Discovery of new biosynthetic pathways: the lipid A story

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Abstract The outer monolayer of the outer membrane of Gram-negative bacteria consists of the lipid A component of lipopolysaccharide (LPS), a glucosamine-based saccharolipid that is assembled on the inner surface of the inner membrane. The first six enzymes of the lipid A pathway are required for bacterial growth and are excellent targets for the development of new antibiotics. Following assembly, the ABC transporter MsbA flips nascent LPS to the periplasmic side of the inner membrane, whereupon additional transport proteins direct it to the outer surface of the outer membrane. Depending on the bacterium, various covalent modifications of the lipid A moiety may occur during the transit of LPS to the outer membrane. These extra-cytoplasmic modification enzymes are therefore useful as reporters for monitoring LPS trafficking. Because of its conserved structure in diverse Gram-negative pathogens, lipid A is recognized as foreign by the TLR4/MD2 receptor of the mammalian innate immune system, resulting in rapid macrophage activation and robust cytokine production.—Raetz, C. R. H., Z. Guan, B. O. Ingram, D. A. Six, F. Song, X. Wang, and J. Zhao. **Discovery of new biosynthetic pathways: the lipid A story. *J. Lipid Res.* 2009.** 50: **S103–S108.**

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DISCOVERY OF LIPID A BIOSYNTHESIS

Although lipid A was recognized over 50 years ago as the hydrophobic moiety of lipopolysaccharide (LPS) (**Fig. 1**), the elucidation of lipid A biosynthesis was delayed until 1983. A key breakthrough was the discovery and structural characterization of a novel *Escherichia coli* lipid, termed 2,3-diacylglucosamine 1-phosphate or "lipid X" (**Fig. 2**) (1). This substance was overlooked in earlier work with *E. coli* because of its low levels in wild-type cells, but it accumulates in certain phosphatidylglycerol-deficient mutants (2). The recognition of lipid X coincided with the determination of the correct structure of lipid A (1, 3, 4). The identification of 2,3-diacylglucosamine 1-phosphate as a precursor of the proximal (right) subunit of lipid A (Fig. 2) was critical for developing testable hypotheses regarding the enzymology and genetics of lipid A assembly (5).

Given the importance of structure determination for the discovery of new pathways, as illustrated by the lipid A story (5), the LIPID MAPS Consortium is attempting to identify additional novel lipids, using mass spectrometry as the starting point (6). Many as yet unknown lipid pathways probably exist in all organisms with possible roles in signal transduction, regulation, and membrane assembly.

THE CONSTITUTIVE ENZYMATIC PATHWAY FOR LIPID A ASSEMBLY

Lipid A, the hydrophobic anchor of LPS, is a glucosamine-based lipid that makes up the outer monolayer of the outer membrane (OM) (Fig. 1) of Gram-negative bacteria (5, 7). An *E. coli* cell contains $\sim 10^6$ lipid A residues and $\sim 10^7$ glycerophospholipids (7). The lipid A and Kdo domains of LPS (Figs. 1 and 2) are usually required for growth (7), but Kdo can be eliminated in the presence of suppressors (8). Wild-type LPS contains additional core and O-antigen sugars, which are not needed for growth, but protect against antibiotics and complement (7).

The constitutive enzymes of the lipid A pathway are conserved (7). They are located in the cytoplasm or on the inner surface of the inner membrane (IM) (7). The first step is the acylation of UDP-GlcNAc (Fig. 2), catalyzed by LpxA. In *E. coli* LpxA is selective for β-hydroxymyristoyl acyl carrier protein (9). The active site of *E. coli* LpxA functions as an accurate hydrocarbon ruler that incorporates a β-hydroxymyristoyl chain two orders of magnitude faster than a β-hydroxylauroyl or a β-hydroxypalmitoyl chain (10). Other LpxA orthologs are designed to incorporate longer or shorter β-hydroxyacyl chains (7).

