not yet available to clarify the mechanism

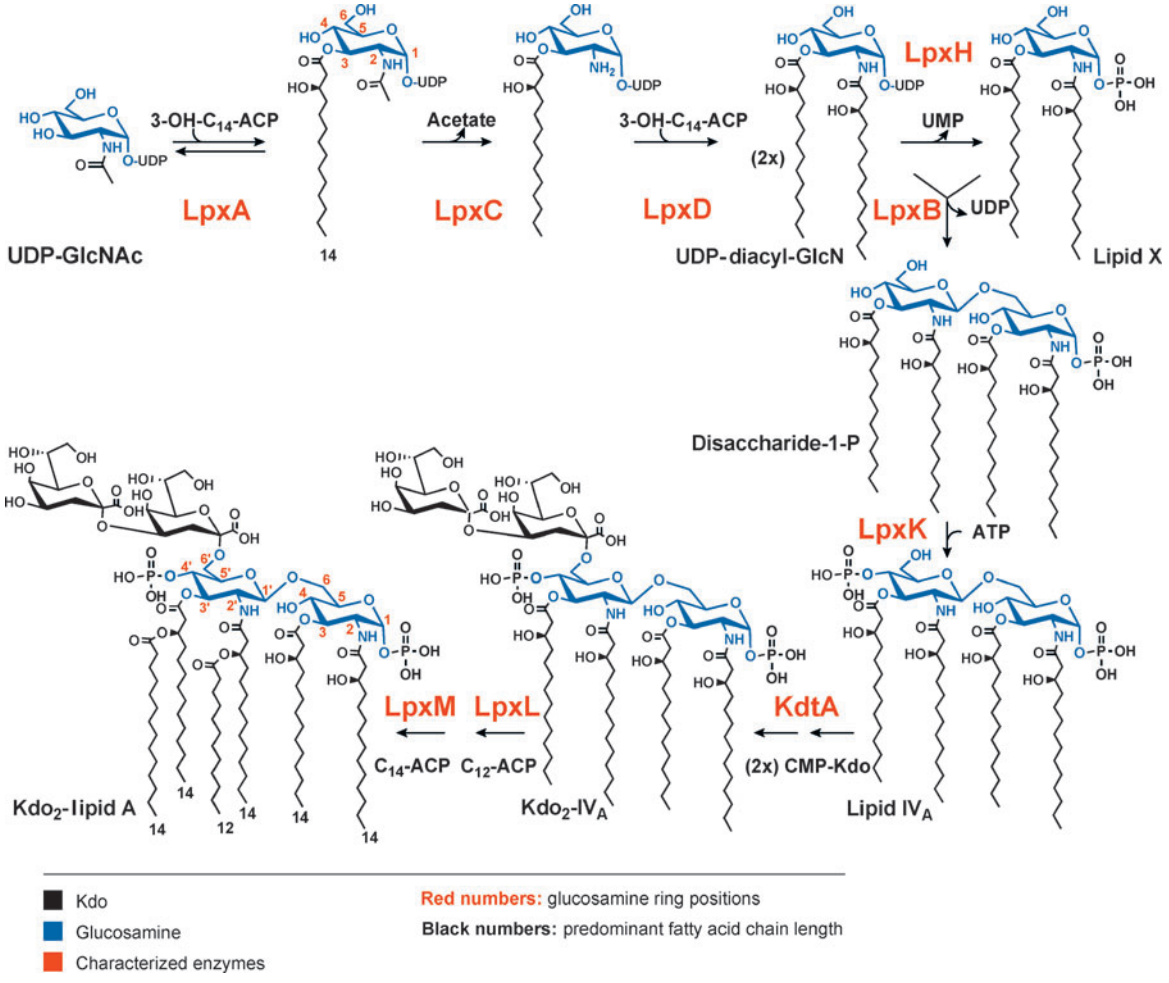

Figure 2

Constitutive pathway for biosynthesis of the Kdo_2-lipid A portion of LPS in *E. coli* K-12. Each enzyme of the constitutive Kdo_2-lipid A pathway is encoded by a single structural gene (2, 69). The glucosamine disaccharide backbone of lipid A and the Kdo disaccharide are shown. LpxA, -C, and -D are soluble cytoplasmic proteins, whereas LpxH and -B are peripheral membrane proteins (2). The distal enzymes of the pathway, starting with LpxK, are integral inner membrane proteins, the active sites of which face the cytoplasm (2). The red numbers specify the glucosamine ring positions of lipid A and its precursors. The black numbers indicate the predominant fatty acid chain lengths found in *E. coli* lipid A. The single molecular species shown at the bottom left represents about 90% of the total lipid A in *E. coli*, with most of the rest bearing a C12 secondary acyl chain at position 3′ (152). Additional minor acyl chain variants can be detected by high-resolution mass spectrometry (245).

of transmembrane signaling (14). However, the crystal structure of the extracellular domain of TLR3 (a TLR4 orthologue that is activated by double-stranded RNA) has recently been reported (37–39).

Discovery and Overview of Kdo_2-Lipid A Biosynthesis

The Kdo_2-lipid A biosynthetic pathway may be viewed as having a conserved and a variable component. The conserved (constitutive)

enzymes (**Figure 2**) are intracellular, present in virtually all gram-negative bacteria, and not generally subject to regulation (2, 40). In contrast, the lipid A modification enzymes, discussed below, are mostly extracytoplasmic and vary from organism to organism. In many instances, the lipid A modification systems are induced or repressed by growth conditions, such as changes in pH, divalent cation concentrations, or the presence of antimicrobial peptides (41–45). Most modification enzymes reside either on the periplasmic surface of the inner membrane or in the outer membrane (46–54). They are excellent markers for following the translocation of nascent LPS from its initial site of biosynthesis on the inner surface of the inner membrane to the outer surface of the outer membrane (55–60) (**Figure 1**).

The systematic elucidation of the constitutive pathway for Kdo_2-lipid A biosynthesis (**Figure 2**) was enabled by the discovery of 2,3-diacylglucosamine 1-phosphate (lipid X) (61, 62), a substance that had been overlooked in earlier work on *E. coli* lipids because it is present at very low levels in wild-type cells (6, 63). However, it accumulates as much as 500-fold, or to about 5% to 10% of the total lipid, in certain kinds of phosphatidylglycerol-deficient mutants (61, 62). The discovery of lipid X (61, 62) coincided with the correct structure determination (64, 65) and chemical synthesis of lipid A (66). Recognition of the existence of an acylated monosaccharide (62) representing a precursor to the proximal (right) subunit of lipid A (**Figure 2**) greatly facilitated the development of testable hypotheses regarding the origin of Kdo_2-lipid A from known lipids and carbohydrates present in *E. coli* (63, 67, 68).

THE CONSTITUTIVE ENZYMATIC PATHWAY OF KDO_2-LIPID A BIOSYNTHESIS

The nine enzymes of the constitutive Kdo_2-lipid A pathway and the single-copy genes encoding them (**Figure 2**) are conserved in most gram-negative bacteria such as *E. coli* (2, 69). The Sphingomonads, which make bioactive sphingolipids instead of Kdo_2-lipid A, are some of the exceptions (70). The sequences of the Kdo_2-lipid A genes are easily recognized when gram-negative genomes are compared (71). LpxA, -C, and -D are soluble proteins (72–74), whereas LpxB and LpxH are peripheral membrane proteins (75–77). LpxK, KdtA, LpxL, and LpxM are integral inner membrane proteins (78–82). Their active sites are presumed to face the cytoplasmic surface of the inner membrane, given that their water-soluble cosubstrates are cytoplasmic molecules (**Figure 2**). Interestingly, higher plants, e.g., *Arabidopsis thaliana*, encode significant orthologues of the constitutive enzymes within their nuclear genomes (2, 83), but lipid A-like molecules have not yet been identified in plants by mass spectrometry or NMR spectroscopy (84).

Fatty Acylation of UDP-GlcNAc

The first step of Kdo_2-lipid A biosynthesis, the fatty acylation of UDP-GlcNAc (**Figure 2**) (67, 85), is catalyzed by LpxA. *E. coli* LpxA requires the thioester *R*-3-hydroxymyristoyl acyl carrier protein (ACP) as its donor substrate (72, 85). It does not recognize *R*-3-hydroxymyristoyl-coenzyme A. The active site of *E. coli* LpxA functions as a precise hydrocarbon ruler that incorporates C14 hydroxyacyl chains two orders of magnitude faster than C12 or C16 chains (86, 87), consistent with the structure of *E. coli* lipid A (**Figure 2**). In *Pseudomonas aeruginosa*, the LpxA ruler is reset to incorporate C10 chains (86, 87), whereas in *Neisseria meningitidis* and *Leptospira interrogans*, it measures C12 chains (88, 89). Strains of *E. coli* in which *P. aeruginosa lpxA* replaces *E. coli lpxA* synthesize hybrid lipid A molecules in which C10 acyl chains are incorporated at positions 3 and 3′ (**Figure 2**, red numbers). The rest of the lipid A molecule is unchanged (90). Single amino acid substitutions can switch *P. aeruginosa* LpxA to a C14- or *E. coli* LpxA to a C10-selective enzyme (87).

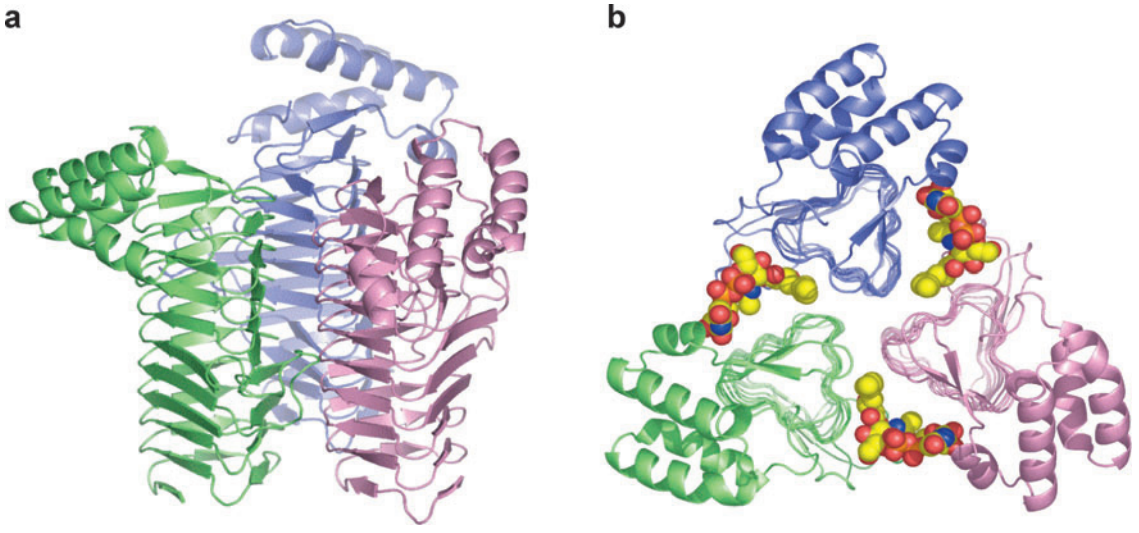

Figure 3

Structure of free LpxA and of LpxA with bound UDP-(3-*O*-acyl)-GlcNAc. The LpxA homotrimer was solved at 2.6 Å (Protein Data Bank code 1LXA) in the absence of bound ligands (91). Each subunit has its own color. The side view (*a*) highlights the β-helix domain (91). The LpxA homotrimer was cocrystallized with a ~25-fold molar excess of UDP-3-*O*-(*R*-3-hydroxydecanoyl)-GlcNAc and solved at 1.8 Å (A. H. Williams & C.R.H. Raetz, in preparation). The top-down view of this complex (*b*) reveals the location of the active site and the positioning of the acyl chain, consistent with previous proposals on the basis of site-directed mutagenesis and NMR studies (94, 246).

The crystal structure of LpxA (91–93) reveals that it is a homotrimer (**Figure 3**), constructed around multiple contiguous hexad repeats. These motifs specify a unique secondary structure consisting of a left-handed helix of short parallel β-sheets. All hexad repeat-containing proteins studied to date are helical homotrimers. Three hexads (18 amino acids) form one coil of the β-helix (91) (**Figure 3**). The three identical active sites of LpxA, which were first proposed on the basis of site-directed mutagenesis, are located at the subunit interfaces (93, 94). A recent X-ray structure of *E. coli* LpxA with bound UDP-3-*O*-(*R*-3-hydroxydecanoyl)-GlcNAc (**Figure 3*b***), a slow substrate in the reverse direction (94), has recently been solved at 1.8 Å (A. H. Williams & C.R.H. Raetz, in preparation). In addition to validating the proposed locations of the LpxA active sites (94), these studies provide a structural explanation for the extraordinary chain length selectivity of these enzymes.

