Chapter 1
Endotoxins: Lipopolysaccharides of Gram-Negative Bacteria

Xiaoyuan Wang and Peter J. Quinn

Abstract Endotoxin refers lipopolysaccharide that constitutes the outer leaflet of the outer membrane of most Gram-negative bacteria. Lipopolysaccharide is comprised of a hydrophilic polysaccharide and a hydrophobic component known as lipid A which is responsible for the major bioactivity of endotoxin. Lipopolysaccharide can be recognized by immune cells as a pathogen-associated molecule through Toll-like receptor 4. Most enzymes and genes related to the biosynthesis and export of lipopolysaccharide have been identified in *Escherichia coli*, and they are shared by most Gram-negative bacteria based on available genetic information. However, the detailed structure of lipopolysaccharide differs from one bacterium to another, suggesting that additional enzymes that can modify the basic structure of lipopolysaccharide exist in bacteria, especially some pathogens. These structural modifications of lipopolysaccharide are sometimes tightly regulated. They are not required for survival but closely related to the virulence of bacteria. In this chapter we will focus on the mechanism of biosynthesis and export of lipopolysaccharide in bacteria.

Keywords Outer membrane · Lipopolysaccharide · Endotoxin · Lipid A · LPS biosynthesis

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

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α-L-Ara4N	4-amino-4-deoxy-α-L-arabinose
Und-P-α-L-Ara4N	undecaprenyl phosphate-L-Ara4N
galacturonic acid	GalA

1.1 Introduction

The discovery of endotoxin in the late nineteenth 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 endotoxins. The historical aspects of the role of endotoxins in bacterial pathogenesis (Beutler and Rietschel, 2003) and their chemical characterization as lipopolysaccharide (LPS) (Raetz et al., 2007; Raetz and Whitfield, 2002) have been the subject of some comprehensive reviews. LPS can be divided into three parts: lipid A, core sugars and O-antigen repeats. Lipid A represents the hydrophobic component of LPS which locates in the outer leaflet of the outer membrane, while core sugars and O-antigen repeats are displayed on the surface of bacteria. Lipid A is known to be responsible for the toxic effects of infections with Gram-negative bacteria (Galanos et al., 1985). The detailed structure of LPS varies from one bacterium to another, and this variation could affect the virulence of bacteria (Wilkinson, 1996). The biosynthetic pathway and export mechanism of LPS has been well characterized in *Escherichia coli*. They are shared to most Gram-negative bacteria, but the exact structures of LPS differ in different bacteria.

LPS can stimulate the transmembrane protein Toll-like receptor 4 (TLR4), a LPS receptor found on the surface of many immune cells such as monocytes, macrophages, neutrophils and dendritic cells (Akira et al., 2006; Poltorak et al., 1998). TLR4 functions as a dimer, and depends on a small protein MD-2 for the recognition of LPS (Triantafilou and Triantafilou, 2002). Other proteins such as CD14 and LBP facilitate the presentation of LPS to MD-2 (Carpenter and O′Neill, 2007; Zhang et al., 1999). After activated by LPS, TLR4 recruits intracellular adapter molecules such as MyD88, Mal, Trif, and Tram (Yamamoto et al., 2002, 2003) which in turn activate other molecules, 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.

The response from the host immune system depends on both the severity of infection and the particular structure of LPS of the invading bacteria. High concentrations of LPS can induce fever, increase heart rate, and lead to septic shock and death (Parillo, 1993). However, in relatively low concentrations some LPSs could be active immuno-modulators, which can induce non-specific resistance to the invading microbe. Some Gram-negative pathogens synthesize LPS molecules that are poorly recognized by human TLR4, these include *Helicobacter pylori* (Suda et al., 2001), *Francisella tularensis* (Ancuta et al., 1996), and *Chlamydia trachomatis* (Heine et al., 2003). The phosphate groups and the length and number of fatty acyl chains

of lipid A play important roles on TLR4 activation (Persing et al., 2002; Rietschel et al., 1994). The *E. coli* lipid A, containing two phosphate groups and six acyl chains composed of 12 or14 carbons, is a powerful activator of the innate immune system (Golenbock et al., 1991).

Since the structure of LPS is closely related to the survival and virulence of Gram-negative bacteria, this chapter will focus on the mechanism of LPS biosynthesis and export to the surface of bacteria.

1.2 Biosynthesize of LPS on the Surfaces of Inner Membrane

The biosynthesis of LPS has been intensively studied in order to develop methods to control Gram-negative pathogens and to cure septic shock. 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 that cause human diseases such as septic shock. Although LPS distributes on the surface of bacterial cells, its synthesis is actually initiated in the cytoplasm. How LPS is synthesized in the cytoplasm and exported to the surface of bacteria has been characterized in *E. coli*. The biosynthesis of LPS is initiated from a small molecule, UDP-*N*-acetylglucosamine (UDP-GlcNAc). Multiplicities of enzymes sequentially function to convert UDP-GlcNAc into core-lipid A, and culminating in LPS. Among the three parts of LPS, the structure of lipid A is more widely conserved in different bacteria than that of core sugars or O-antigen, so are the enzymes involved in the biosynthesis of lipid A, core sugars and O-antigen.

