

Review

Lipopolysaccharide: Biosynthetic pathway and structure modification

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ABSTRACT

Lipopolysaccharide that constitutes the outer leaflet of the outer membrane of most Gram-negative bacteria is referred to as an endotoxin. It is comprised of a hydrophilic polysaccharide and a hydrophobic component referred to as lipid A. Lipid A is responsible for the major bioactivity of endotoxin, and is recognized by immune cells as a pathogen-associated molecule. Most enzymes and genes coding for proteins responsible for the biosynthesis and export of lipopolysaccharide in *Escherichia coli* have been identified, and they are shared by most Gram-negative bacteria based on genetic information. The detailed structure of lipopolysaccharide differs from one bacterium to another, consistent with the recent discovery of additional enzymes and gene products that can modify the basic structure of lipopolysaccharide in some bacteria, especially pathogens. These modifications are not required for survival, but are tightly regulated in the cell and closely related to the virulence of bacteria. In this review we discuss recent studies of the biosynthesis and export of lipopolysaccharide, and the relationship between the structure of lipopolysaccharide and the virulence of bacteria.

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Abbreviations: LPS, lipopolysaccharide; TLR4, toll-like receptor 4; Kdo, 3-deoxy-D-manno-octulosonic acid; Hep, L-glycero-D-manno-heptose; CAMPs, cationic antimicrobial peptides; α -L-Ara4N, 4-amino-4-deoxy- α -L-arabinose; Und-P- α -L-Ara4N, undecaprenyl phosphate-L-Ara4N.

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1. Introduction

Gram-negative bacteria have two distinct membranes: an inner membrane and an outer membrane. A prominent constituent of the outer leaflet of the outer membrane is lipopolysaccharide (LPS). The LPS components of many bacteria are toxic. The

discovery of endotoxin in the late 19th century was based on the demonstration that heat-killed cholera bacteria were themselves toxic rather than causing toxicity by secretion of a product from the living organism. Secreted toxins became broadly known as “exotoxins”, and the toxic materials of bacteria as “endotoxin”. The historical aspects of the role of endotoxins in bacterial patho-

genesis and their chemical characterization as LPS have been the subject of a comprehensive review [1].

The LPS molecule can be divided into three parts: lipid A, core polysaccharides and O-antigen repeats (Fig. 1). Lipid A represents the hydrophobic component of LPS which locates in the outer leaflet of the outer membrane, while core polysaccharides and

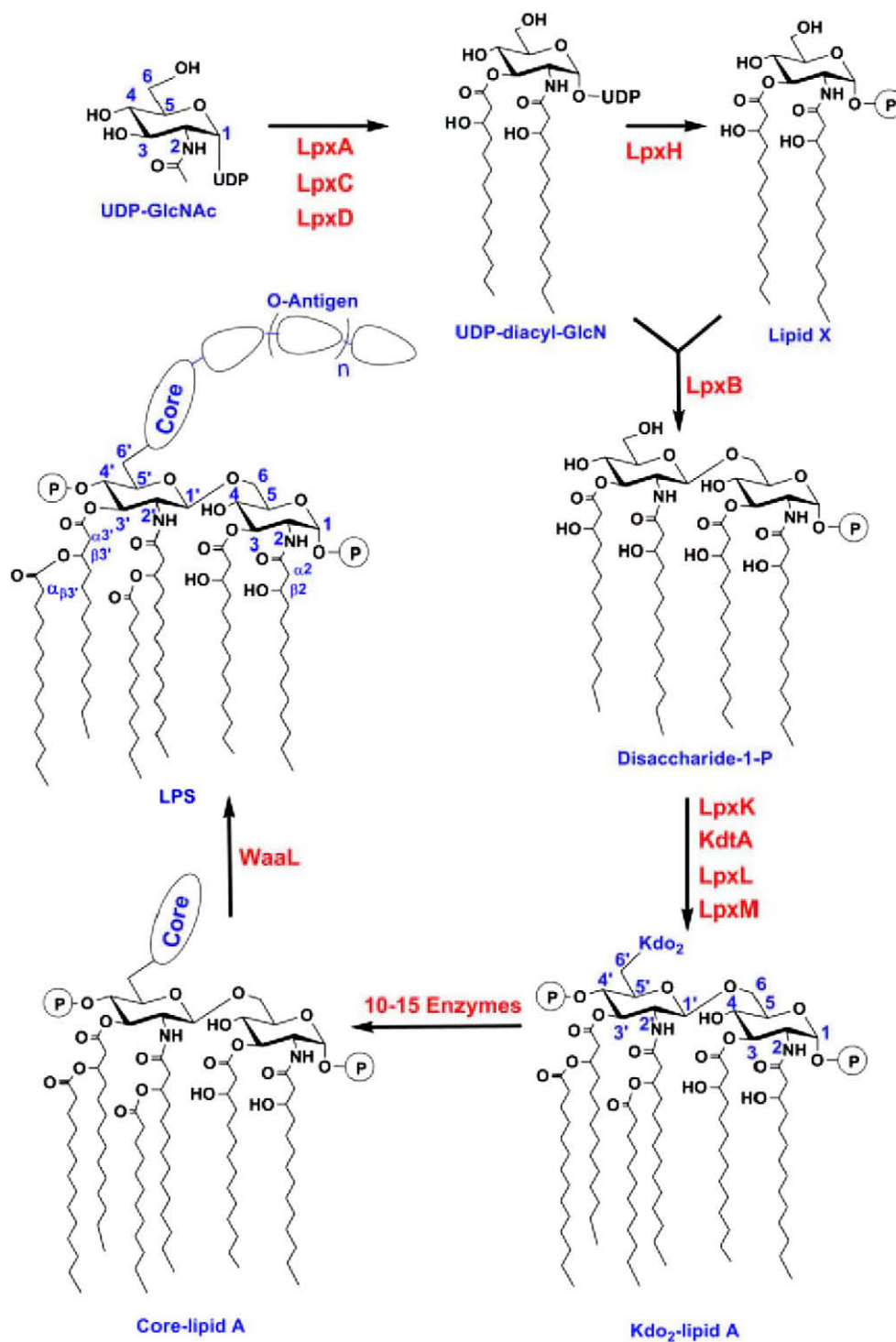


Fig. 1. Structure and biosynthetic pathway of LPS in *E. coli*. Each reaction is catalyzed by a single enzyme. The name of the enzyme is highlighted in red, and the name of the substrate in blue. The structure of lipid A is shown in detail, but structures of core oligosaccharides and O-antigen are simplified as symbols since there are many variations in these two regions. The genes encoding the enzymes of lipid A biosynthesis are present in single copy and highly conserved among bacteria [2,3]. The core region usually contains 10–15 monosaccharides. The O-antigen usually contains only a few monosaccharides, but can be repeated many times in LPS. Noncarbohydrate components are also found in these regions.