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Abbreviations: GalN, galactosamine; IM, inner membrane; LPS, lipopolysaccharide; OM, outer membrane.

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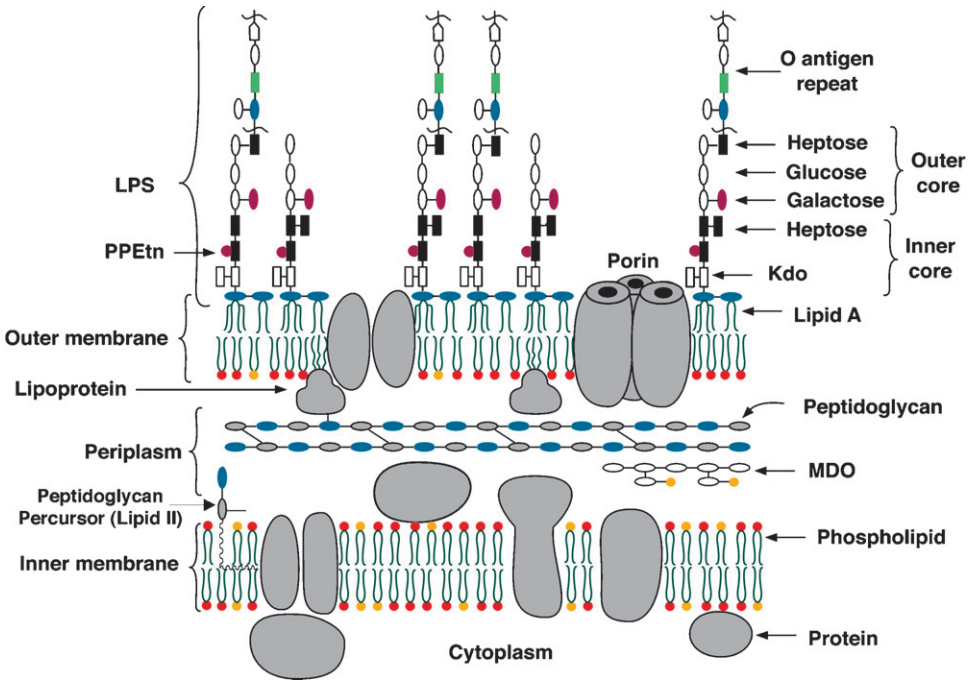

Fig. 1. Schematic representation of the *E. coli* cell envelope.

The equilibrium constant (~0.01) for UDP-GlcNAc acylation is unfavorable (11). The deacetylation of UDP-3-*O*-(acyl)-GlcNAc by LpxC is therefore the committed step. LpxC is a Zn^{2+}-dependent enzyme (12), conserved in diverse Gram-negative bacteria, and is an excellent target for new antibiotics (13, 14). Slow, tight-binding inhibitors of LpxC, such as CHIR-090, have antibiotic activity comparable to ciprofloxacin and are effective against almost all Gram-negatives (14), including *E. coli*, *Pseudomonas aeruginosa*, and *Francisella novicida*. Following deacetylation, a second β-hydroxymyristoyl chain is added by *E. coli* LpxD to make UDP-2,3-diacyl-GlcN (Fig. 2) (15), which is cleaved by LpxH to form 2,3-diacyl-GlcN-1-phosphate (lipid X) (16). A β,1′-6 linked disaccharide is then generated by LpxB, which condenses another molecule of UDP-2,3-diacyl-GlcN with lipid X (17). LpxA, LpxC, and LpxD are soluble proteins with known structures (18–21). LpxH and LpxB are peripheral membrane proteins; their structures have not been reported (16, 17).