An Analogue of UDP-GlcNAc in Which NH_2 Replaces the GlcNAc 3-OH Group

Many bacteria (94a), including *L. interrogans* and *Acidithiobacillus ferrooxidans*, contain a dehydrogenase (GnnA) and a transaminase (GnnB) that convert UDP-GlcNAc to the analogue UDP-GlcNAc3N, in which the GlcNAc 3-OH group is replaced with an amine (**Figure 4**) (89, 95). LpxA of *L. interrogans*, which is absolutely selective for a C12 chain (89, 95) (**Figure 4**), acylates UDP-GlcNAc3N but not UDP-GlcNAc (89, 95). This remarkable selectivity accounts for the fact that *L. interrogans* lipid A molecules contain four *N*-linked hydroxyacyl chains (96), as the rest of the pathway is conserved (not shown in **Figure 4**). A crystal structure of *L. interrogans* LpxA has recently been solved at 2.2 Å (A. H. Williams & C.R.H. Raetz, in preparation); this structural data should help elucidate the mechanism by which the

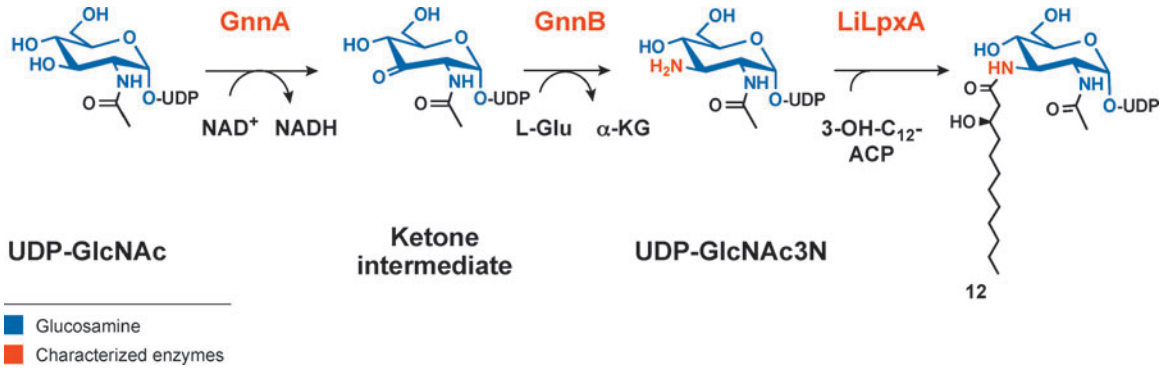

Figure 4

Biosynthesis and acylation of UDP-GlcNAc3N in *L. interrogans*. The sugar nucleotide UDP-GlcNAc3N is synthesized in two reactions from UDP-GlcNAc. The intermediate ketone has not yet been characterized, but UDP-GlcNAc3N generated in vitro by GnnA and GnnB can be isolated in milligram quantities (89, 95). LpxA from *L. interrogans* is 41% identical to *E. coli* LpxA at the protein level, and its X-ray structure has recently been determined (A. H. Williams & C.R.H. Raetz, in preparation). *L. interrogans* LpxA does not catalyze the acylation of UDP-GlcNAc and is absolutely selective for C12 hydroxyacyl chains (89, 95).

L. interrogans enzyme differentiates sugar nucleotides. *A. ferrooxidans* LpxA can utilize both UDP-GlcNAc and UDP-GlcNAc3N for Kdo_2-lipid A biosynthesis, resulting in lipid A mixtures containing two, three, or four *N*-linked acyl chains (89). Like *A. ferrooxidans* LpxA, *E. coli* LpxA cannot discriminate between UDP-GlcNAc and UDP-GlcNAc3N (89, 95), but the latter substrate is not available in *E. coli*. The presence of additional *N*-linked acyl chains may increase the stability of lipid A in some bacteria to acid or base hydrolysis—or may prevent its degradation by lipases.

Deacetylation of UDP-3-*O*-(acyl)-GlcNAc

The equilibrium constant (~0.01) for UDP-GlcNAc acylation by *E. coli* LpxA is unfavorable (72, 94). Thus, the deacetylation of UDP-3-*O*-(acyl)-GlcNAc by LpxC is the actual committed step of Kdo_2-lipid A biosynthesis (74, 97). LpxC is a Zn^{2+}-dependent enzyme that is highly conserved in all gram-negative bacteria (98, 99). It displays no sequence similarity to other deacetylases or amidases. It is an excellent target for the development of novel antibiotics (10, 100, 101). Slow, tight-binding inhibitors of LpxC with low nM affinity have recently been reported (**Figure 5*a***). These compounds are *N*-aroyl-L-threonine hydroxamates (**Figure 5*a***). They possess antibiotic activity comparable to ciprofloxacin (102). The hydroxamate group presumably binds to the catalytic Zn^{2+} ion in a stereospecific manner. The recent X-ray (103–106) and NMR (107, 108) structures (**Figure 6**) of LpxC with the bound substrate-mimetic hydroxamate inhibitor TU-514 (**Figure 5*b***), the fatty acyl chain of which occupies a hydrophobic tunnel leading away from the LpxC active site (**Figure 6**), may facilitate the design of inhibitors with even greater antibiotic activity. Clinical applications would include the treatment of cystic fibrosis patients infected with multidrug-resistant *P. aeruginosa*.

LpxC levels increase five- to tenfold in cells treated with sublethal doses of LpxC inhibitors (109). Induction is not associated with increased transcription (109) but may be due to reduced LpxC turnover when the early steps of Kdo_2-lipid A biosynthesis are curtailed. LpxC induction is also seen in temperature-sensitive LpxA mutants in the absence of LpxC inhibitors (109). Although the signaling mechanisms controlling

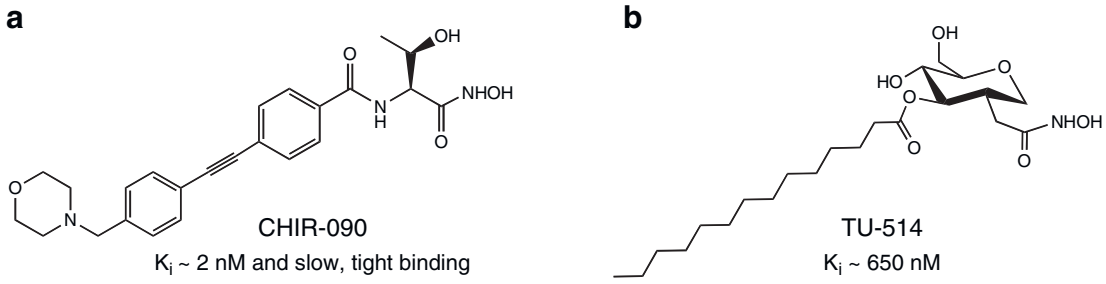

Figure 5

Structures of LpxC inhibitors CHIR-090 and TU-514. (*a*) The slow, tight-binding inhibitor CHIR-090 inhibits diverse LpxC orthologues in the low nM range and displays potent antibiotic activity against many gram-negative bacteria (102). (*b*) The substrate mimetic TU-514 inhibits *E. coli* LpxC competitively with $K_i \sim 650$ nM but has little or no antibiotic activity (100).

LpxC induction are unknown, two amino acids at the C terminus of LpxC are critical for this regulation (110). The FtsH protease is partially responsible for regulating LpxC turnover in vivo (111), but additional processes cannot yet be excluded.

Following deacetylation, a second *R*-3-hydroxymyristate chain is added by LpxD to make UDP-2,3-diacyl-GlcN (**Figure 2**) (73). The X-ray structure of LpxD shows that it, like LpxA, is a homotrimer constructed around multiple contiguous hexad repeats (111a).

Formation of the Lipid A Disaccharide

The pyrophosphate linkage of UDP-2,3-diacyl-GlcN is cleaved by LpxH, which catalyzes the attack of water on the α-phosphorus

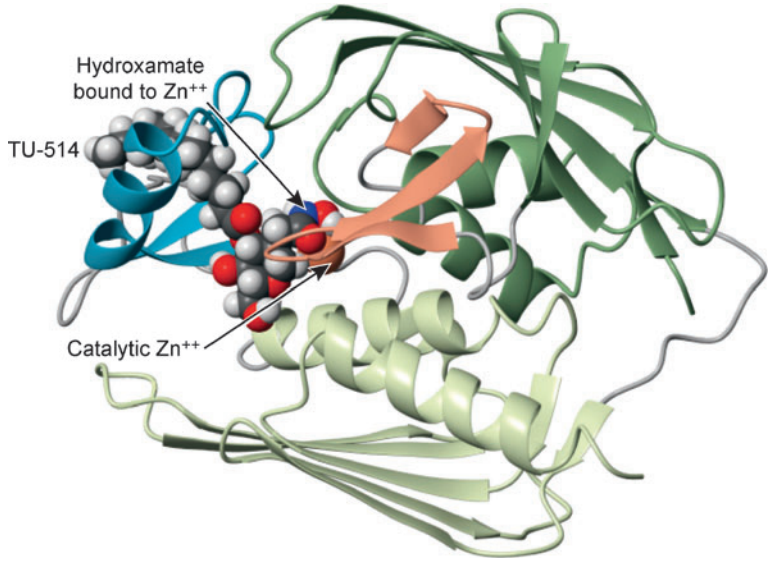

Figure 6

NMR structure of LpxC with bound substrate-mimetic inhibitor TU-514. This ribbon diagram is based on the NMR studies of Coggins et al. (107, 108). The recent crystal structure of the same complex is similar, except for slight differences in the orientation of the tetrahydropyran ring (105).

atom of the UDP moiety to form 2,3-diacyl-GlcN-1-phosphate (lipid X) (76, 77) and UMP (**Figure 2**). LpxH is unusual in that it is missing in about one third of the gram-negative genomes. An alternative pyrophosphatase of this kind must exist in these strains because all of them contain LpxD and LpxB (**Figure 2**), but the relevant gene has not been identified. LpxH is a peripheral membrane protein that is stimulated in vitro by Mn^{2+}. This enzyme is distantly related to the phosphoprotein phosphatase family. Its structure is not yet available.

The β,1′-6-linked disaccharide that is characteristic of all lipid A molecules is generated by LpxB, which condenses UDP-2,3-diacyl-GlcN with lipid X (68, 75) and releases UDP (**Figure 2**). Like LpxH, LpxB is a peripheral membrane protein. It is a member of a unique family of glycosyltransferases, some of which are distantly related to MurG (112), as judged by PSI-BLAST analysis (113). LpxB is very useful for the chemoenzymatic synthesis of Kdo_2-lipid A analogues (114, 115). Its crystal structure has not been reported. A second LpxB orthologue of unknown function is present in strains of *Legionella*, where it is required for growth inside of *Acanthamoeba* (116).

Kdo Incorporation and Secondary Acylation

The integral inner membrane proteins LpxK (78, 117), KdtA (WaaA) (118, 119), LpxL (HtrB), and LpxM (MsbB) (79, 80, 82, 120) catalyze the last four steps of the constitutive pathway in *E. coli* (**Figure 2**). Each protein contains one predicted membrane-spanning segment at its N terminus. The active sites likely face the cytoplasm. LpxK phosphorylates the 4′-position of the disaccharide 1-phosphate generated by LpxB (**Figure 2**) to form lipid IV_A (121). This important precursor is an excellent endotoxin antagonist in human cells but an agonist of reduced potency in the mouse (33). This unusual pharmacology is determined by the source of the TLR4/MD2 complex (14, 122–124). Hexa-acylated lipid A dimerizes human TLR4/MD2, whereas lipid IV_A does not (14, 122–124).