1.2.1 Beginning in the Cytoplasm to Form Kdo_2-Lipid A

For studies of LPS biosynthesis, *E. coli* has been the most favoured Gram-negative bacterium. The first stage of LPS biosynthesis is to synthesize Kdo_2-lipid A (Doerrler, 2006; Raetz and Whitfield, 2002). This involves nine enzymes (Table 1.1), takes place in the cytoplasm at first and end up on the cytoplasmic surface of the 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. 1.1).

The first reaction catalyzed by LpxA is reversible; therefore, the second reaction catalyzed by LpxC is a committed step. Recently, LpxA, LpxC and LpxD have been isolated and their structure characterized by X-ray diffraction and NMR methods (Barb et al., 2007; Buetow et al., 2007; Williams and Raetz, 2007; Bartling and Raetz, 2009). Both LpxA and LpxD are acyltransferase and their active forms are homotrimers. LpxC, however, is a Zn^{2+}-dependent deacetylase which has no sequence homology with other deacetylases. This makes LpxC a promising target for development of novel antibiotics (Barb et al., 2009). The active site of *E. coli*

Table 1.1 Information on nine enzymes required for the biosynthesis of Kdo_2-lipid A in *E. coli*

Enzyme	Gene	Function	Substate	Donor	Reference
LpxA	*lpxA*	Acyltransferase	UDP-GlcNAc	R-3-hydroxymyristoyl ACP	Williams and Raetz (2007)
LpxC	*lpxC*	Deacetylase	UDP-3-O-(acyl)-GlcNAc	None	Barb et al. (2007)
LpxD	*lpxD*	Acyltransferase	UDP-3-O-(acyl)-GlcN	R-3-hydroxymyristoyl ACP	Buetow et al. (2007)
LpxH	*lpxH*	Pyrophosphatase	UDP-2,3-diacyl-GlcN	None	Babinski et al. (2002a, b)
LpxB	*lpxB*	Disaccharide synthase	UDP-2,3-diacyl-GlcN; Lipid X	None	Crowell et al. (1986; 1987)
LpxK	*lpxK*	4′-Kinase	Disaccharide 1-phosphate	None	Garrett et al. (1997; 1998)
KdtA	*kdtA*	Kdo transferase	Lipid IV_A	CMP-Kdo	Brozek et al. (1989)
LpxL	*lpxL*	Acyltransferase	Kdo_2-lipid IV_A	Lauroyl ACP	Brozek and Raetz (1990)
LpxM	*lpxM*	Acyltransferase	Kdo_2-penta-lipid A	Myristoyl ACP	Brozek and Raetz (1990)