The IM proteins LpxK, KdtA, LpxL, and LpxM catalyze the last five steps (Fig. 2) (7). Each contains one predicted membrane-spanning segment. LpxK phosphorylates the 4′-position (Fig. 2) to generate the intermediate lipid IV_A (22), which is an endotoxin antagonist in human cells, but an agonist in mouse (23). This differential pharmacology is determined by the lipid A receptor of the mammalian innate immune system, the TLR4/MD2 complex (24, 25). Next, two Kdo residues are incorporated by the bifunctional enzyme KdtA (Fig. 2) (26). The labile nucleotide CMP-Kdo, derived from arabinose 5-phosphate (not shown), is the Kdo donor (26). The last steps of *E. coli* lipid A biosynthesis involve the addition of the secondary laurate and myristate chains (Fig. 2) by LpxL and LpxM (27), which display sequence similarity to each other and are related to the lysophosphatidic acid acyltransferases (28). The product, hexa-acylated lipid A (Fig. 2), is a TLR4/MD2 agonist in diverse animal species. The *lpxM* gene is usually nonessential (27). *Salmonella typhimurium* and *Shigella lpxM* mutants make penta-acylated lipid A and are attenuated (29).

Why lipid A structure is relatively conserved and required for growth in most Gram-negatives remains uncertain. *Neisseria meningitidis* is unusual in that its *lpxA* gene can be deleted; these mutants grow slowly without lipid A or LPS, but still assemble an OM (30).

UNUSUAL STRUCTURE OF *FRANCISELLA* LIPID A

Despite the conservation of the constitutive pathway in diverse bacterial genomes, lipid A structure can vary considerably. In *F. novicida*, a mouse-specific strain that is a model for the type A human pathogen *Francisella tularensis*, the 4′-phosphate moiety and the 3′-hydroxyacyl chain are missing (**Fig. 3A** versus 3B) (31–33). Furthermore, the phosphate group at the 1-position is modified with a galactosamine (GalN) unit (Fig. 3B) (32, 33). We have discovered that over 90% of the lipid A in *F. novicida* is in a "free" form, i.e., not linked to Kdo, core sugars, and O-antigen (33). The small amount of LPS that is present in *F. novicida* contains only a single Kdo residue (31). A portion of the LPS (31), but not the free lipid A (33), also lacks the usual lipid A 1-phosphate group (**Fig. 4**).

We have recently elucidated the enzymatic basis for many of the structural differences between *F. novicida* and *E. coli* lipid A. Like *E. coli*, *F. novicida* first makes lipid