Next, two Kdo residues are incorporated by the bifunctional enzyme KdtA (WaaA) (118, 119, 125). The labile sugar nucleotide CMP-Kdo is the Kdo donor (40, 126, 127). The second Kdo unit is incorporated much more rapidly than the first, and therefore the intermediate with a single Kdo residue does not accumulate (**Figure 2**). However, in *Hemophilus influenzae*, *Vibrio cholerae*, *Bordetella pertussis*, and several other organisms, KdtA incorporates only one Kdo residue (128). A special kinase (KdkA), unique to these bacteria (128, 129), then incorporates a phosphate group at the same position where the outer Kdo residue is added by *E. coli* KdtA (**Figure 2**). *Hemophilus kdtA* and *kdkA* in combination can functionally complement a knockout mutation of the *E. coli kdtA* gene (130). When the heptose region of the core is intact, however, a monofunctional Kdo transferase can rescue a KdtA deletion mutant (131). KdtA of *Chlamydia trachomatis* incorporates at least three Kdo residues and can also functionally substitute for *E. coli* KdtA (9, 132).

The last steps of *E. coli* Kdo_2-lipid A biosynthesis involve the addition of the secondary lauroyl and myristoyl residues to the distal glucosamine unit (**Figure 2**) (120) by LpxL and LpxM, which require the Kdo disaccharide moiety in their substrates for activity (82, 120). LpxL and LpxM prefer acyl-ACP donors but can also function with acyl-coenzyme A substrates (D.A. Six & C.R.H. Raetz, in preparation). LpxL and LpxM display significant sequence similarity to each other (79, 80) but not to LpxA or LpxD. LpxL and LpxM are distantly related to the lysophosphatidic acid acyltransferase family (D.A. Six & C.R.H. Raetz, in preparation). The *lpxM* gene is not required for growth in *E. coli* (82, 133, 133a). *Salmonella typhimurium* and *Shigella lpxM* mutants are attenuated in their ability to cause inflammation (134–136). Outer membrane permeability and growth

defects observed in *S. typhimurium lpxM* mutants have been found to select for extragenic suppressor muations in genes unique to *S. typhimurium* (133a). These phenotypes and suppression phenomena are not seen in *E. coli* K-12 *lpxM* mutants. Both *lpxL* and *lpxM* can be deleted in *E. coli*, provided the cells are grown on minimal medium or at low temperature (82). Tetra-acylated core-lipid A, which accumulates in *lpxL* mutants, is not rapidly transported from its site of biosynthesis on the inner surface of the inner membrane to the outer membrane (82).

The *E. coli* chromosome encodes an additional gene homologous to *lpxL*, termed *lpxP*, which is expressed at low temperature (12°C) (81, 137). LpxP incorporates palmitoleate in place of laurate (not shown in **Figure 2**), perhaps reflecting the need to adjust outer membrane fluidity (81, 137). In *Yersinia pestis*, *lpxL* is missing, but *lpxM* and *lpxP* are present (138). Consequently, *Y. pestis* synthesizes tetra-acylated Kdo_2-lipid A at 37°C but makes hexa-acylated Kdo_2-lipid A at 25°C (138). As noted above, tetra-acylated lipid A is a relatively weak TLR4/MD2 agonist in the mouse and an antagonist in humans (138). Introduction of the *E. coli lpxL* gene into *Y. pestis* permits the synthesis of hexa-acylated Kdo_2-lipid A (a potent TLR4/MD2 agonist) at all temperatures (138). Such constructs are fully attenuated in a mouse infection model and provide immunity against a subsequent challenge with wild-type *Y. pestis* (138).

Why Kdo_2-lipid A is essential for growth in most gram-negative bacteria remains uncertain. It may be required for the proper folding of some outer membrane proteins (139, 140). Recently, Nishiyama et al. (141) have reported a lipid A-like factor required for signal recognition particle/SecYEG-dependent and –independent membrane protein integration in *E. coli*. Because mass spectrometry and NMR spectroscopy were not used for structural analysis, the chemical nature of this factor and its identification as lipid A-related remain in question (141).

N. meningitidis is unusual in that its *lpxA* gene can be inactivated (142); such mutants grow slowly without Kdo_2-lipid A but nevertheless can assemble a functional outer membrane, albeit missing some lipoproteins (7).

EXPORT OF NEWLY SYNTHESIZED LPS AND PHOSPHOLIPIDS

How *E. coli* lipids cross the inner membrane and are transported to the outer membrane (**Figure 7**) is not fully understood (55, 143). A clue to bacterial lipid transport emerged from studies of *lpxL* mutants (**Figure 2**) and their suppression by multiple copies of *msbA* (144–146). LpxL is the lauroyl transferase of lipid A biosynthesis (**Figure 2**) (79). LPS with tetra-acylated lipid A accumulates in inner membranes of *lpxL* mutants at 42°C, and growth on broth is inhibited (146). MsbA is an essential ABC transporter (**Figure 7**), closely related to eucaryotic Mdr proteins (144). MsbA overexpression restores the growth of *lpxL* mutants at 42°C without restoring laurate addition, resulting in export of LPS with tetra-acylated lipid A to the outer membrane (146). *E. coli msbA* knockouts are not viable (144), but their analysis is complicated by two factors. First, long times (4–8 h) are needed to dilute out preexisting MsbA supplied in *trans* from a temperature-sensitive plasmid (146). Second, the *lpxK* gene (**Figure 2**), which is immediately downstream in an operon with *msbA*, is also essential for growth (117, 146).

To gain a clearer understanding of MsbA function, a temperature-sensitive point mutant of *E. coli* (WD2) was isolated in which there is a single A270T substitution in the fifth predicted membrane-spanning segment (143), located near the proposed MsbA dimer interface on the periplasmic side of the inner membrane (147–149). This mutant protein is rapidly inactivated at 44°C. Export of all major lipids (both LPS and phospholipids) to the outer membrane is inhibited by ~90% in WD2 after 30 min at 44°C (143), as judged by pulse-labeling studies.

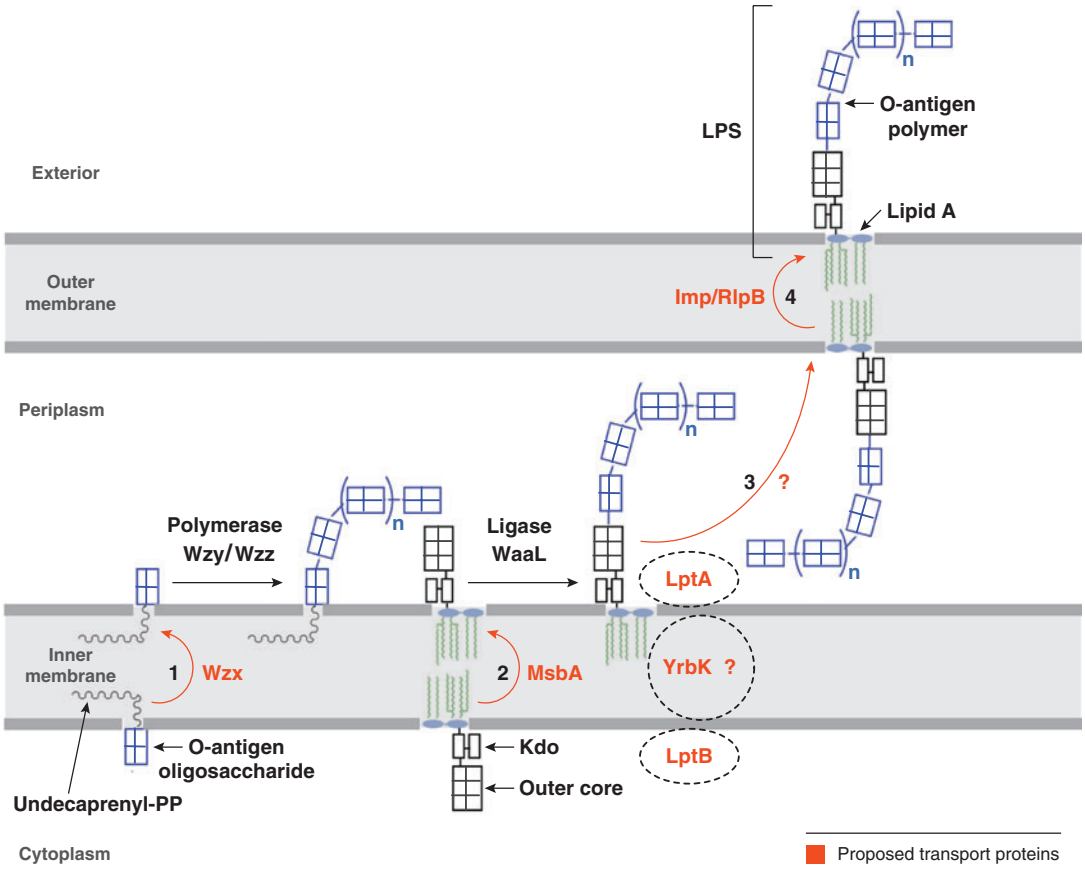

Figure 7

Export of nascent core-lipid A and O-antigen precursors, and the assembly of lipopolysaccharide (LPS) in *E. coli* K-12. The proteins are involved in the export of LPS. The ABC transporter MsbA flips newly synthesized core-lipid A to the outer surface of the inner membrane (55, 143). O-antigen is assembled separately on undecaprenyl diphosphate and is flipped by the putative transporter Wzx (247). O-antigen oligosaccharides are polymerized on the periplasmic surface of the inner membrane by Wzy and Wzz and then transferred to nascent core-lipid A by WaaL (2). In vitro systems for the polymerase and ligase have not been reported. The periplasmic protein LptA (159), which is part of an ABC transporter that might include LptB and YrbK (159), may somehow shuttle LPS from the outer surface of the inner membrane to the inner surface of the outer membrane. Here, the essential outer membrane protein complex Imp/RlpB is required for the assembly of LPS into the outer surface of the outer membrane, as judged by lipid A accessibility to the ectoenzymes PagL or PagP (56, 60, 197, 209). With the exception of the lipid-activated ATPase activity of MsbA (154), no in vitro assays have been developed for any of the proposed transporters.

Kdo_2-lipid A biosynthesis, phospholipid biosynthesis, and export of major outer membrane proteins are not immediately affected (143). However, minor protein species have not been examined (143). The cells do not undergo rapid lysis, suggesting that peptidoglycan assembly is not inhibited (143). Electron microscopy reveals inner membrane invaginations in WD2 at 44°C, consistent with increased surface area secondary to a selective block of lipid export (143). However, the covalent modifications of newly synthesized Kdo_2-lipid A with 4-amino-4-deoxy-L-arabinose (L-Ara4N) or phosphoethanolamine moieties

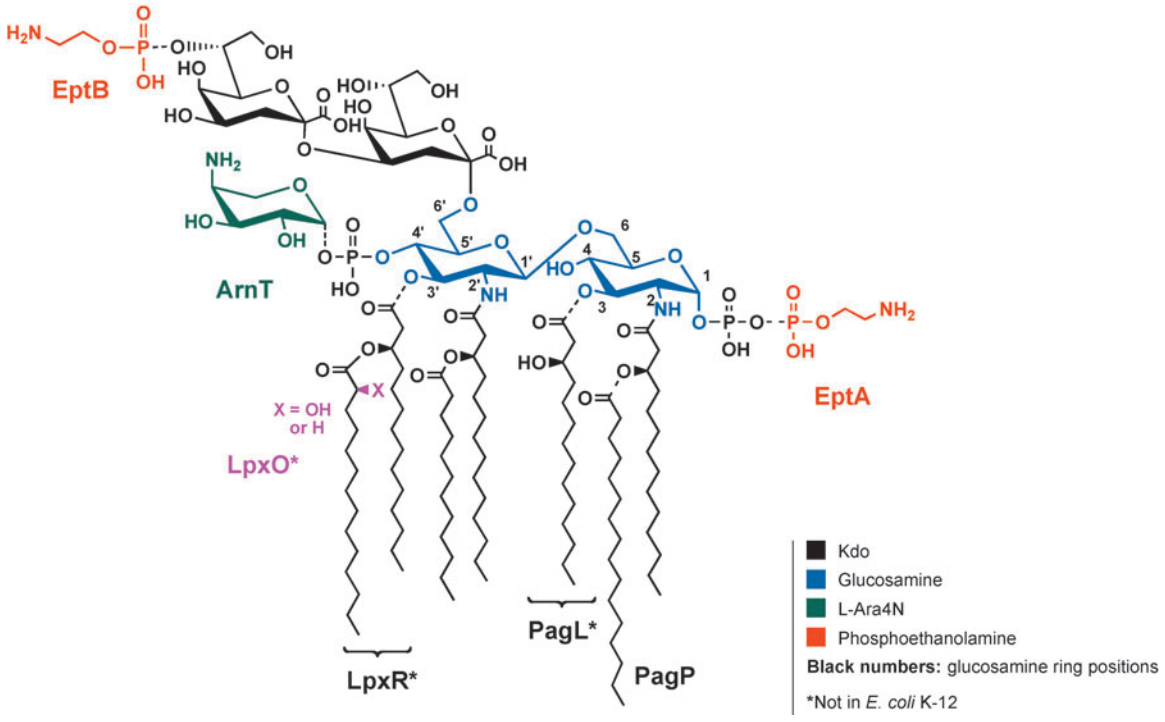

Figure 8

Covalent modifications of Kdo_2-lipid A in *E. coli* K-12 and *Salmonella*. The known covalent modifications of Kdo_2-lipid A (3) are indicated by the substituents with the dashed bonds. Under some conditions, the positions of the phosphoethanolamine and L-Ara4N substituents are reversed (not shown) (248, 249). Lipid A species with two phosphoethanolamine units or two L-Ara4N moieties may also be present (44). Expression of the enzymes ArnT (47) and EptA(PmrC) is under the control of PmrA/B (50, 52). PagP and PagL are regulated by PhoP/Q (46, 47). LpxO, LpxR, and EptB are not regulated by either PhoP/Q or PmrA/B (44, 53, 54). Asterisks indicate modification enzymes not found in *E. coli* K-12. Transfer of the *Salmonella* genes encoding these enzymes to *E. coli* results in the expected lipid A modifications. When grown with high concentrations of divalent cations, both *E. coli* and *Salmonella* make a subset of lipid A species with a diphosphate group at the 1-position (not shown), which is generated by the enzyme YeiU (M.S. Trent, in preparation).