O-antigen repeats are displayed on the surface of the bacterial cells [2,3]. Lipid A is known to be responsible for the toxic effects of infections with Gram-negative bacteria [4]. The detailed structure of LPS varies from one bacterium to another, and this variation could affect the virulence of the bacterium [5]. The biosynthetic pathway of LPS has been well characterized in *E. coli*. The biosynthetic pathway and export mechanisms of LPS are common to most Gram-negative bacteria, but some bacterial pathogens can further modify the basic structure of their LPS.

LPS can be recognized by toll-like receptor 4 (TLR4), a receptor found on the surface of different immune cells of host organisms such as monocytes, macrophages, neutrophils and dendritic cells [6,7]. TLR4 functions as a dimer, and depends on a small protein MD-2 for the recognition of LPS [8]. Other proteins such as CD14 and LBP facilitate the presentation of LPS to MD-2 [9,10]. After activation by LPS, TLR4 recruits adapter molecules such as MyD88, Mal, Trif, and Tram within the cytoplasm of cells to propagate a signal [11,12]. These adapter molecules in turn activate other molecules within the cell, including protein kinases IRAK1, IRAK4, TBK1, and IKKi, to amplify the signal, and result in the induction or suppression of genes that orchestrate the inflammatory response. High concentrations of LPS can induce fever, increase heart rate, and lead to septic shock and death following lung or kidney failure. However, in relatively low concentrations lipid A is an active immuno-modulator, which can induce non-specific resistance to both bacterial and viral infections. Due to its importance, the biosynthetic pathway and structure modification of lipid A and LPS have been the subject of considerable interest. Here we summarize recent studies of the biosynthesis and structure modification of lipid A and LPS, and discuss the relationship between the structure of lipid A and the virulence of the bacteria.

2. Biosynthetic pathway of lipopolysaccharide

LPS molecules are major constituents of the outer leaflet of the outer membranes in most Gram-negative bacteria. They are essential for the survival of bacteria, including some pathogens. Some LPS molecules can cause human diseases such as septic shock. The biosynthesis of LPS has been intensively studied in order to develop methods to control Gram-negative pathogens and to cure septic shock. Although LPS distributes on the surface of bacterial cells, its synthesis is initiated in the cytoplasm. How LPS is synthesized in the cytoplasm and exported to the surface of bacteria has been studied most extensively in *E. coli*. The biosynthesis of LPS in *E. coli* is initiated from a small molecule, UDP-*N*-acetylglucosamine (UDP-GlcNAc). A multiplicity of enzymes sequentially function to convert UDP-GlcNAc into disaccharide-1-P, Kdo₂-lipid A, core-lipid A, and culminating in LPS (Fig. 1). The structure of lipid A is more widely conserved in different bacteria than are the core oligosaccharides or the O-antigen repeats of LPS. This is also the case for the enzymes involved in the biosynthesis of LPS.

2.1. Biosynthesis of Kdo₂-lipid A

E. coli is the most favoured Gram-negative bacterium for studies of LPS biosynthesis. The first stage of the biosynthetic pathway is the synthesis of Kdo₂-lipid A [3,13]. The pathway is mediated by nine enzymes (Table 1) and takes place in the cytoplasm and on the inner surface of inner membrane. The initial building block of lipid A is UDP-GlcNAc. The first three reactions are catalyzed by soluble enzymes LpxA, LpxC and LpxD, resulting in the addition of two 3-OH fatty acid chains to the 2- and 3-positions of the UDP-GlcNAc to form UDP-diacyl-GlcN (Fig. 1). The first reaction catalyzed by LpxA is reversible; the second reaction catalyzed by LpxC is a committed step. LpxA, LpxC and LpxD have been isolated and their structure characterized by X-ray diffraction and NMR methods [14–16]. The active forms of LpxA and LpxD are homotrimers. LpxC is a Zn²⁺-dependent enzyme which has no sequence homology with other deacetylases which makes it a promising target for development of novel antibiotics. The active site of *E. coli* LpxA functions as a precise hydrocarbon ruler and is manifest by incorporation of C14 hydroxyacyl chains at a rate two orders of magnitude faster than C12 or C16 chains, consistent with the structure of lipid A (Fig. 1).

The UDP-diacyl-GlcN is next hydrolyzed by LpxH to form lipid X [17,18]. LpxB condenses lipid X and its precursor UDP-diacyl-GlcN to form disaccharide-1-P [19,20]. Both LpxH and LpxB enzymes catalyzing the reactions are peripheral membrane proteins. The enzymes that catalyze the followed reactions in the pathway, LpxK, KdtA, LpxL and LpxM, respectively, are integral proteins of the inner membrane. LpxK is a kinase that phosphorylates the 4'-position of the disaccharide-1-P to form lipid IV_A [21,22]. KdtA is a bifunctional enzyme that incorporates two 3-deoxy-D-manno-octulosonic acid (Kdo) residues at the 6'-position of the lipid IV_A, using the sugar nucleotide CMP-Kdo as the donor [23]. The resulting Kdo₂-lipid IV_A undergoes further reactions catalyzed by LpxL and LpxM to form Kdo₂-lipid A (Fig. 1). LpxL adds a secondary lauroyl residue and LpxM adds a myristoyl residue to the distal glucosamine unit, respectively [24]. These subsequent acylations do not depend on Kdo *in vivo* [25]. The nine enzymes involved in the biosynthesis of Kdo₂-lipid A all have relatively high specificity for their respective substrates (Table 1). For example, LpxA, LpxD, LpxL and LpxM are all acyltransferases, but they selectively catalyze different substrates and employ different acyl donors. The minimal LPS structure needed for the viability of *E. coli* is lipid IV_A although such *E. coli* mutants exhibit highly attenuated growth [25]. *E. coli* mutants able to synthesize Kdo₂-lipid IV_A are able to grow more vigorously than the lipid IV_A mutant. The slow growth of these mutants appears to be due to defects in the export of such minimal LPS molecular species [25].