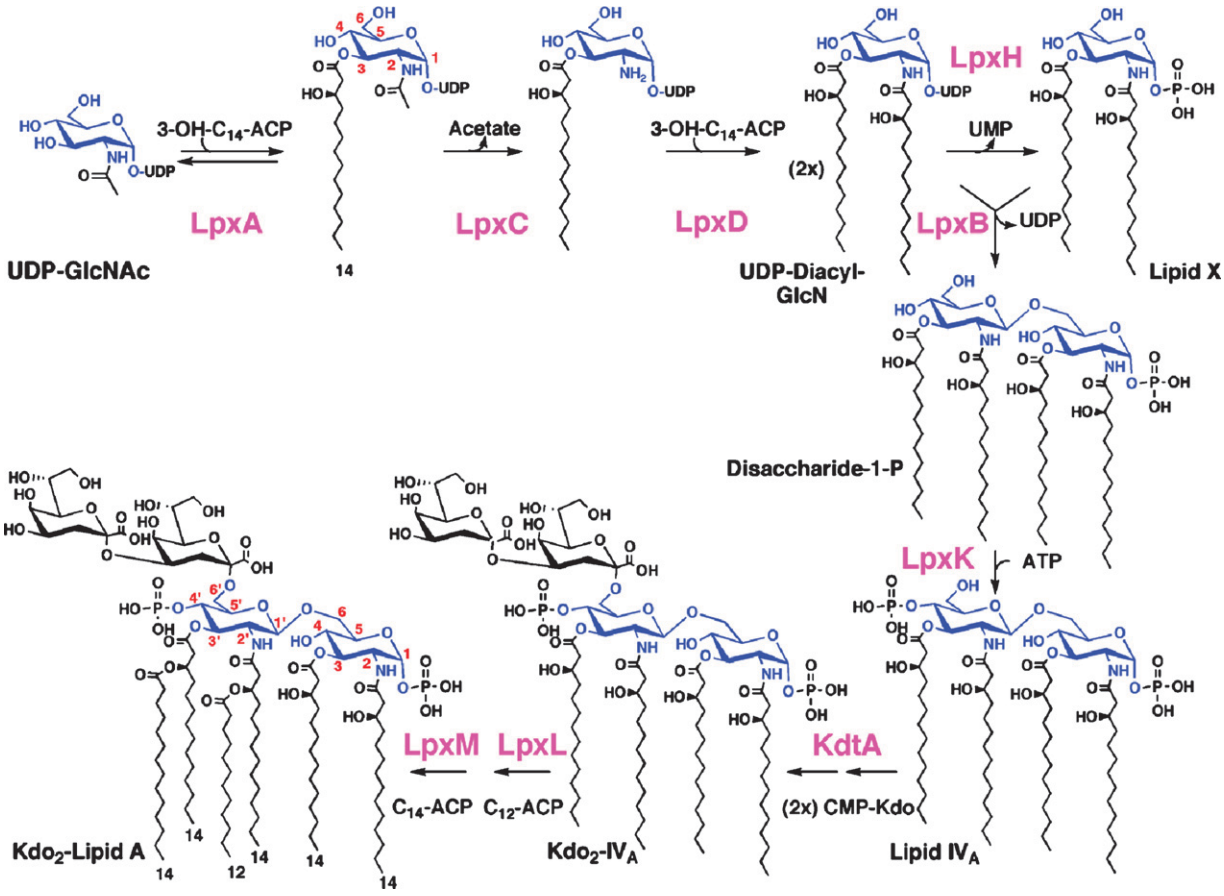

Fig. 2. The constitutive pathway of lipid A biosynthesis in *E. coli.*

IV_A (Fig. 2), albeit with longer hydroxyacyl chains (5). If the lipid IV_A is glycosylated by KdtA, it is then acylated by the Kdo-dependent acyltransferase LpxL1 and converted to LPS (Fig. 4, right side). Alternatively, *F. novicida* lipid IV_A can acquire its 2′ secondary acyl chain directly without Kdo addition (Fig. 4, left side), because of a second LpxL ortholog (LpxL2), which is Kdo-independent (D. Six and C. Raetz, unpublished observations). LpxL2