(**Figure 8**), which occur on the outer surface of the inner membrane, are inhibited at 44°C in temperature-sensitive MsbA mutants (55), consistent with the idea that MsbA is the flippase for LPS (**Figure 7**).

Several recent X-ray structures of the homodimeric MsbA protein at 4.2–4.5 Å (147–149) support the proposed flippase function of MsbA (143) and suggest the existence of multiple conformational states. However, the relatively low resolution of these structures (147–149), compared with those of other ABC transporters, has hampered structural interpretation and raised serious issues regarding the published conformations of the MsbA protein (150, 151). An improved, high-resolution structure of MsbA, preferably with a well-defined ligand such as Kdo_2-lipid A (152), would greatly facilitate further mechanistic studies of MsbA.

Studies of MsbA-mediated LPS flip-flop in *E. coli* membrane vesicles or in purified, reconstituted systems have not been reported. Why phospholipid transport to the outer

membrane is blocked in *msbA* mutants is also unclear (143). There is evidence that phospholipid flip-flop is not ATP dependent in bacterial inner membrane vesicles (153). Although purified MsbA is a lipid-activated ATPase (154), phospholipid flip-flop could not be demonstrated in liposomes containing MsbA alone (155). MsbA may indeed be a specific flippase primarily for LPS, or inhibition of phospholipid export in *msbA* mutants (143) might be secondary to LPS accumulation. Alternatively, other proteins may be needed together with MsbA to catalyze phospholipid and LPS flip-flop. In some marine bacteria, MsbA is fused via its C terminus to LpxK (156).

Recently, *msbA* has been identified as a multicopy suppressor of Kdo-deficient mutants of *E. coli* (8), including strains with deletions in the Kdo transferase (**Figure 2**) (9) (C.M. Reynolds & C.R.H. Raetz, in preparation). Additional uncharacterized suppressor mutations and multicopy suppressors have also been reported (8); their analysis should provide exciting new insights into lipid A trafficking and function. In Kdo-deficient strains harboring the appropriate suppressor, lipid IV_A is sufficient for outer membrane biogenesis and cell viability (8), but the cells are still sensitive to the LpxC inhibitor CHIR-090 (C.M. Reynolds & C.R.H. Raetz, in preparation). The outer membrane protein profile of Kdo-deficient strains is remarkably similar to that observed with wild-type *E. coli* (8). It appears that MsbA overproduction can overcome the transport defect associated with both underacylated (146) and Kdo-deficient (157) LPS precursors.

In *N. meningitidis* slow growth is possible without LPS when *lpxA* is deleted (142). Consequently, one can also delete the *msbA* gene in *N. meningitidis* without loss of viability and yet still assemble an outer membrane (158). Only small amounts of LPS are present in *msbA* mutants of *N. meningitidis*, suggesting downregulation of Kdo_2-lipid A biosynthesis when LPS export is blocked at the inner membrane (158). In contrast to *E. coli*, phospholipid export is not inhibited in *N. meningitidis* MsbA mutants (158), showing that MsbA is not required for phospholipid flipping or export in *N. meningitidis*. *N. meningitidis msbA* can partially complement the temperature-sensitive *E. coli msbA* mutant WD2 (158). It remains possible that *E. coli* MsbA is required for the rapid flipping of phospholipids, given the fast growth of *E. coli* versus *N. meningitidis*.

Additional proteins are required to assemble and attach O-antigen (2–4), and to shuttle nascent LPS across the periplasm and into the outer membrane (**Figure 7**). The essential periplasmic protein YhbN(LptA) has recently been implicated in this process (159). A linked cytoplasmic ABC transporter subunit homologue, designated LptB, also plays a role (159), perhaps in conjunction with the additional transmembrane protein YbrK (159). The LptA/YbrK/LptB transporter complex is thought to function after MsbA-catalyzed LPS flipping, possibly extracting nascent LPS from the periplasmic surface of the inner membrane on its way to the outer membrane (159). Next, the outer membrane protein Imp (56, 160) and its lipoprotein partner RlpB (60) are thought to flip nascent LPS within the outer membrane, bringing it to the exterior. Depletion of LptA/B (159) or Imp/RlpB (56, 160) causes the accumulation of aberrant heavy membranes of unknown composition. As with MsbA, in vitro transport assays with pure proteins have yet to be developed to validate these proposals.

Although eukaryotic Mdr proteins are thought to catalyze phospholipid flip-flop in vitro, mouse mutants lacking the three major Mdr proteins are viable and show no generalized defects in lipid trafficking (161, 162). Given the multitude of Mdr-like proteins in mammalian genomes, functional redundancy may account for the lack of phenotype. However, mouse Mdr2 knockouts display a specific lipid transport deficiency in that they cannot pump phosphatidylcholine into their bile (163). Many of the additional Mdr-like proteins present in animal cells have recently been implicated in the transport of specific

lipids (164). As with MsbA, however, simple and direct in vitro assays for Mdr-catalyzed lipid flip-flop are not well developed.

LIPID A MODIFICATION SYSTEMS IN GRAM-NEGATIVE BACTERIA

E. coli K-12 and *S. typhimurium* contain enzymes for modifying lipid A with phosphoethanolamine (in red) (**Figure 8**) (50, 52, 53, 165), L-Ara4N (in green) (**Figure 8**) (41, 47, 48, 165, 166) and/or palmitate (in black) (**Figure 8**) (46, 167, 168). Two selective deacylases and a dioxygenase are also present in *S. typhimurium* (**Figure 8**). Many of these enzymes are regulated in response to changes in growth conditions. For instance, the addition of palmitate by PagP and the removal of an acyl chain by PagL can be activated by cationic antimicrobial peptides acting through the PhoP transcription factor (45). Changes to the acylation pattern of lipid A can provide resistance to some cationic antimicrobial peptides and/or attenuate the endotoxic properties of lipid A (168). The attachment of phosphoethanolamine by the enzyme EptA (50) and L-Ara4N by the enzyme ArnT (**Figure 8**) (47) is induced by activation of the PmrA transcription factor, either by exposure of cells to mild acid or by *pmrA* constitutive mutations (44, 169, 170). The L-Ara4N group is positively charged at pH 7. It neutralizes the negative charge of the lipid A 4′-phosphate group (**Figure 8**), thereby reducing bacterial susceptibility to cationic antimicrobial peptides and polymyxin (41, 169–171).

Addition of Polar Groups to *E. coli* and *Salmonella* Lipid A

The biosynthesis of L-Ara4N and the mechanism of its attachment to core-lipid A have recently been elucidated. The process starts with the oxidative decarboxylation of UDP-glucuronic acid by the C-terminal domain of ArnA (PmrI) (**Figure 9**) (172). The resulting UDP-4-ketopentose is transaminated by ArnB (PmrH) (173) to generate UDP-L-Ara4N, which is then formylated by the N-terminal domain of ArnA (174). X-ray structures of ArnA (175, 176) and ArnB (177) are available because both are soluble proteins. How they interact and transfer their products between their active sites is unknown. Only the *N*-formyl derivative of UDP-L-Ara4N (magenta modification) (**Figure 9**) can be transferred to undecaprenyl phosphate by ArnC (PmrF) (174). The subsequent deformylation by ArnD to generate undecaprenyl phosphate L-Ara4N (**Figures 9** and **10**) renders the pathway irreversible (174). After transport of undecaprenyl phosphate-L-Ara4N to the outer surface of the inner membrane, ArnT (PmrK) transfers the L-Ara4N residue to the 4′-phosphate group of core-lipid A (**Figure 9**) (47). A possible inner membrane transport system for undecaprenyl phosphate-L-Ara4N, encoded by the *arnE* and *arnF* genes (**Figure 9**), has recently been identified (A. Yan & C.R.H. Raetz, in preparation).

Phosphoethanolamine transfer to core-lipid A by the enzyme EptA (**Figure 8**), predominantly to the 1 phosphate group, likewise occurs on the outer surface of the inner membrane (50, 52, 55). Under certain growth conditions or in the absence of L-Ara4N, EptA can also modify the lipid A 4′-position with a second phosphoethanolamine moiety (not shown in **Figure 8**) (44). Phosphatidylethanolamine serves as the phosphoethanolamine donor substrate. In contrast to the L-Ara4N group, which is critical for polymyxin resistance (41, 174, 178), the roles of the phosphoethanolamine modifications remain uncertain.

E. coli and *S. typhimurium* both contain a related enzyme, designated EptB, which is homologous to EptA but is not regulated by PmrA. Instead, EptB is induced by the addition of 5 mM Ca^{2+} to the growth medium (53, 179) and is under the control of the σ^E transcription factor (180). EptB transfers a phosphoethanolamine moiety from phosphatidylethanolamine to the outer Kdo