The gene cluster, *lpxD-fabZ-lpxA-lpxB*, encoding proteins LpxD, FabZ, LpxA and LpxB has been characterized in *E. coli* and several other species of bacteria [26,27]. Proteins LpxA, LpxB and LpxD

Table 1

Nine enzymes required for the biosynthesis of lipid A and the single-copy genes encoding them in *E. coli*.

Enzymes	Genes	Function
LpxA	<i>lpxA</i>	LpxA catalyzes the fatty acylation of UDP-GlcNAc. It requires the thioester R-3-hydroxymyristoyl acyl carrier protein as its donor [14]
LpxC	<i>lpxC</i>	LpxC catalyzes the deacetylation of UDP-3-O-(acyl)-GlcNAc which is the actual committed step of lipid A biosynthesis [15]
LpxD	<i>lpxD</i>	LpxD adds a second R-3-hydroxymyristate chain to make UDP-2,3-diacyl-GlcN [16]
LpxH	<i>lpxH</i>	LpxH cleaves the pyrophosphate linkage of UDP-2,3-diacyl-GlcN to make lipid X [17,18]
LpxB	<i>lpxB</i>	LpxB condenses UDP-2,3-diacyl-GlcN with lipid X to form the 1'-6-linkage in lipid A [19,20]
LpxK	<i>lpxK</i>	LpxK phosphorylates the 4'-position of the disaccharide 1-phosphate generated by LpxB to form lipid IV _A [21,22].
KdtA	<i>kdtA</i>	KdtA incorporates two Kdo residues to the 6'-position of lipid IV _A [23]
LpxL	<i>lpxL</i>	LpxL adds a secondary lauroyl residue to the fatty acid chain at 2'-position of lipid A. It prefers acyl-ACP donors [24]
LpxM	<i>lpxM</i>	LpxM adds a secondary lauroyl residue to the fatty acid chain at 3'-position of lipid A. It prefers acyl-ACP donors [24]

catalyze early steps in the lipid A pathway using (3R)-hydroxyacyl-ACP as a donor; while FabZ catalyzes the dehydration of (3R)-hydroxyacyl-ACP to trans-2-acyl-ACP [28], which is further utilized as a fatty acid donor in the biosynthesis of phospholipids. Therefore, this gene cluster could be important for regulating the proportions of LPS and phospholipids in membranes of the bacteria. Another gene cluster, *msbA-lpxK*, also exists in many Gram-negative bacteria, and these two genes are even found to be fused together in some marine bacteria [29]. MsbA is known as a specific transporter primarily for LPS, while LpxK is a kinase that adds a phosphate group to the 4'-position of lipid A [21,22]. Why these two genes are always in the same cluster is not clear.

2.2. Connection of the core oligosaccharides

The structure of lipid A is highly conserved, while the structure of the core oligosaccharides shows some variations. The core oligosaccharides are sequentially assembled on lipid A at the cytoplasmic surface of the inner membrane in a process that involves a number of membrane-associated glycosyltransferases, using nucleotide sugars as donors. The biosynthesis of core oligosaccharides is rapid and efficient, suggesting that the glycosyltransferases function as a coordinated complex. Core oligosaccharides can be divided into two structurally distinct regions: the inner core which connects to lipid A and the outer core which connects to the O-antigen repeats. The inner core oligosaccharides typically contain residues of Kdo and α -glycero-D-manno-heptose (Hep). The Kdo residue is the most conserved component found in the core region of LPS. The outer core oligosaccharides show more structural diversity than those of the inner core. Structures of core oligosaccharides in *E. coli* strains R1, R2, R3, R4, and K-12 are different [30,31], but the basic backbones are all a linear oligosaccharide of six units which can be attached to other units to create branches. The common sugars found in the core oligosaccharides are Kdo, Hep, D-glucose and D-galactose.

In *E. coli* and *Salmonella*, genes required for the biosynthesis of core oligosaccharides exist in three operons: *gmhD*, *waaQ* and *kdtA* operons [32]. In *E. coli* K-12, the *gmhD* operon contains four genes *gmhD-waaF-waaC-waaL* that are required for the biosynthesis of inner core oligosaccharides [33]. The *gmhD*, *waaF* and *waaC* genes

encode proteins involved in the biosynthesis and transfer of Hep, whereas the *waaL* gene encodes a ligase enzyme required for the attachment of O-antigen to the core-lipid A [34] (Fig. 2). The *waaQ* operon contains 7–9 genes that code for enzymes that are responsible for the biosynthesis of outer core oligosaccharides and its modification. The gene *kdtA* in the *kdtA* operon encodes KdtA that can add two Kdo residues to the lipid IV_A [23].

2.3. Addition of O-antigen to form lipopolysaccharide

O-antigen, similarly to the core oligosaccharides, is synthesized on the cytoplasmic surface of the inner membrane. Using the sugar nucleotides as donors, the units of O-antigen are assembled by glycosyltransferase enzymes on the membrane-bound carrier, undecaprenyl phosphate which is also used for synthesis of peptidoglycan and capsular polysaccharides. The *rfb* gene cluster in both *E. coli* and *Salmonella enterica* encodes the enzymes required for the synthesis of the sugar-nucleotide precursors that are unique to O-antigens, the glycosyltransferases and polymerases needed for the assembly of the O-antigen and the components required for the transfer of O-antigen polymers across the inner membrane [3].