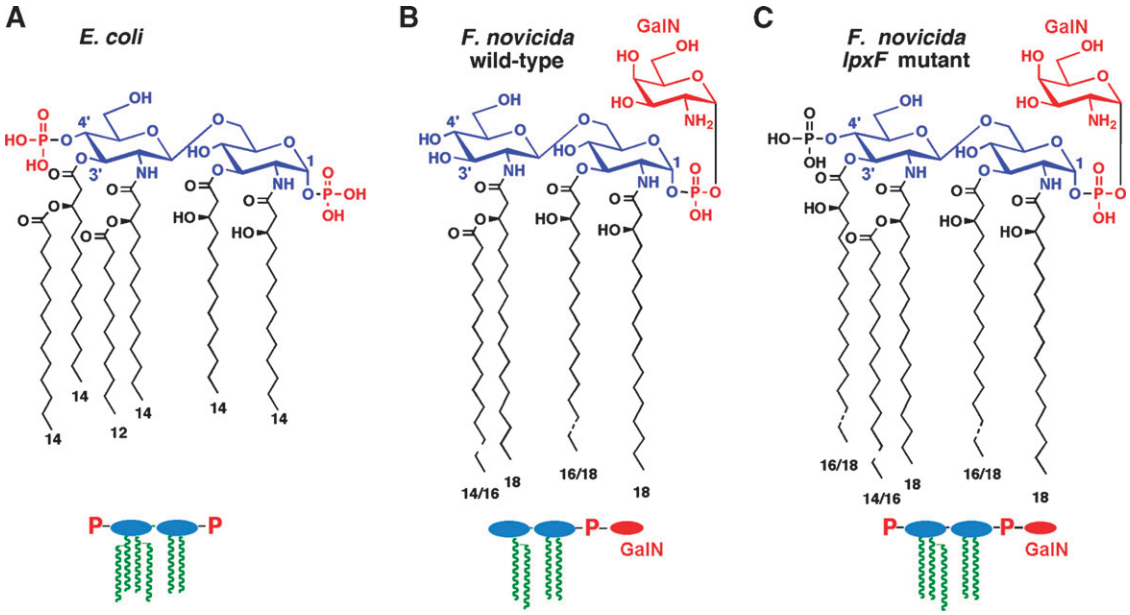

Fig. 3. The structure of lipid A in *E. coli* versus *F. novicida.*

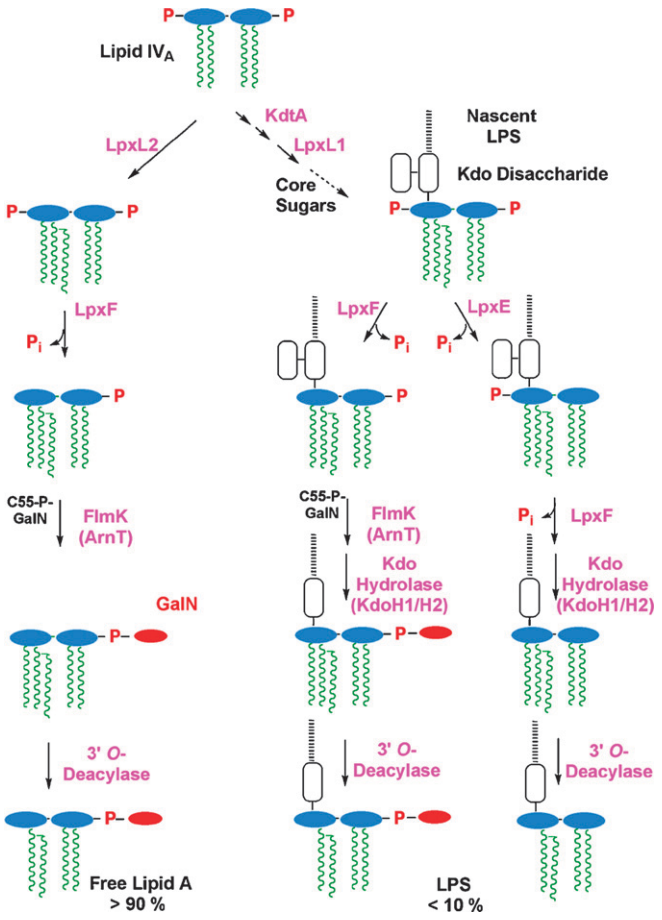

Fig. 4. Lipid A modification enzymes in *F. novicida*.

may function more rapidly than KdtA in *F. novicida*. The LpxL2 penta-acylated product is exported by MsbA (**Fig. 5**), possibly accounting for the large pool of free lipid A. No hexa-acylated lipid A is made because *F. novicida* lacks LpxM (Fig. 2).

Removal of the phosphate groups by LpxF (34) and LpxE (35) (Figs. 4 and 5) occurs on the outer surface of the IM. The order of processing is not fully established. The phosphatase genes have been cloned and expressed in *E. coli*, where their function in cells requires MsbA (the IM flippase for LPS) (34, 35). In *F. novicida*, the 4′-phosphate group is removed quantitatively in both free lipid A (33) and LPS-bound lipid A (Fig. 4) (31, 32). However, LpxE, the 1-phosphatase, is Kdo-dependent (Fig. 4) (35); thus, the 1-phosphate group is present in free lipid A, but is missing in some of the LPS (Fig. 4, right bottom) (31). Deletion of *lpxF* results in retention of the 4′-phosphate group and the 3′-hydroxyacyl chain (36) (Fig. 3C), implying sequential processing (Fig. 4, bottom). LpxF mutants are highly attenuated (36) and can be used to immunize mice against wild-type *F. novicida* (R. Ernst and C. Raetz, unpublished observations).