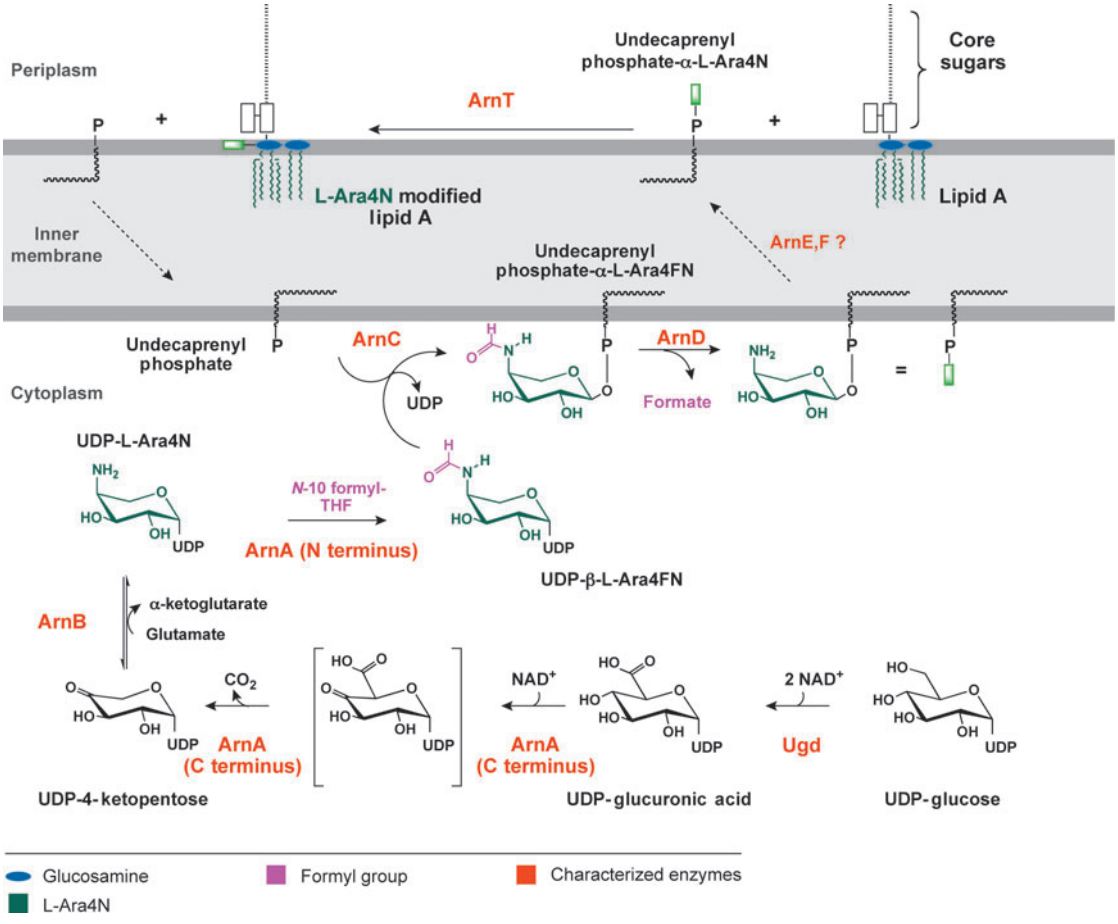

Figure 9

Biosynthesis of the L-Ara4N unit and its attachment to core-lipid A. Formation of the L-Ara4N moiety begins with the oxidation of UDP-glucose to UDP-glucuronic acid (172), as first proposed by Zhou et al. (165). Next, the C-terminal domain of ArnA catalyzes an NAD^+-dependent oxidative decarboxylation to yield an unusual UDP-4-ketopentose, which is converted to UDP-L-Ara4N (*green* moiety) by the transaminase ArnB (173). The N-terminal domain of ArnA then uses *N*-10-formyltetrahydrofolate to add a formyl group to UDP-L-Ara4N (174). Next, ArnC, a distant orthologue of dolichyl phosphate-mannose synthase, selectively transfers the formylated L-Ara4N residue to undecaprenyl phosphate (174). The ArnD-dependent deformylation of this lipid to make undecaprenyl phosphate-α-L-Ara4N (which accumulates in polymyxin-resistant mutants) likely occurs on the inner leaflet of the inner membrane and may prevent reversal of the ArnC reaction (174). After transport to the outer surface of the inner membrane by a process that may involve the inner membrane proteins ArnE and ArnF (A. Yan & C.R.H. Raetz, in preparation), the polytopic membrane protein ArnT transfers the L-Ara4N moiety (shown as a *green rectangle*) to core-lipid A. Given the dual function the ArnA holoenzyme (174–176), the possibility of substrate channeling from ArnA to ArnC via ArnB deserves consideration. However, ArnA and ArnB do not associate with each other in vitro.

residue (**Figure 8**). Diacylglycerol is generated as the by-product (53). Heptose-deficient mutants lacking EptB are killed by the presence of 5 mM Ca^{2+} in the growth medium (53), suggesting a function in the maintenance of outer membrane stability.

Additional Ept-related enzymes are likely necessary to control the incorporation of

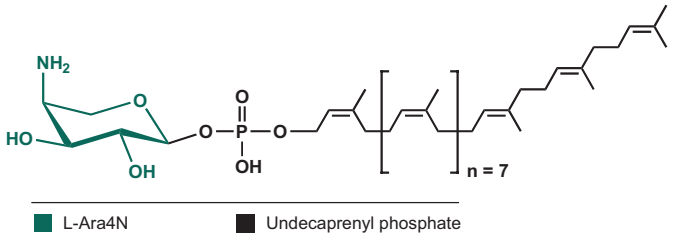

Figure 10

Structure of undecaprenyl phosphate-α-L-Ara4N. This lipid accumulates in polymyxin-resistant mutants of *E. coli* and *Salmonella* (48).

phosphoethanolamine into other cell envelope components, including the first inner core heptose sugar, which is modified in a PmrA-dependent manner by an *ept* gene homologue in *S. typhimurium* (181). Phosphoethanolamine is not normally incorporated into the lipid A moiety of *E. coli* K-12 until the cells are exposed to mildly acidic growth conditions (44), but pathogenic *E. coli* O157:H7 expresses this modification constitutively (182). The latter organism harbors an *ept* gene homologue in the plasmid pO157-encoded *shf* locus (183, 184), but the corresponding gene is replaced by *virK*, an unrelated gene needed for intercellular spreading in *Shigella flexneri* (136). The plasmid-borne *shf* loci of *E. coli* O157:H7 and *S. flexneri* also encode a second *lpxM* gene homologue, which must be inactivated together with the chromosomal copy in order to achieve virulence attenuation (136, 183). The *shf* loci also encode the *wabB* glycosyltransferase gene that controls attachment onto the third heptose sugar of GlcNAc, which is a unique feature of the R3 inner core (184).

When grown in the presence of 10 mM Mg^{2+} at neutral pH, *E. coli* and *S. typhimurium* synthesize a subset of core-lipid A molecules (20%–30%) in which a diphosphate group is present at position 1 of lipid A (not shown in **Figures 2** or **8**) (166). The diphosphate residue is generated on the periplasmic surface of the inner membrane by YeiU, an undecaprenyl diphosphate-specific phosphotransferase (185) (M.S. Trent, in preparation). YeiU also functions as an undecaprenyl diphosphate phosphatase. Undecaprenyl diphosphate is generated on the outer surface of the inner membrane during the polymerization of peptidoglycan (**Figure 1**) (186). The lipid A diphosphate groups, synthesized by YeiU and EptA, might function to stabilize and/or balance the surface electrostatics of the outer membrane depending on environmental conditions. However, YeiU is not essential for cell growth on nutrient broth.

Modification of the Fatty Acyl Chains of *E. coli* and *Salmonella* Lipid A

Modification of the lipid A moiety of LPS with palmitate by PagP (CrcA) (**Figure 8**) is under control of the PhoP/PhoQ system, which is activated by low Mg^{2+} concentrations or cationic antimicrobial peptides (42, 44, 168). An acidic patch on the surface of the periplasmic domain of the PhoQ sensor kinase may orient parallel to the membrane plane, which would allow Mg^{2+} to bridge the acidic patch with anionic phospholipid polar head groups and maintain a repressed regulatory state (45, 187). The PhoP/PhoQ system is thought to be activated either by growing cells under Mg^{2+}-limited conditions, or under Mg^{2+}-replete conditions found during growth within macrophage phagosomal vacuoles (188), upon displacement of Mg^{2+} by cationic antimicrobial peptides (45). In *S. typhimurium*, PhoP/PhoQ activation triggers the PmrA/PmrB pathway via a posttranslational mechanism using an effector known as PmrD, but the *pmrD* gene is nonfunctional in *E. coli* (189). Consequently, PmrA/PmrB-dependent modification of the lipid A phosphate groups can be uncoupled from the PhoP/PhoQ-dependent incorporation of palmitate only in *E. coli* (57).

S. typhimurium mutants unable to add palmitate to lipid A are sensitive to certain cationic antimicrobial peptides, including representatives of amphipathic α-helical (C18G) and β-sheet (protegrin) structural classes, but not including polymyxin

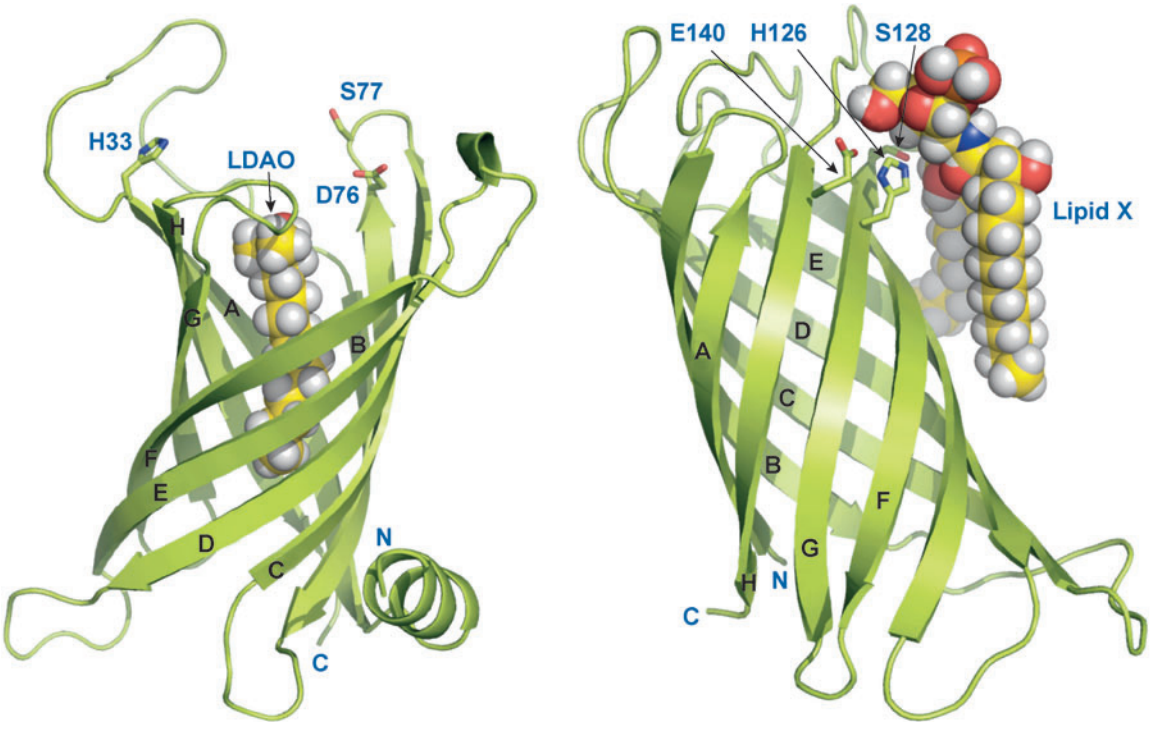

Figure 11

The outer membrane lipid A palmitoyltransferase PagP (*left*) and the lipid A 3-*O*-deacylase PagL (*right*). The disordered loop connecting the first and second β-strands of PagP (Protein Data Bank code 1THQ), and the bound lipid X with PagL (Protein Data Bank code 2ERV) were introduced subsequently and energy minimized. The authors thank Chris Neale and Régis Pomès (University of Toronto) and Lucy Rutten and Jan Tommassen (Utrecht University) for providing the coordinates of energy-minimized PagP and PagL, respectively. The β-strands (A-H) (*black letters*), and the amino- (N) and carboxyl- (C) termini (*blue letters*) are indicated on both structures. Abbreviation: LDAO, lauroyldimethylamine-*N*-oxide.

(168). Although the amphipathic α-helical peptides LL37 and C18G are both inducers of the PhoP/PhoQ system (45), PagP only provides measurable resistance to the latter (190). PagP is required for animal infections caused by *Legionella pneumophila* and *Bordetella bronchiseptica*, where it provides resistance to cationic antimicrobial peptides and antibody-mediated complement lysis, respectively (191–193). The *pagP* gene is distributed among a narrow group of primarily pathogenic bacteria, and its regulation often correlates with their pathogenic lifestyle (194). For example, the Bvg virulence regulator controls *B. bronchiseptica* PagP expression (192), and the incorporation of palmitate into *Yersinia pseudotuberculosis* lipid A predominates at the body temperature of the infected host (195).

Palmitate transfer to the lipid A moiety of LPS occurs on the outer surface of the outer membrane where PagP uses phospholipids as palmitoyl donors (46). Both X-ray and NMR structures of *E. coli* PagP (**Figure 11**) have been reported (196, 197). The active site of PagP faces the exterior (197), suggesting that its activity is regulated by phospholipid access because phospholipids are not always present on the outer surface of the outer membrane. PagP is an eight-stranded

antiparallel β-barrel, preceded by an N-terminal amphipathic α-helix (**Figure 11**). The β-barrel axis is tilted by ~25° with respect to the membrane plane (196, 198). Like most β-barrel outer membrane proteins, the interior of the inner leaflet-exposed half of the molecule is largely polar, but the interior of the outer LPS-exposed half is decidedly hydrophobic and lined by a bound molecule of the detergent lauroyldimethylamine-*N*-oxide (**Figure 11**). Proline residues punctuate the β-strands at two opposing sites and disrupt the continuity of β-barrel hydrogen bonding around the bound detergent, thus providing obvious routes for lateral access of lipid substrates from within the outer leaflet of the membrane.