The O-antigens of LPS exhibit considerable diversity. The O-antigen can be homopolymers or heteropolymers. The connection of units in O-antigen may be linear or branched. The unit structures of O-antigen can differ in the monomer type as well as the position and stereochemistry of the O-glycosidic linkages. The numbers of O-antigen groups can be up to 60 in *S. enterica* and 164 in *E. coli*, but only three structures are shared by the two genera.

After synthesis on the cytoplasmic face, both core-lipid A and O-antigen are transported to the periplasmic face of the inner membrane, where O-antigen is polymerized by Wzy and Wzz and ligated to the core-lipid A by WaaL, resulting in a nascent LPS [35] (Fig. 2).

3. Transport of lipopolysaccharide

LPS molecules are essential for the survival of most Gram-negative bacteria. They are synthesized in the cytoplasm and peri-

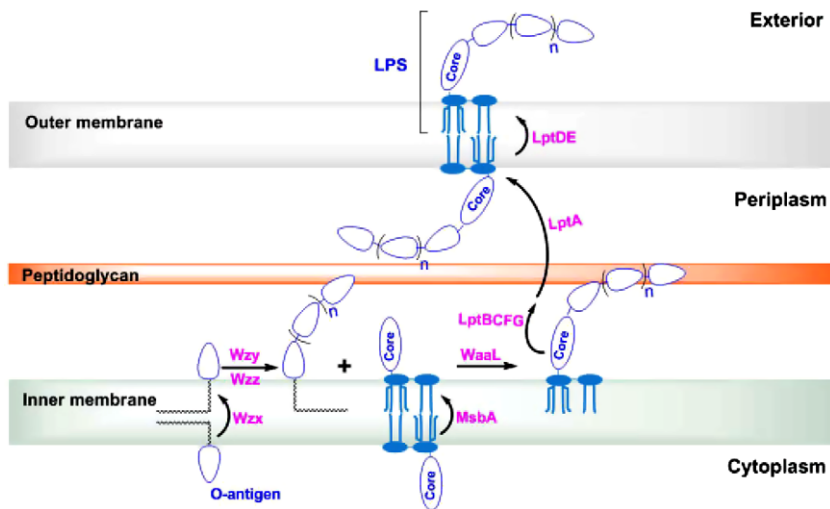


Fig. 2. Export of LPS and its precursors in *E. coli*. The ABC transporter MsbA flips the core-lipid A from the inner surface to the outer surface of the inner membrane. O-antigen oligosaccharides is assembled separately on undecaprenyl diphosphate, flipped from the cytoplasmic face to the periplasmic face of the inner membrane by the transporter Wzx, and polymerized on the periplasmic face of the inner membrane by Wzy and Wzz and then transferred to the core-lipid A by WaaL. The protein LptA, LptB, LptC, LptF and LptG might shuttle the nascent LPS from the periplasmic face of the inner membrane to the inner layer of the outer membrane. The outer membrane proteins LptD and LptE are required for the assembly of LPS into the outer surface of the outer membrane [48,49].

plasm, and exported to the outer leaflet of outer membranes. The transport of LPS is critical because defects in the export of LPS are known to be lethal. The enzymes involved in the export of lipid A and LPS and their genes are listed in Table 2. The processes associated with the export of lipid A and LPS are briefly outlined in Fig. 2. Wzx flips the O-antigen from the cytoplasmic face to the periplasmic face of the inner membrane, and MsbA flips the core-lipid A in the same way. In the periplasmic face of the inner membrane, the O-antigen is polymerized by Wzy and Wzz to form O-antigen repeats which are, in turn, transferred to the core-lipid A by WaaL resulting in the nascent LPS. The proteins LptA, LptB, LptC, LptF and LptG then shuttle the nascent LPS from the periplasmic face of the inner membrane to the inner surface of the outer membrane, where LptD and LptE assemble LPS into the outer surface of the outer membrane.

3.1. Crossing the inner membrane

LPS is assembled in the periplasm but precursors of LPS, the core-lipid A and the O-antigen, are synthesized at the cytoplasmic surface of the inner membrane. Therefore, the core-lipid A and the O-antigen must be transported across the inner membrane [13].

The transport of core-lipid A is carried out by a membrane protein MsbA [36,37]. In *E. coli*, MsbA is a homodimer and each monomer contains six transmembrane helices and a cytosolic ATP-binding domain [38]. MsbA is highly conserved in Gram-negative bacteria and shares homology with the multidrug resistance (MDR) proteins of eukaryotes.

The transport of the O-antigen across the inner membranes is mediated by Wzx protein [39,40]. Wzx proteins from different bacteria have similar hydrophathy profiles [41] and can complement each other in the translocation of different O-antigen sugar precursors, but no sequence homology or conserved residues are found amongst Wzx proteins [42]. There is evidence that Wzx proteins might function by recognizing the first sugar phosphate bound to

the undecaprenyl-P [43]. ATP-binding domains do not exist in the primary sequences of Wzx proteins [43,44].

3.2. Reaching the surface

The next question is how does LPS cross the periplasmic space and reach to the outer surface of the outer membrane? Several proteins have been reported to function in this process (Fig. 2). They are the periplasmic protein LptA, the cytosolic protein LptB, the inner membrane proteins LptC, LptF and LptG, and the outer membrane proteins LptD and LptE [45–47]. It appears that some of these proteins may function as complexes [50]. The ABC transporter LptBFG, functioning with LptC and LptA, translocates LPS to the inner leaflet of the outer membrane [45,46]. When LptA, LptB, or both were depleted, LPS was found to accumulate in the periplasm [45]. In the outer membrane, nascent LPS is exported to the outer leaflet by LptD and LptE [45,46,48–50]. Further *in vitro* studies are needed to confirm the functions of these LPS transporters.