The GalN moiety (Fig. 3), which is present on both free lipid A and on LPS-bound lipid A (Fig. 4), is attached by FlmK (an ortholog of *E. coli* ArnT) on the outer surface of the IM (33, 37). The GalN donor is undecaprenyl-phosphate (C55-P)-GalN (Fig. 4), which is generated from C55P and UDP-GalNAc (X. Wang, F. Song, and C. Raetz, unpublished observations). The removal of the 3′-hydroxyacyl chain (Fig. 4) probably occurs in the OM (Fig. 5), as in the case with *Salmonella* LpxR (38), but the relevant *F. novicida* 3′-*O*-deacylase gene has not yet been identified. Interestingly, *F. novicida* KdtA is bifunctional (J. Zhao and C. Raetz, unpublished observations), but its LPS contains only one Kdo residue (31). This anomaly is explained in part by the presence of a Kdo hydrolase in *F. novicida* (35), which consists both of a catalytic and a membrane-anchoring subunit, termed KdoH1 and KdoH2, respectively (J. Zhao and C. Raetz, unpublished observations). This type of hydrolase is also found in *Helicobacter* (39). Deletion of KdtA in *F. novicida* is not lethal, but it eliminates LPS and causes modest accumulation of free lipid A (J. Zhao and C. Raetz, unpublished observations).

DIVERSITY AND INTERCHANGEABILITY OF LIPID A MODIFICATION SYSTEMS

Whereas the constitutive lipid A pathway is conserved, the systems for lipid A modification are diverse and are usually not required for growth (5). What the modification enzymes have in common is their localization (Fig. 5) (5). With few exceptions (5), removal or modification of the lipid A phosphate groups occurs on the outer surface of the IM, whereas removal of acyl chains generally occurs in the OM (5) (Fig. 5). The distinct localization of the modification enzymes, in contrast to the intracellular constitutive enzymes (Fig. 2), makes the former excellent reporters for LPS trafficking (40, 41).

Lipid A modification enzymes can be transferred from one bacterial species to another. Expression of LpxE in *E. coli* or *Salmonella* results in nearly quantitative 1-dephosphorylation of lipid A without obvious growth impairment (35). Expression of *F. novicida* LpxF in *E. coli* or *Salmonella* LpxM mutants (which synthesize penta-acylated lipid A) results in the complete removal of the 4′-phosphate group (34). However, wild-type *F. novicida* LpxF cannot dephosphorylate hexa-acylated lipid A (34).

The attenuation of *F. novicida* LpxF mutants results from their hypersensitivity to cationic antimicrobial peptides, because of the negative charge presented by the LpxF mutant lipid A (Fig. 3) (36). Consequently, it is now possible to construct novel attenuated bacterial strains by interchanging or mutating lipid A modification enzymes (34, 36).

LIPID A AS AN ACTIVATOR OF INNATE IMMUNITY

Many Gram-negatives synthesize lipid A species resembling those of *E. coli* (Fig. 2) (5). As noted above, lipid A is detected by the TLR4/MD2 receptor, the crystal structure of which was recently determined (24, 25). *E. coli* lipid A induces macrophages to make potent mediators of inflammation, such as tumor necrosis factor-α and interleukin-1β