The mechanism by which PagP selects palmitate in preference to other fatty acyl chains involves another "hydrocarbon ruler," which can be reset to recognize shorter fatty acids by means of single amino acid substitutions (196). Substitution of Gly88 lining the floor of the lauroyldimethylamine-*N*-oxide binding pocket can make the pocket shallower by the same length as the introduced side chain, and this affords a corresponding shortening of the acyl chain that is selected by the enzyme (196). The Gly88-proximal aromatic side chains of Tyr26 and Trp66 undergo a rare exciton interaction, which can be detected by circular dichroism spectroscopy and provides a sensitive probe to gauge methylene unit resolution of acyl chain selection (198a). The ability to modulate PagP acyl chain selection might be important for the preparation of endotoxin antagonists and adjuvants (30, 31, 199).

The amino acid residues implicated in catalysis Asp76, His33, and Ser77 (**Figure 11**) are not organized into a catalytic triad characteristic of serine esterases (196, 197), but PagP can exist in a conformational equilibrium between two dynamically distinct states (200). Although the known structure represents an inhibited R state, an ordering of residues in and around the disordered cell-surface loop that connects the first two β̃–strands is thought to be necessary to afford a catalytically competent T state, the structural details of which remain to be elucidated. Knowledge of the T-state structure would likely reveal details of the PagP catalytic mechanism.

PagP can function as a membrane-intrinsic probe to monitor either the transport of LPS to the outer membrane (158) or the translocation of phospholipids into the outer leaflet, which occurs when LPS organization is disrupted by mutations that affect the presentation of LPS on the cell surface (60) or by EDTA that chelates the Mg^{2+} needed to neutralize negative charge repulsions between neighboring LPS molecules (57). Phospholipid accumulation in the outer leaflet can render cells sensitive to hydrophobic antibiotics and detergents, which are normally impermeable when lipid asymmetry is maintained. An outer membrane permeability defect observed in an LpxM-deficient mutant of *E. coli* O157:H7 was recently associated with PagP activation through a lipid perturbation mechanism, which revealed that PagP can contribute to the restoration of the permeability barrier (S.-H. Kim, W. Jia, E. Vinogradov, C.L. Gyles, & R.E. Bishop, in preparation). Interestingly, PagP activation in this mutant also induced a truncation of the R3 core at the level of the first outer core glucose unit, which could be rescued by restoring the cytosolic pool of UDP-glucose. The implication that PagP activation in the outer membrane can control cytoplasmic functions is consistent with observations that LPS modifications, including palmitoylation of lipid A, can initiate signal transduction across the bacterial cell envelope to the transcription factor σ^E (201).

S. typhimurium contains several additional lipid A modification enzymes that are not present in wild-type *E. coli* K-12. PagL is an outer membrane lipase that is regulated by PhoP/PhoQ and removes the *R*-3-hydroxymyristoyl chain at position 3 of the lipid A moiety (**Figure 8**) (49). LpxR, a distinct outer membrane lipase, cleaves the

intact 3′-acyloxyacyl moiety of Kdo_2-lipid A (**Figure 8**) (54). In vitro studies have shown that LpxR activity is Ca^{2+} dependent and Kdo activated (54). Finally, LpxO is an inner membrane enzyme that hydroxylates the 3′ secondary acyl chain of Kdo_2-lipid A in the presence of O_2 (**Figure 8**), using Fe^{2+} and α-ketoglutarate as cofactors (202, 203). LpxO is not under the control of PhoP/PhoQ, and its active site faces the cytoplasm (44, 55). The crystal structures of LpxO and LpxR have not yet been determined.

Expression of LpxO, LpxR, or PagL in *E. coli* K-12 leads to the expected lipid A modifications (**Figure 8**) (49, 54, 202). Interestingly, PagL and LpxR are latent in *S. typhimurium*, unless overexpressed (49, 54), suggesting the presence of endogenous inhibitors such as the L-Ara4N moiety (204). The functions of these enzymes are unknown, but PagL and LpxR would likely attenuate the cytokine-inducing ability of LPS (205–207).

Despite its absence from *E. coli*, PagL homologues are more widely distributed than PagP, although PagL is not primarily restricted to pathogenic organisms (208). The crystal structure of PagL from *P. aeruginosa* reveals an overall fold that is similar to PagP (209), and its active site likewise faces the outer surface of the outer membrane (**Figure 11**). Both molecules are eight-stranded antiparallel β-barrels and are strikingly tilted in the outer membrane. However, PagL differs in the presentation of its lipid substrate to a distinct catalytic triad formed by Glu140, His126, and Ser128 on the β-barrel exterior. Energy minimization of the model substrate lipid X reveals that acyl chains likely bind into hydrophobic grooves on the β-barrel exterior (209). Although the *P. aeruginosa* enzyme normally encounters *R*-3-hydroxydecanoyl chains in its substrates in vivo, it also utilizes *R*-3-hydroxymyristoyl chains in vitro, which indicates that acyl chains encountered on the β-barrel exterior cannot be measured with the same precision as performed by PagP. The potential of PagL to dimerize at an interface between active sites suggests a potential mechanism to inhibit activity in the outer membrane (209).

Modification of *Francisella* and *Helicobacter* Lipid A

Francisella tularensis is the cause of tularemia, a highly contagious pulmonary disease of humans and animals (210). *Francisella novicida* U112, a related environmental organism, does not infect humans and affords a practical model system for laboratory investigation (211). Strains of *Francisella* synthesize relatively little LPS but do contain significant amounts of free lipid A, which can be extracted with chloroform-methanol together with the glycerophospholipids (212). The biological significance of free lipid A, which lacks Kdo and other core sugars, is unclear (212). The genomes of *Francisella* encode all the enzymes of the constitutive Kdo_2-lipid A biosynthetic pathway, as well as enzymes for core sugar addition (213). It may be that nascent lipid A is pumped out of the cell by MsbA more rapidly than it is glycosylated by KdtA. Alternatively, free lipid A may arise from nascent LPS by the action of an unusual Kdo hydrolase present in *Francisella* membranes (58). However, a Kdo hydrolase is also present in membranes of *Helicobacter pylori* (214), but free lipid A is not abundant. The gene encoding the Kdo hydrolase has not yet been identified.

The predominant lipid A species present in *Francisella* are shown in **Figure 12** (upper right). Interesting features (also seen with the portion of the lipid A that is linked to *Francisella* LPS) include the absence of the 4′-phosphate group and the 3′-acyl chain (212, 215). A galactosamine residue, which is a cationic sugar resembling L-Ara4N, is attached to the 1-phosphate group of *Francisella* lipid A (212, 215). Under some conditions, the 1-phosphate and galactosamine groups appear to be missing (**Figure 12**, lower right structure) (216).

Some of the novel enzymatic reactions that account for these interesting lipid A modifications have been identified (**Figure 12**). The

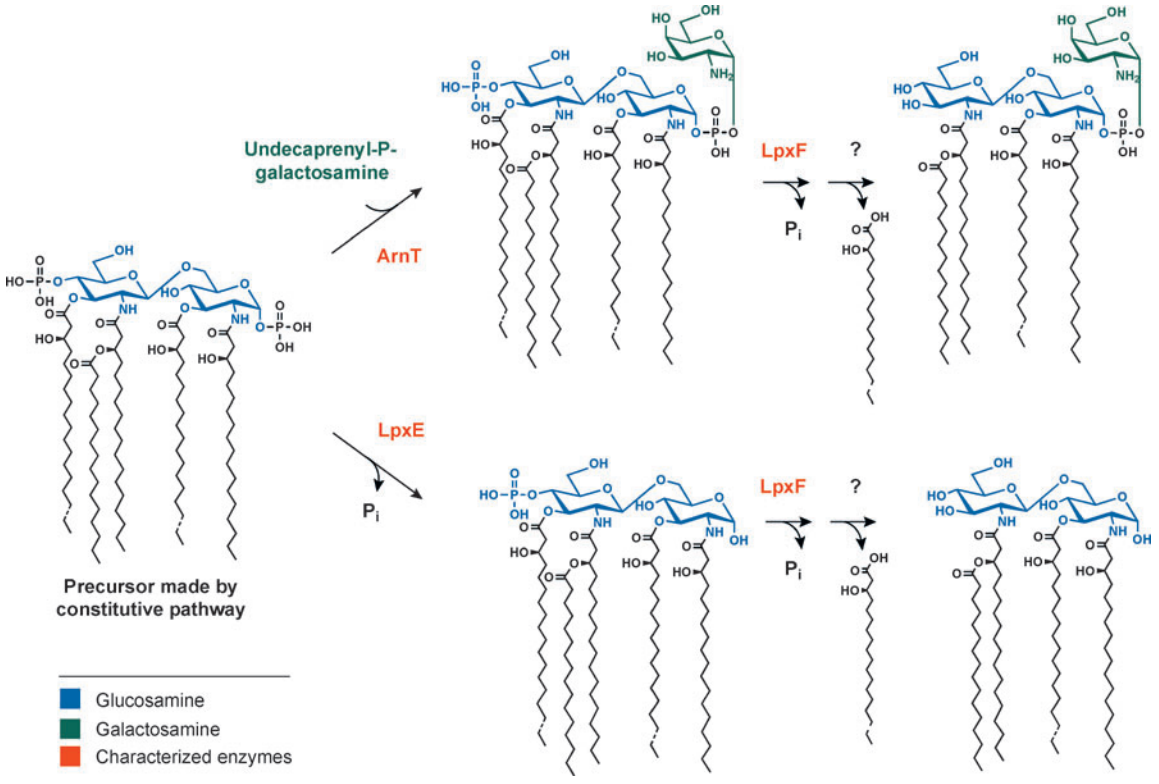

Figure 12

Extracellular covalent modifications of lipid A in *Francisella tularensis*. The proposed modification pathway is based on the genetic characterization of the two inner membrane phosphatases, LpxE and LpxF (58, 59), which are present in many bacteria that synthesize phosphate-deficient lipid A. The attachment of galactosamine to *F. tularensis* lipid A involves a polyisoprene phosphate donor, analogous to undecaprenyl phosphate-L-Ara4N in *E. coli* (X. Wang & C.R.H. Raetz, in preparation). The *F. tularensis* system is also unusual in that much of its lipid A is "free," i.e., not covalently attached to LPS (212). The origin and function of free lipid A have not been firmly established. LpxE strongly prefers substrates that contain a Kdo disaccharide (not shown), indicating that it targets LPS but not free lipid A.

lipid A phosphate groups are removed by two distinct inner membrane phosphatases, designated LpxE and LpxF (**Figure 12**), the active sites of which face the periplasmic surface of the inner membrane (58, 59). Expression of LpxE in strains of *E. coli* or *Salmonella* leads to nearly quantitative dephosphorylation at the 1-position (58). Expression of LpxF in wild-type *E. coli* has no effect because LpxF does not dephosphorylate lipid A species containing a secondary acyl chain at the 3′-position (59). However, LpxF expression leads to complete loss of the 4′-phosphate moiety in *lpxM* mutants of *E. coli* or *Salmonella*, which synthesize mainly penta-acylated lipid A species (**Figure 2**) (59). Cells of *Salmonella* expressing *lpxE* synthesize the nontoxic, adjuvant form of lipid A (also known as monophosphoryl lipid A) (30, 31), which is usually prepared by mild acid hydrolysis of LPS or by chemical synthesis. LpxE-expressing strains may be useful as live oral vaccines, assuming that the excessive inflammation that is normally caused by *Salmonella* lipid A is indeed suppressed.

Disruption of the *lpxF* gene in *F. novicida* (**Figure 12**) results in the quantitative retention of the lipid A 4′-phosphate group

with the accumulation of the species shown at the top center of **Figure 12** (217). When the 4′-phosphate moiety is left in place, the 3′-acyl chain is not removed (217), suggesting an obligatory order of processing. Because there is no *lpxR* gene in *Francisella* (213), it is unclear how the 3′-acyl chain is actually removed. *H. pylori* lipid A, like that of *F. novicida*, lacks both the 4′-phosphate group and the 3′-acyl chain(s). There is no *lpxF* gene in *H. pylori* (218), but an orthologue of *lpxR* is present. When *lpxR* is inactivated in *H. pylori*, a hexa-acylated lipid A species lacking the 4′-phosphate group accumulates (M.S. Trent, in preparation), suggesting that *H. pylori* contains a distinct 4′-phosphatase that can dephosphorylate lipid A molecules containing a secondary 3′-acyl chain.