4. Structural modification of lipopolysaccharide

Bacteria not only require numerous genes to synthesize and transport LPS, but have also evolved mechanisms to modify their LPS structure, even the most conserved part, lipid A. Modification of lipid A has been the subject of many studies over recent years and it has been shown that this can involve both the hydrophilic disaccharide region as well as the hydrophobic acyl chain domain. Table 3 lists the enzymes that modify the structure of lipid A and their genes. Orthologs of the genes required for the biosynthesis of lipid A in *E. coli* exist in most Gram-negative bacteria, suggesting that lipid A synthesis is separated from the modifications *in vivo*. Modifications of lipid A usually occur at the periplasmic face of the inner membrane or in the outer membrane. The structure modification of lipid A might help the bacteria to resist the cationic

Table 2
Enzymes involved in the transport of LPS and its precursors in *E. coli*.

Enzymes	Genes	Function
MsbA	<i>msbA</i>	MsbA is an essential ABC transporter. It exports core-lipid A from the cytoplasmic to the periplasmic face of the inner membrane [36–38]
Wzx	<i>wzx</i>	Wzx exports undecaprenol phosphate O-antigen from the cytoplasmic to the periplasmic face of the inner membrane [39,40]
LptA	<i>lptA</i>	LptA is a periplasmic protein required for LPS transport across the periplasm [45]
LptB	<i>lptB</i>	LptB is a cytoplasmic protein associated with the inner membrane. It is required for LPS transport across the periplasm [45]
LptC	<i>lptC</i>	LptC is an inner membrane protein required for LPS transport across the periplasm [45,46]
LptD	<i>lptD</i>	LptD is an essential β -barrel outer membrane protein. It is required for LPS assembly at the outer surface of the outer membrane [48,49]
LptE	<i>lptE</i>	LptE is an essential outer membrane lipoprotein. It is required for LPS assembly at the outer surface of the outer membrane [48,49]
LptF	<i>lptF</i>	LptF is an inner membrane protein required for LPS transport across the periplasm [46,47]
LptG	<i>lptG</i>	LptG is an inner membrane protein required for LPS transport across the periplasm [46,47]

Table 3
Enzymes involved in the structural modification of lipid A in Gram-negative bacteria. The structure and numbering scheme of lipid A are shown in Fig. 1.

Enzymes	Genes	Function
LpxE	<i>lpxE</i>	LpxE removes the phosphate group from the 1-position of lipid A [92]
LpxF	<i>lpxF</i>	LpxF removes the phosphate group from the 4'-position of lipid A [91]
LpxO	<i>lpxO</i>	LpxO adds a OH group to the $\alpha_{\beta 3}$ -position [83,84]
ArnT	<i>arnT</i>	ArnT transfers the L-Ara4N unit to lipid A [97]
LpxR	<i>lpxR</i>	LpxR catalyzes the removal of the 3'-acyloxyacyl moiety [81]
PagL	<i>pagL</i>	PagL removes the 3-O-linked acyl chain of lipid A [80]
PagP	<i>pagP</i>	PagP transfers a palmitate from glycerophospholipids to the 2-position of lipid A [77,78]
PmrC	<i>pmrC</i>	PmrC adds a phosphoethanolamine to 1-position of lipid A [98]
LpxXL	<i>lpxXL</i>	LpxXL adds a very long fatty acid chain to the β_2 -position [88]
LpxT	<i>lpxT</i>	LpxT transfers a phosphate group to the 1-phosphate of lipid A [101]
LpxQ	<i>lpxQ</i>	LpxQ oxidizes the proximal glucosamine of lipid A to form an aminogluconate unit [103]
LmtA	<i>lmtA</i>	LmtA catalyzes the methylation of 1-phosphate of lipid A [102]

antimicrobial peptides (CAMPs) released by the host immune system, or to evade recognition by the innate immune receptor TLR4.

4.1. Regulation of lipid A modification

Some modifications of the lipid A are under control of the PhoP–PhoQ system and/or PmrA–PmrB system [51]. PhoP–PhoQ is a two-component system that governs virulence, mediates the adaptation to Mg^{2+} -limiting environment and regulates numerous cellular activities in Gram-negative bacteria [52–54]. It consists of an inner membrane sensor PhoQ and a cytoplasmic regulator PhoP (Fig. 3). PhoQ contains an acidic patch on the surface of its periplasmic domain. Mg^{2+} bridges the acidic patch with anionic phospholipid polar head groups to maintain a repressed regulatory state [55,56]. The PhoP–PhoQ system can also be activated when the bacterium is exposed to CAMPs [56–58]. The activation of the PhoP–PhoQ system can lead to the activation or repression of over 40 genes [59,60].

A PmrA–PmrB two-component system is also required for *S. enterica* virulence in mice [61]. It is usually induced by high Fe^{3+} , the specific signal recognized by the sensor PmrB [62]. It can also be induced by low Mg^{2+} , which is detected by the sensor PhoQ of the PhoP–PhoQ system [63]. The activation by low Mg^{2+} requires PhoP, PhoQ, PmrA and PmrB proteins [64] as well as the PhoP-activated PmrD protein [65] (Fig. 3). In *E. coli*, the PmrA–PmrB pathway cannot be triggered by the PhoP–PhoQ system because the PmrD is not functional [66].