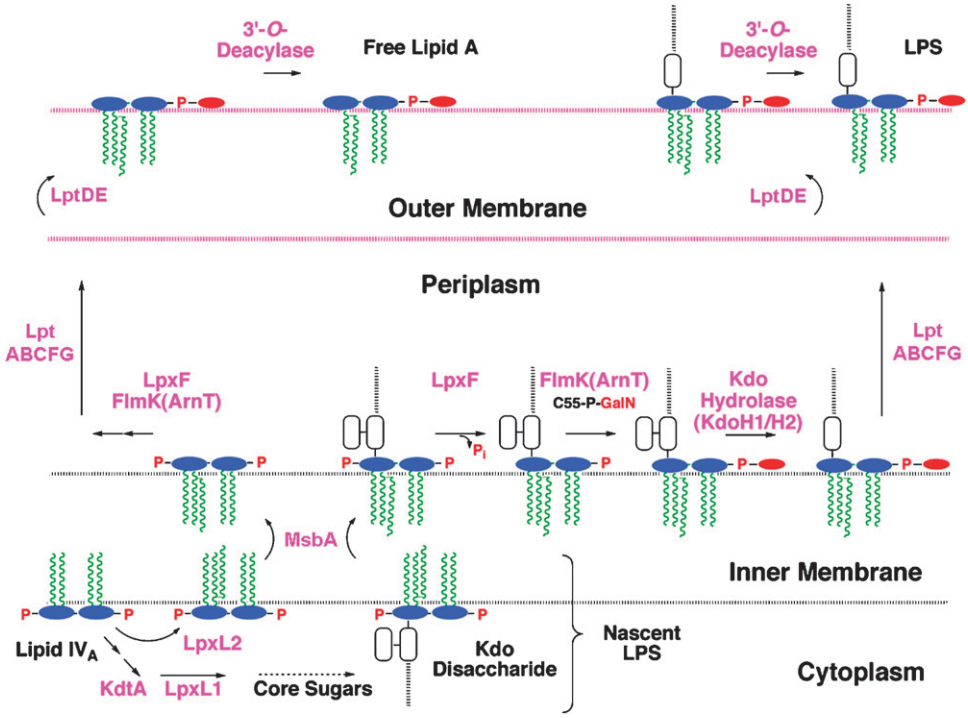

Fig. 5. Topography of lipid A export and modification in *F. novicida*.

(4). These events are desirable for clearing local infections, but when overproduced systemically during sepsis, these proteins can damage small blood vessels and contribute to septic shock (42). The characteristic structural features of *E. coli* lipid A (Fig. 2), especially its two phosphate groups and acyloxyacyl chains, are needed to trigger full TLR4/MD2 activation (4). With the availability of modification enzymes like LpxE, the lipid A moiety of live bacteria can now be altered by heterologous expression (35) to be a TLR4/MD2 partial agonist, thereby retaining its desirable adjuvant properties while greatly reducing its toxicity (43).

DIRECTIONS FOR THE FUTURE

The following questions regarding lipid A biology and chemistry remain to be answered: *1*) Why is the lipid A moiety of LPS required for growth in most Gram-negative bacteria? Studies of unusual systems, like *N. meningitidis*, in which lipid A is not required for growth, might provide the answer (30); *2*) Can in vitro assays for LPS flip-flop and intermembrane transport be developed? The genetic evidence for the role of the MsbA transporter and the Lpt protein complex is compelling, but it needs to be strengthened with in vitro biochemistry employing purified components; *3*) Can new antibiotics be developed by targeting LpxC and other enzymes of the lipid A pathway? The best available LpxC inhibitors (14) are potent enough for clinical trials, but studies of bioavailability, pharmacokinetics and toxicology have not been published; and *4*) Can lipid A modification enzymes be exploited for vaccine development? The opportunities afforded by attenuated *lpxF* mutants of *F. tularensis* (36) or strains lacking *lpxM* (*msbB*) are under investigation (29). The properties of *Salmonella* strains expressing LpxE, which synthesize the mono-phosphorylated, nontoxic adjuvant form of lipid A, will be of great interest.

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