The mutant of *F. novicida* lacking *lpxF* is hypersensitive to cationic antimicrobial peptides and is avirulent in a mouse infection model (217). Following short-term intraperitoneal injection, the *lpxF* mutant bacteria trigger the production of a subset of cytokines, suggestive of TLR2 activation, whereas wild-type cells do not (217). Unlike the *Y. pestis* construct described above (138), the lipid A of *Francisella lpxF* mutant bacteria does not activate TLR4 (217), and *lpxF* mutant cells do not trigger the production of TNFα (217). Instead, the hypersensitivity of the *lpxF* mutant to cationic antimicrobial peptides (217) may cause damage to the bacterial envelope and expose other ligands, such as membrane lipoproteins for TLR2 or bacterial DNA for TLR9 (17). The potential of *lpxF* mutants as novel vaccines for the prevention of tularemia has not yet been explored.

Deletion of the single *arnT* gene homologue present in *F. novicida* leads to the production of lipid A molecules lacking the galactosamine modification (212), consistent with the finding that undecaprenyl phosphate-galactosamine (**Figure 12**) is present among the minor lipids of *F. novicida* (X. Wang & C.R.H. Raetz, in preparation). The galactosamine modification pathway in *Francisella* therefore appears to be analogous to the L-Ara4N pathway (**Figure 9**) in *E. coli*. Interestingly, the live vaccine strain of *F. tularensis*, like the *arnT* mutant of *F. novicida*, lacks the galactosamine modification on its free lipid A (212). Whether this feature is due to the absence of ArnT in the live vaccine strain or the inability to synthesize the undecaprenyl phosphate-galactosamine donor substrate (**Figure 12**) is unclear.

Lipid A Processing in *Rhizobium leguminosarum* and *Rhizobium etli*

The plant endosymbionts *R. leguminosarum* and *R. etli* synthesize a complex mixture of lipid A molecules that lack the 1- and 4′-phosphate groups found in most other gram-negative bacteria (**Figure 13**) (219–221). In a subset of molecular species, the anomeric carbon atom of the proximal unit is oxidized to a carboxylic acid (**Figure 13**) (219–221). Galacturonic acid residues are attached to the outer Kdo moiety (not shown) and to the 4′-position of lipid A (219–221). Both organisms synthesize mainly penta-acylated core-lipid A molecules (220, 221) with an unusually long secondary acyl chain at the 2′-position (**Figure 13**) (220, 221); this is a characteristic structural feature of the lipid A from many Rhizobiaciae (222). A portion of the lipid A molecules are deacylated at position 3 (223), and there is considerable fatty acid chain length heterogeneity (**Figure 13**), when contrasted with *E. coli* lipid A (152, 220, 221). The core sugars beyond the Kdo region of *Rhizobium* LPS are also very different from those of *E. coli* (224, 225) in that the heptose units are replaced by mannose, galactose, and Kdo (not shown in **Figure 13**).

The enzymology, genetics, and topography of *R. leguminosarum* and *R. etli* lipid A biosynthesis have been investigated in considerable detail. These bacteria contain orthologues of the first seven *lpx* genes found in *E. coli* (**Figure 2**) (226), and therefore they initially synthesize the tetra-acylated precursor Kdo_2-lipid IV_A, shown in **Figures 13** and **14**. The diverse lipid A molecular species of

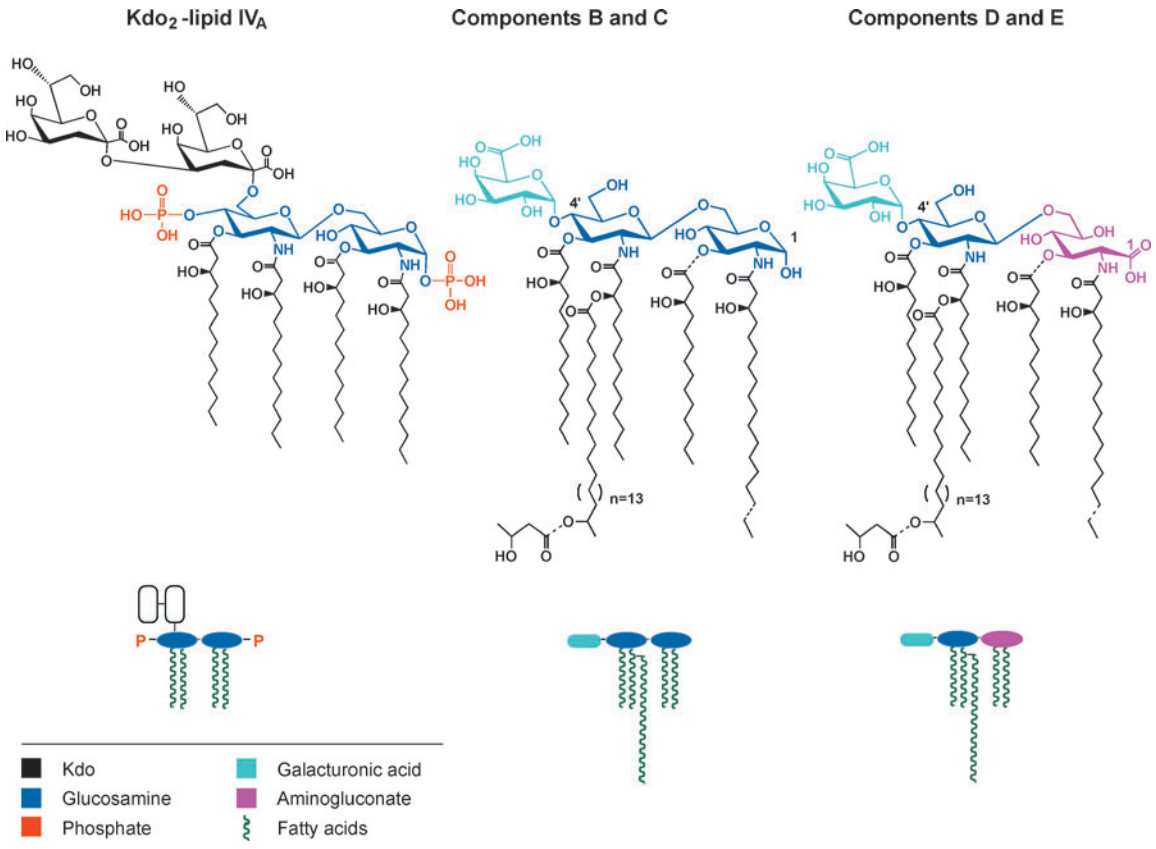

Figure 13

Kdo_2-lipid IV_A versus the mature lipid A moiety of *Rhizobium etli* and *Rhizobium leguminosarum* LPS. These bacteria make phosphate-deficient lipid A molecules from Kdo_2-lipid IV_A (226, 250), using the lipid A phosphatases LpxE and LpxF, as described in the text. Lipid A molecules of *Rhizobium* typically contain a very long secondary acyl chain at position 2′ (222). Additional unique features include the presence of galacturonic acid in place of phosphate at position 4′ and oxidation of the proximal glucosamine unit in a portion of the molecules to aminogluconate (219–221). Partial substituents and microheterogeneity of acyl chains lengths are indicated by dashed bonds. Components C and E lack the 3-*O*-linked hydroxyacyl chain. The schematic representations of these structures are shown below the actual chemical structures.

Rhizobium (**Figure 13**) are generated from Kdo_2-lipid IV_A by enzymes that are not present in *E. coli*. For instance, the long secondary acyl chain is incorporated by LpxXL, a distant orthologue of LpxL (227), requiring a special acyl carrier protein termed ACP-XL (**Figure 14**) (228). *R. leguminosarum* and *R. etli* also contain the phosphatases LpxE (58, 229) and LpxF (59), which catalyze the removal of the 1- and 4′-phosphate groups respectively (**Figure 14**), as in *Francisella*. Following removal of the 1-phosphate moiety, LpxQ (230, 231) can oxidize the proximal glucosamine of *Rhizobium* lipid A in the presence of O_2 to form an aminogluconate unit. Removal of the 4′-phosphate group by LpxF is necessary for the incorporation of the 4′-galacturonic acid moiety (**Figure 14**), which appears to involve a polyisoprene phosphate sugar donor (rather than a sugar nucleotide) (B.O. Ingram & C.R.H. Raetz, unpublished results). Similarly, the incorporation of galacturonate into

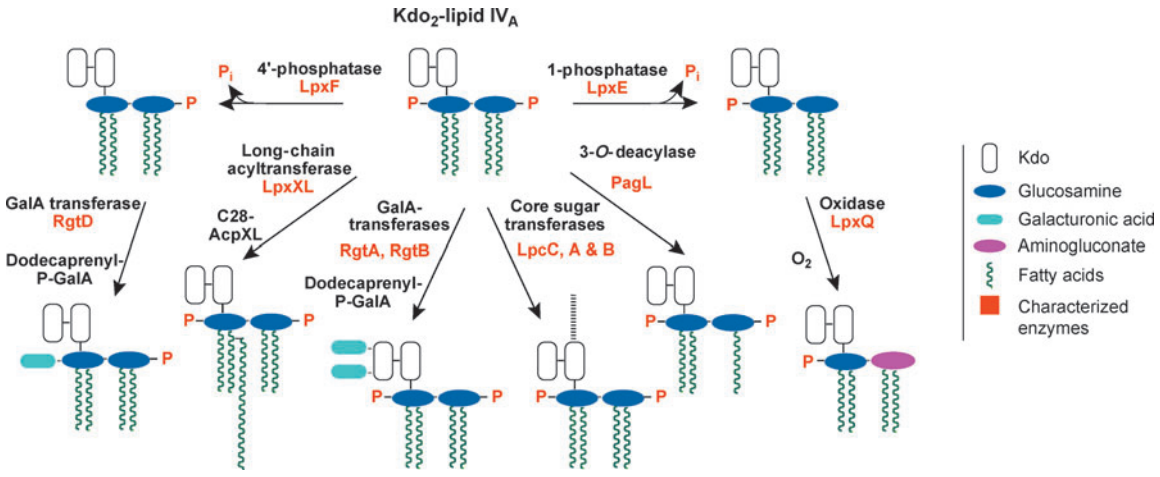

Figure 14

Kdo_2-lipid IV_A processing in *Rhizobium etli* and *Rhizobium leguminosarum* membranes. The processing enzymes unique to the *Rhizobium* system can be assayed using *E. coli* Kdo_2-lipid IV_A as the model substrate (223, 225, 227–229, 232, 233, 250–252). The enzymes that have been characterized to date are labeled in red according to the genes that encode them. Other colors as labeled: glucosamine, galacturonic acid, aminogluconate, Kdo, other core sugars (*black dashed line*), fatty acids.

the Kdo region of *Rhizobium* LPS by the enzymes RgtA and RgtB (**Figure 14**) requires dodecaprenyl phosphate galacturonic acid as the donor substrate (232, 233). RgtA and RgtB (**Figure 14**) are distantly related in sequence and membrane topography to ArnT (232) (**Figure 9**).

The precise order of Kdo_2-lipid IV_A modification in *Rhizobium* (**Figure 14**) is not fully established. In vitro there is no obligatory order of enzymatic processing, with the exception of LpxE preceding LpxQ, and LpxF preceding RgtD (**Figure 14**). However, several lines of evidence strongly support the enzymatic topography shown in **Figure 15**, in which LpxE, LpxF, and the Rgt enzymes function on the outer surface of the inner membrane, and LpxQ and PagL act in the outer membrane. Heterologous expression of the inner membrane phosphatases LpxE or LpxF in the appropriate strains of *E. coli* results in nearly complete and selective lipid A dephosphorylation, provided that the LPS flippase MsbA (**Figures 7** and **15**) is functional (58, 59). The involvement of a dodecaprenyl phosphate-linked galacturonic acid donor, instead of a sugar nucleotide, is likewise consistent with the addition of galacturonate residues to core-lipid A occurring on the outer surface of the inner membrane (**Figure 15**) (232, 233), analogous to L-Ara4N in polymyxin-resistant *E. coli* (**Figures 8** and **9**) (48). PagL and LpxQ are recovered in the outer membrane when expressed in *E. coli* (49, 230, 231). LpxQ efficiently generates lipid A species containing aminogluconate when coexpressed with LpxE in *E. coli* (B.O. Ingram & C.R.H. Raetz, unpublished results).