The best example of the regulation of PmrA is the *arn* operon [67–70] and *ugd* gene [71]. Protein products encoded by these genes can synthesize and incorporate a 4-amino-4-deoxy- α -L-arabinose (α -L-Ara4N) into the lipid A [67,70,72]. This modification can assist the bacteria resist the antibiotic polymyxin B [73]. The *arn* operon contains *arnB*–*arnC*–*arnA*–*arnD*–*arnT*–*arnE*–*arnF* genes that encode seven enzymes, ArnB, ArnC, ArnA, ArnD, ArnT, ArnE and ArnF, respectively [74]. Ugd initiates the pathway by converting UDP-glucose to UDP-glucuronic acid. The C-terminal domain of ArnA catalyzes the oxidative decarboxylation of UDP-glucuronic acid to generate UDP-4-keto-pyranose. ArnB then catalyzes a transamination using glutamic acid as the amine donor to form UDP-L-Ara4N. Subsequently, the N-terminal domain of ArnA uses N-10-formyltetrahydrofolate to synthesize N-formylate UDP- α -L-Ara4N, which is, in turn, transferred by ArnC to undecaprenyl phosphate. Then ArnD catalyzes deformylation of this substrate

to undecaprenyl phosphate- α -L-Ara4N (Und-P- α -L-Ara4N). ArnE and ArnF flip the Und-P- α -L-Ara4N from the cytoplasmic face to the periplasmic face of the inner membrane [75], where ArnT transfers the L-Ara4N unit to the core-lipid A (Fig. 3).

4.2. Modification in the fatty acyl chain region

Several enzymes have been reported to modify the fatty acyl chain region of lipid A. They are membrane proteins PgaP, PagL, LpxR and LpxO.

PagP is a palmitoyl transferase which locates in the outer membrane; it transfers a palmitate from glycerophospholipids to the β 2-position of lipid A, resulting in a hepta-acylated structure [76]. PagP is regulated by PhoP–PhoQ system. It was originally identified in *Salmonella* as a protein that is important for resistance to certain CAMPs. The hepta-acylated structure of lipid A might prevent the insertion of CAMPs. PagP has been well characterized in both *E. coli* and *Salmonella*, and the structure has been determined by both NMR spectroscopy and X-ray crystallography [77,78].

PagL is a lipase that removes the 3-O-linked acyl chain of lipid A but plays no role in antimicrobial peptide resistance [79]. Like PagP, PagL is also located in the outer membrane. The *pagL* mutant of *Salmonella typhimurium* displays no obvious phenotypes in a murine model. Although PagL is under the control of the PhoP–PhoQ system it is not active in the outer membrane of *Salmonella* when grown under Mg^{2+} -limiting conditions. PagL might be post-translationally inhibited within the outer membrane because it could be activated in mutants of *Salmonella* that were unable to modify their lipid A with L-Ara4N. PagL from *Pseudomonas aeruginosa* consists of an eight-stranded beta-barrel with the axis tilted by approximately 30 degrees with respect to the lipid bilayer. It contains an active site with a Ser–His–Glu catalytic triad and an oxyanion hole that comprises the conserved Asn [80]. Molecules of lipid A, PagL and PagP all locate in the outer membrane of bacteria, which facilitates the rapid modification of the lipid A structure.

LpxR is another outer membrane protein that removes the 3'-acyloxyacyl moiety of *Salmonella* lipid A [81]. Orthologs of *Salmonella* LpxR can be found in various Gram-negative bacteria such as *Helicobacter pylori*, *Yersinia enterocolitica*, *E. coli* O157:H7, and *Vibrio cholerae*. LpxR usually remains inactive in *Salmonella* outer membrane, but appears to be activated in *H. pylori* since the major lipid A species of *H. pylori* is completely 3'-O-deacylated. LpxR is

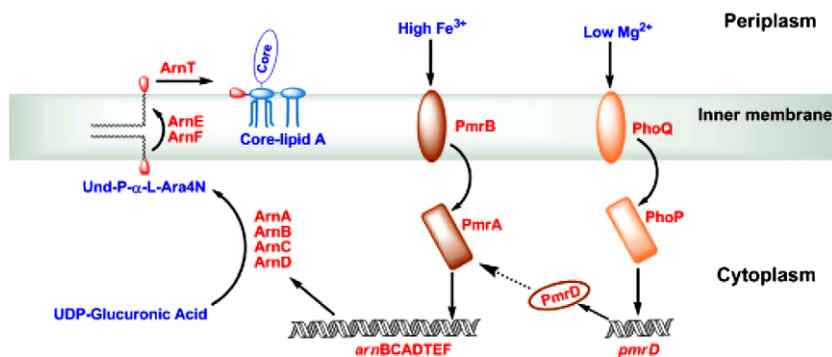


Fig. 3. Biosynthesis of undecaprenyl phosphate- α -L-Ara4N and transfer of the L-Ara4N moiety to lipid A are regulated by the PhoP–PhoQ and PmrA–PmrB systems. In *S. typhimurium*, transcription of PmrA-activated genes is promoted during growth in low Mg^{2+} via the PhoP–PhoQ system, and the PmrA–PmrB system; and in the presence of high Fe^{3+} via the PmrA–PmrB system, independently of PhoP–PhoQ and PmrD. In *E. coli*, transcription of PmrA-activated genes is promoted in the presence of Fe^{3+} via the PmrA–PmrB system. The PmrD protein is produced in low Mg^{2+} in a PhoP-dependent manner, but it cannot activate the PmrA–PmrB system. The *arn* operon contains seven genes required for the biosynthesis of Und-P- α -L-Ara4N and transfer of the L-Ara4N moiety to lipid A is regulated by the activated PmrA. Soluble proteins ArnA, ArnB, ArnC and ArnD convert UDP-glucuronic acid to Und-P- α -L-Ara4N on the cytoplasmic face of the inner membrane. Membrane proteins ArnE and ArnF then transport Und-P- α -L-Ara4N to the periplasmic face of the inner membrane, where the membrane protein ArnT transfers the L-Ara4N moiety (shown as a red strawberry shape) to the core-lipid A.

not regulated by either PhoP–PhoQ or PmrA–PmrB, but requires the divalent cation Ca^{2+} for enzymatic activity. The crystal structure of *S. typhimurium* LpxR revealed that it is a 12-stranded beta-barrel and its active site is located between the barrel wall and an alpha-helix formed by an extracellular loop [82].

LpxO is an inner membrane protein that can generate a 2-OH at the $\alpha\beta_3$ -position of *Salmonella* lipid A [83,84]. This hydroxylation is independent of MsbA transport, indicating a cytoplasmic active site for LpxO. LpxO is not regulated by either the PhoP–PhoQ or the PmrA–PmrB systems.

The length of the fatty acid chains in the lipid A differs in different Gram-negative bacteria [85]. For example, the fatty acyl chains of *E. coli* lipid A are 12 or 14 carbons long, while that of *Francisella novicida* lipid A are 16–18 carbons long [86]. Interestingly, the *Rhizobium etli* lipid A contains a very long fatty acyl chain which has 28 carbons and is attached to lipid A by acyltransferase LpxXL [87]. LpxXL plays an important role in bacterial development [88].

4.3. Modification in the hydrophilic region

In addition to changes in the fatty acid region, the hydrophilic region of lipid A molecule can also be modified. Lipid A usually contains two phosphate groups which impart a net negative charge to the molecule. The negative charges of lipid A allow the binding of positively charged CAMPs. To evade the attack by the immune system some bacterial pathogens have evolved a less negatively-charged variation of lipid A. Modification to the hydrophilic region of lipid A focuses on the removal or decoration of the phosphate groups at the 1- and 4'-positions. The modification can include the addition of the amine-containing residues such as α -L-Ara4N and phosphoethanolamine. These modifications result in resistance to CAMPs and to polymyxin B and are controlled by the PmrA–PmrB two-component system.

The removal of phosphate groups to reduce the overall negative charge of lipid A occurs in several bacterial pathogens or endosymbionts. For example, *R. etli* lipid A does not contain phosphate [89], while *Francisella tularensis* lipid A contains only one phosphate group [90]. The absence of a phosphate group would greatly decrease the surface negative charge of these bacteria. Two genes *lpxE* and *lpxF* encoding the lipid A phosphatases have been identified in *F. novicida* [91,92]. LpxE selectively removes the phosphate group at the 1-position of lipid A, while LpxF selectively removes the phosphate group at the 4'-position. The *lpxF* deletion mutant of *F. novicida* no longer infects host mice [93], suggesting that the phosphate group on lipid A is closely related to the infectivity of bacteria. Orthologs of LpxE also exist in *R. etli* and in *H. pylori* [94–96].

The addition of amino groups on lipid A is believed to be another strategy that bacteria employ to escape the immune system. ArnT is an amino-arabinose transferase found in *S. typhimurium* and transfers L-Ara4N to the 4'-phosphate of lipid A [97]. PmrC encodes a protein necessary for addition of phosphoethanolamine to the 1-phosphate of lipid A [98]. Under some conditions, the positions of phosphoethanolamine and L-Ara4N substituents are reversed, and lipid A species with two phosphoethanolamine units or two L-Ara4N moieties may be present. Expression of the enzymes ArnT and PmrC is under the control of PmrA. *F. novicida* lipid A contains galactosamine attached to the 1-phosphate group, which is added by an enzyme encoded by an ortholog gene of *arnT* [90]. Recently, a pathway for the synthesis and incorporation of the galactosamine to lipid A has been characterized in *F. novicida* [99,100].

The 1-position of lipid A can also be modified by enzymes LpxT, LmtA and LpxQ. Using undecaprenyl pyrophosphate as the substrate donor, LpxT adds a second phosphate group at 1-phosphate of lipid A, therefore one-third of the lipid A in *E. coli* contains a

diphosphate unit at 1-position [101]. LmtA is a membrane enzyme in *Leptospira interrogans* that transfers a methyl group from S-adenosylmethionine (SAM) to the 1-phosphate of lipid A [102]. LpxQ can oxidize the proximal glucosamine of *Rhizobium* lipid A in the presence of O_2 to form an aminogluconate unit [103].

5. Structure of lipid A and virulence of bacteria

LPS is present on the surface of Gram-negative bacteria and is responsible for activation of the innate immune system. In limited infections, the response to LPS is beneficial, helping to clear the invading microbe. However, in overwhelming infections, high levels of circulating cytokines might cause the syndrome of septic shock [104]. Lipid A is the bioactive component of LPS [4]. The response from the host immune system depends on both the severity of infection and the particular structure of lipid A of the invading bacteria. Some Gram-negative pathogens synthesize lipid A molecules that are poorly recognized by human TLR4, these include *H. pylori* [105,106], *F. tularensis* [107,108], *L. pneumophila* [109], *Porphyromonas gingivalis* [110], and *Chlamydia trachomatis* [111]. The phosphate groups and the length and number of fatty acyl chains of lipid A play important roles on TLR4 activation [112–114]. The *E. coli* lipid A, containing two phosphate groups and six acyl chains composed of 12 or 14 carbons (Fig. 1), is a powerful activator of the innate immune system [115].

F. tularensis, a highly infectious category A human pathogen, can synthesize LPS without core-oligosaccharide and O-antigens [90]. The *F. novicida* lipid A is a disaccharide of glucosamine, acylated with primary 3-hydroxystearoyl chains at 2-, 3-, and 2'-positions, and a secondary palmitoyl residue at 2'-position. The 4- and 3'-positions of lipid A are not derivatized. *F. novicida* lipid A cannot activate TLR4. Several genes have been identified as responsible for the unique structure of lipid A in *Francisella* [91,92,100]. For example, the gene *lpxF* encodes a protein LpxF responsible for removal of the phosphate group of *E. coli* lipid A. The mutant of *F. novicida* lacking *lpxF* synthesizes a lipid A molecule with an additional phosphate group at 4'-position and an additional fatty acid group at 3'-position when compared with the wild type lipid A (Fig. 4A). The *lpxF* mutant of *F. novicida* is avirulent in a mouse infection model (Fig. 4B) and is hypersensitive to cationic antimicrobial peptides (Fig. 4C and D). Following short-term intraperitoneal injection, the *lpxF* mutant bacteria triggers the production of a subset of cytokines, whereas wild-type cells do not [93]. The lipid A of *Francisella lpxF* mutant does not activate TLR4, and *lpxF* mutant cells do not trigger the production of $\text{TNF}\alpha$. The hypersensitivity of the *lpxF* mutant to CAMPs may cause damage to the bacterial envelope and expose other ligands.