The biological significance of the lipid A modification systems of *Rhizobium* has been evaluated by genetics. In each instance studied to date, the relevant enzymes were first identified by the development of in vitro biochemical assays, followed by the expression cloning of the corresponding structural genes (59, 225, 227–229, 231, 232). Subsequent deletion of these structural genes caused the accumulation of the predicted structurally altered lipid A species, demonstrating the biological relevance of the enzymatic approach. For instance, deletion of *lpxXL* or *acpXL*

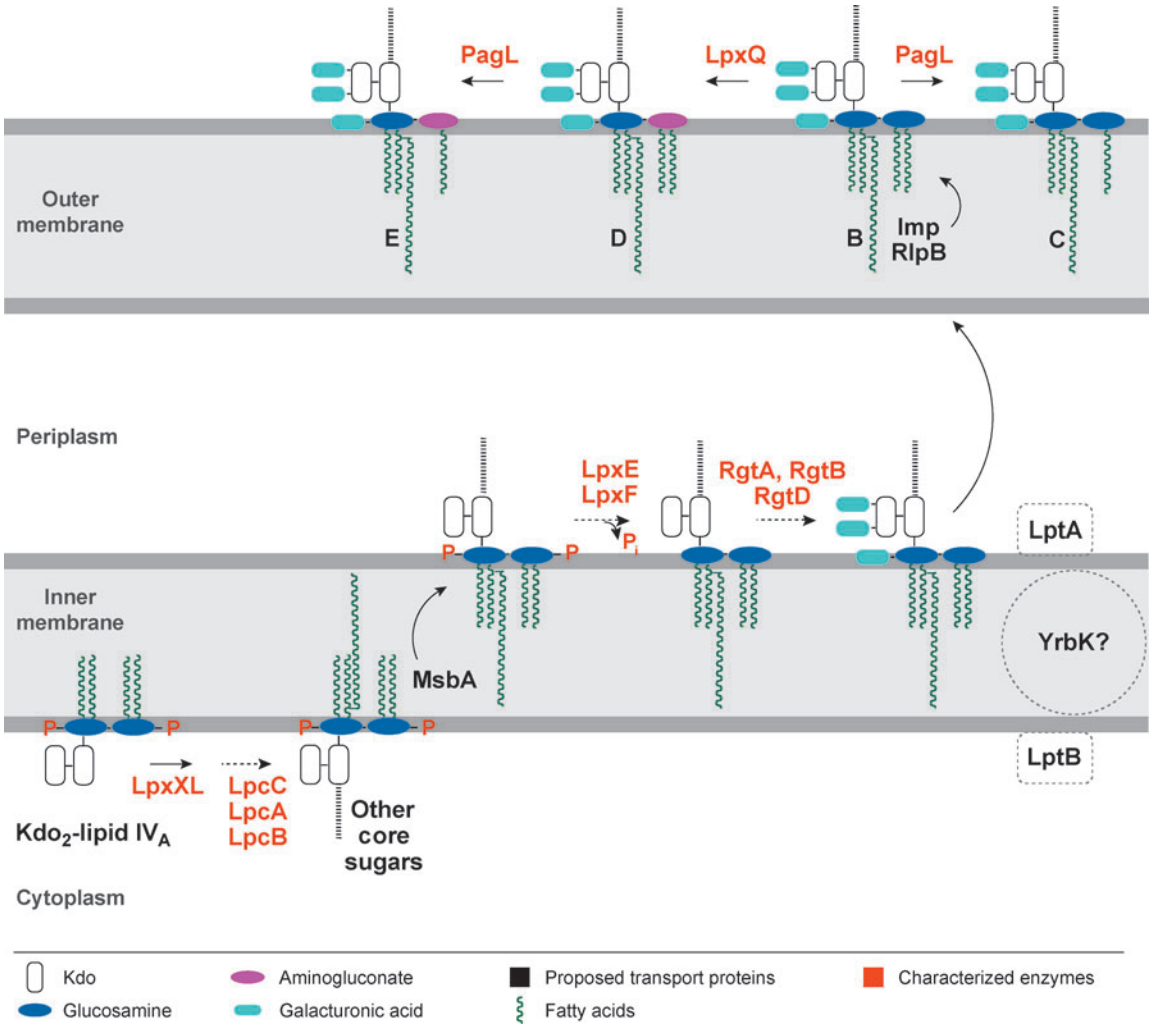

Figure 15

Topography of lipid A modifications in *Rhizobium etli* and *Rhizobium leguminosarum*. The proposed topography of the processing enzymes is based on the finding that lipid A dephosphorylation by LpxE and LpxF requires a functional *msbA* gene when the *lpxE* or *lpxF* genes are expressed in *E. coli* (51, 58, 59). The Rgt proteins require a polyisoprene donor as their cosubstrate, consistent with a periplasmic localization (232, 233). PagL and LpxQ are known to be outer membrane proteins (47, 231). The X-ray structure of PagL shows that its active site is oriented toward the outside (209). The orientation of LpxQ is unknown (231). PagP is not present in *Rhizobium*. Enzymes are indicated in red, and putative transport proteins are shown in black. Letters in the outer membrane refer to lipid A components B, C, D, and E, shown in **Figure 13**. Other colors as labeled: glucosamine, galacturonic acid, aminogluconate, Kdo, other core sugars (*black dashed line*), and fatty acids.

results in the failure to incorporate the long secondary acyl chain (**Figure 13**) (234, 235). These mutant bacteria grow slowly, and they are hypersensitive to detergents and low pH (234, 235). However, these mutants are nevertheless able to form partially functional nodules in their host plants. Deletion of *Rhizobium* LpxE or LpxF results in the

retention of the 1- or 4′-phosphate moieties, respectively (C. Sohlenkamp, B.O. Ingram, & C.R.H. Raetz, in preparation). In the *lpxE* mutant, the aminogluconate cannot be generated, whereas in the *lpxF* mutant the 4′-galacturonate residue is not incorporated. Studies of the phenotypes of the mutants should provide insights into the functions of these structural features.

Unusual Lipid A Modifications in Other Bacteria

Given the diversity of gram-negative bacteria, it is likely that many additional lipid A modification enzymes exist. Some modification enzymes are present in only a few types of organisms. For instance, *L. interrogans* contains a lipid A methyl transferase (LmtA) that uses *S*-adenosylmethionine to methylate the 1-phosphate group (236). LmtA is distantly related to eukaryotic methyl transferases involved in the C-terminal processing of farnesylated proteins (236). LmtA can be expressed in *E. coli*, resulting in methylation of about half the lipid A (236). The function of lipid A methylation is unknown.

Some strains of *Acinetobacter* make LPS containing an analogue of Kdo, known as Ko, in which the CH_2 moiety at the Kdo 3-position is hydroxylated (237). Interestingly, the KdtA orthologue of this organism utilizes CMP-Kdo in vitro (238), raising the possibility Ko is formed after the addition of Kdo to lipid IV_A. A Kdo-selective dioxygenase, analogous to LpxO (**Figure 8**), might account for these observations. The relevant gene has not yet been identified.

SUMMARY POINTS

1. All gram-negative bacteria, with the exception of the few that make sphingolipids in place of LPS, synthesize Kdo_2-lipid A by minor variations on the constitutive *E. coli* pathway shown in **Figure 2**. The constitutive enzymes are cytoplasmic or associated with the inner surface of the inner membrane. Crystal structures of the soluble enzymes LpxA, LpxC, and LpxD have been determined.
2. The lipid A moiety of LPS is required for the viability of most gram-negative bacteria, possibly because it is needed for the proper folding of some outer membrane proteins. The early steps of lipid A biosynthesis are excellent targets for the design of new antibiotics, as illustrated by the potent LpxC inhibitor CHIR-090 (**Figure 5*a***).
3. Newly synthesized core-lipid A is flipped to the outer surface of the inner membrane in *E. coli* by the essential ABC transporter MsbA (**Figure 7**). Additional proteins have recently been identified that are required for LPS transport across the periplasm and for assembly into the outer membrane. Reconstituted in vitro systems for studying LPS transport have not yet been reported.
4. Enzymes that modify the lipid A moiety of LPS are highly variable from organism to organism and generally are located on the outer surface of the inner membrane or in the outer membrane. The modification enzymes can be used as markers for following LPS transport from the cytoplasmic surface of the inner membrane to the outer membrane (**Figure 15**). Structures of several outer membrane modification enzymes have recently been determined by X-ray crystallography and NMR (**Figure 11**).
5. Lipid A modification enzymes are not required for growth under laboratory conditions but confer selective advantages in some instances, such as resistance to cationic antimicrobial peptides due to charge neutralization.

6. The genes encoding lipid A modification enzymes can be mutated or heterologously expressed in foreign bacterial hosts, facilitating the modification of lipid A structure in living cells. Some gram-negative pathogens with re-engineered lipid A structures display attenuated virulence and have potential utility as vaccines.

FUTURE ISSUES

The following questions and research directions will dominate lipid A and LPS research in the coming years.

1. Why is the lipid A moiety of LPS essential for growth in most gram-negative bacteria? Careful genetic and biochemical examination of unusual systems like *N. meningitidis*, in which lipid A is not required for growth (142), might prove informative. The powerful approach of searching for second-site suppressor mutations in *E. coli* has recently provided new insights into the role of the Kdo disaccharide (8) but has not yet been applied systematically to all the genes of the constitutive pathway.
2. What is the relevance of the *lpx* gene orthologues present in higher plants? Mutants of *Arabidopsis* lacking *lpxA* are viable but lack fine root hairs (D. Liu, B. J. Nikolau, & C.R.H. Raetz, in preparation). Despite suggestive recent immunochemical studies (84), no lipid A-like molecules have been isolated from plants and characterized by chemical methods. Such molecules might have been overlooked if localized to root hairs or stem cells. However, *Arabidopsis lpxA* can rescue a temperature-sensitive *lpxA* mutant of *E. coli*, showing that plant LpxA can catalyze UDP-GlcNAc acylation (83).
3. Can in vitro assays for LPS flipping in membrane vesicles be developed? The genetic and biochemical evidence for the involvement of proteins such as MsbA (55, 143) and Imp/RlpB (56, 60) in catalyzing transmembrane movement of LPS in cells is very compelling, but it needs to be bolstered by the development of in vitro assays in reconstituted liposomes with purified components. The use of lipid A modification enzymes, e.g., LpxE or PagL, as reporters for the transmembrane movement of LPS in vitro should be explored because this approach may facilitate development of robust in vitro systems.
4. Can crystal structures of the inner membrane proteins involved in lipid A biosynthesis and modification be obtained? A great deal of progress has already been made with some soluble proteins, i.e., LpxA (91, 93) and LpxC (103, 107), but the other constitutive enzymes are also of great interest. A structure of TLR4/MD2 with a bound ligand, such as Kdo_2-lipid A, should provide important new insights into LPS signaling mechanisms in animal systems. Methods for crystallizing cytoplasmic membrane proteins have improved in recent years. However, the problems encountered with the structural studies of MsbA show how difficult this approach can be (150, 151).
5. Will lipid A-like molecules that block the TLR4/MD2 receptor be useful for the treatment of gram-negative sepsis? Many clinical trials have failed in the arena of sepsis (25), but the current work with the antagonist E5564 is unique with regard to potency and selectivity of this LPS antagonist in humans (36). Phase III trials are in progress.

6. Can clinically useful antibiotics be developed around the constitutive lipid A pathway? The best available LpxC inhibitors (**Figure 5*a***) (102) may be effective enough for clinical trials, pending improvements in bioavailability, pharmacokinetics and toxicology.
7. Can lipid A modification systems be exploited for vaccine development? Work with *Y. pestis* expressing *lpxL* is the most advanced in this regard (138). The opportunities afforded by attenuated *lpxF* mutants of *F. tularensis* (59, 217) or strains lacking *lpxM* (*msbB*) (135, 136) are under active investigation. The properties of *Salmonella* strains expressing LpxE (58), which synthesize the monophosphorylated, nontoxic adjuvant form of lipid A, will also be of great interest.

Answers to many of the above questions should emerge from careful studies of the unique proteins and enzymes that assemble and modify the lipid A moeity of LPS and its precursors. A combination of genetics and biochemistry will be required to bridge the remaining gaps between lipid A structural diversity and biological function.

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