The fatty acid chains in lipid A are also related to the infectivity of bacteria. *Yersinia pestis* causes infection through flea bites. In fleas which have a body temperature around 21–27 °C, *Y. pestis* synthesizes lipid A containing six fatty acid chains, but in the human host (37 °C) *Y. pestis* synthesizes lipid A containing four fatty acid chains [116]. The lipid A with six fatty acid chains can activate the immune system through TLR4, but the lipid A with four fatty acid chains cannot [116]. Therefore, *Y. pestis* can escape attack by the immune system because of its unique molecular structure of lipid A.

Modification of the acylation pattern of *Salmonella* lipid A by either PagP or PagL also results in attenuation of lipid A signaling through the TLR4 pathway and, therefore, may promote evasion of the innate immune system during infection [79]. The lipid A molecules of *Sinorhizobium meliloti*, a legume symbiont and *Brucella abortus*, a phylogenetically related mammalian pathogen, are unusually modified with a very-long-chain fatty acid. This

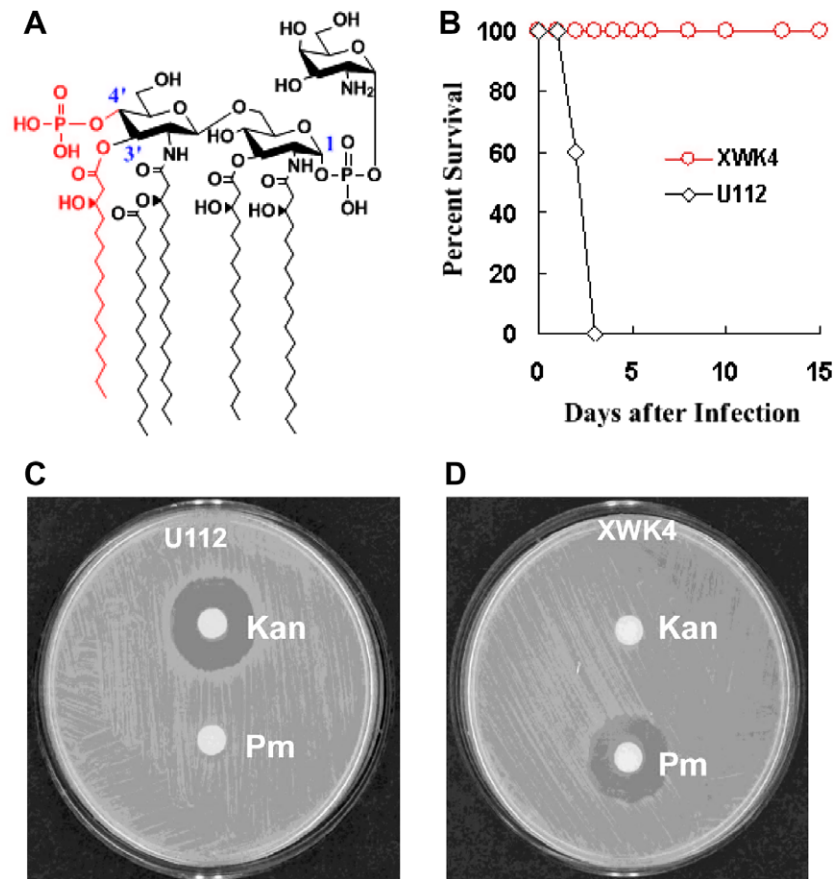


Fig. 4. Virulence of bacteria depends on the detailed structure of its lipid A. LpxF is an enzyme that removes the phosphate group at 4'-position of lipid A [91]. A *F. ovicida* mutant XWK4 was constructed by replacing the *lpxF* gene with kanamycin resistance gene in the chromosome of *F. novicida* U112 [93]. (A) The structure of lipid A synthesized by XWK4 contains an additional phosphate group at 4'-position and an additional fatty acid chain at 3'-position of lipid A (shown in red), compared with the lipid A synthesized by wild type U112 which is shown in black. (B) The mice infected with 10^6 viable *F. novicida* U112 can only survive 3 days, but those infected with 10^6 viable mutant XWK4 can survive more than 15 days (shown in red circles), suggesting that XWK4 had lost its virulence completely because of the structure modification of lipid A. (C) Disc diffusion tests showed that *F. novicida* U112 is sensitive to kanamycin (Kan) but resistant to polymyxin B (Pm). (D) Disc diffusion tests showed that *F. novicida* mutant XWK4 is resistant to kanamycin but sensitive to polymyxin B, consistent with its more negatively-charged lipid A structure.

unusual lipid A modification could be crucial for the chronic infection of both *S. meliloti* and *B. abortus* [117].

6. Conclusion

As the major component of the outer membrane, LPS is essential for the survival of most Gram-negative bacteria. Therefore, the enzymes involved in the biosynthesis and transport of lipid A and LPS have become targets for the development of new antibiotics. At present, the first three enzymes LpxA, LpxC and LpxD of the lipid A biosynthetic pathway have been purified, and their structures have been characterized by X-ray diffraction and NMR methods [14,16,118]. Based on the structural information from these proteins, research into developing new antibiotics has been initiated [119,120].

More enzymes have been identified that modify the inner core and lipid A regions of LPS. Diverse biochemical structures of lipid A have been found on the outer surface of different bacteria [5]. Some modifications to the lipid A structure are regulated by two-component regulatory systems in response to specific environmental stimuli [51] while other bacteria appear to modify their lipid A constitutively [90]. LPS or lipid A can cause diseases such as septic shock, multiple organ dysfunction and failure. Understanding the biochemistry of lipid A modifications and their impact on pathogenesis could lead to novel treatment options for these diseases. By modifying the lipid A structures, we could develop new LPS im-

mune adjuvant or antagonists [113,121,122], or improve the traditional Gram-negative bacterial live vaccines.

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