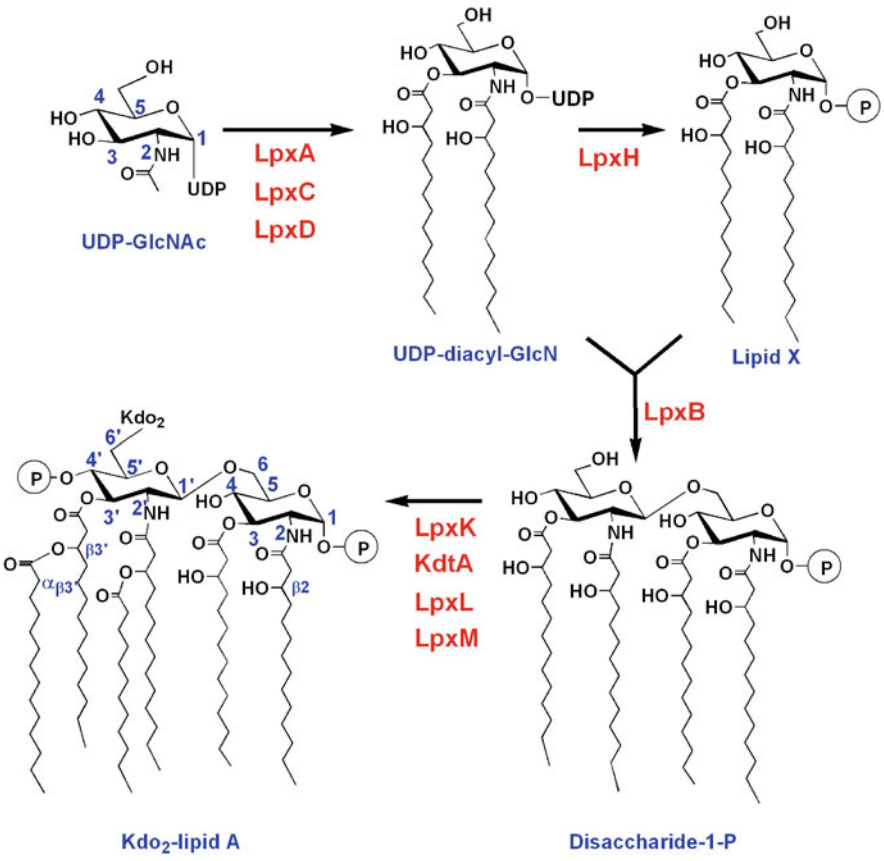

Fig. 1.1 Structure and biosynthetic pathway of Kdo_2-lipid A in *E. coli*. Each reaction is catalyzed by a single enzyme. The names of the enzyme and substrate are highlit. The carbon position and the carbon number of fatty acid chains in lipid A are labeled. The genes encoding the enzymes of Kdo_2-lipid A biosynthesis are present in single copy and highly conserved among bacteria (Raetz et al., 2007; Raetz and Whitfield, 2002)

LpxA functions as a precise hydrocarbon ruler and is manifested by incorporation of C14 hydroxyacyl chains at a rate two orders of magnitude faster than C12 or C16 chains. This explains why most of fatty acids of lipid A contain 14 carbons (Fig. 1.1).

The UDP-diacyl-GlcN is next hydrolyzed by LpxH to form lipid X (Babinski et al., 2002a, b), which is further condensed with its precursor UDP-diacyl-GlcN by LpxB to form disaccharide-1-P (Crowell et al., 1986, 1987; Metzger and Raetz, 2009). Both LpxH and LpxB enzymes are peripheral membrane proteins, while enzymes that catalyze next reactions in the pathway, LpxK, KdtA, LpxL and LpxM, are all integral proteins in the inner membrane. LpxK is a kinase that phosphorylates the 4′-position of the disaccharide-1-P to form lipid IVA (Garrett et al., 1997, 1998). KdtA is a bifunctional enzyme that incorporates two 3-deoxy-D-manno-octulosonic

acid (Kdo) residues at the 6′-position of the lipid IVA, using a sugar nucleotide CMP-Kdo as the donor (Brozek et al., 1989). The resulting Kdo_2-lipid IVA undergoes further reactions catalyzed by LpxL and LpxM to form Kdo_2-lipid A (Fig. 1.1). LpxL adds a secondary lauroyl residue and LpxM a myristoyl residue to the distal glucosamine unit, respectively (Brozek and Raetz, 1990). These subsequent acylations do not depend on Kdo in vivo (Klein et al., 2009). The nine enzymes involved in the biosynthesis of Kdo_2-lipid A all have relatively high specificity for their respective substrates (Table 1.1). For example, LpxA, LpxD, LpxL and LpxM are all acyltransferases, but they selectively catalyze different substrates and employ different acyl donors.

LpxD, FabZ, LpxA and LpxB are encoded by a gene cluster *lpxD-fabZ-lpxA-lpxB* in *E. coli* and several other bacteria (Mohan et al., 1994; Steeghs et al., 1997). Proteins LpxA, LpxB and LpxD 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 (Heath and Rock, 1996), 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 the bacterial membranes. 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 (Venter et al., 2004). MsbA is known as a specific transporter for LPS, while LpxK is a kinase that adds a phosphate group to the 4′-position of lipid A (Garrett et al., 1997, 1998). Why these two genes are always in the same cluster is not clear.

1.2.2 Connecting the Core Oligosaccharides to Lipid A

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; the outer core which connects to the O-antigen. Although the structure of lipid A is highly conserved, the structure of the core oligosaccharides shows more variations. The inner core oligosaccharides typically contain residues of Kdo and L-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 (Muller-Loennies et al., 2002, 2003), but the basic backbones are all a linear oligosaccharide of six units. The common sugars found in the core oligosaccharides are Kdo, Hep, D-glucose and D-Galactose.

Genes required for the biosynthesis of core oligosaccharides exist in three operons: *gmhD* and *waaQ* operons in *E. coli* and *Salmonella* (Roncero and Casadaban, 1992). The *gmhD* operon contains four genes *gmhD-waaF-waaC-waaL* that are required for the biosynthesis of inner core oligosaccharides (Schnaitman and Klena, 1993). 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 (Whitfield et al., 1997). The *waaQ* operon contains 7–9 genes that code for enzymes responsible for the biosynthesis of outer core oligosaccharides and their modification.

1.2.3 Synthesizing the O-antigen at the Cytoplasmic Surface of the Inner Membrane

Similarly to the core oligosaccharides, O-antigen is synthesized at 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 *S. 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 (Raetz and Whitfield, 2002). The O-antigens of LPS exhibit considerable diversity. The unit structures of O-antigen differ in the monomer type as well as the position and stereochemistry of the O-glycosidic linkages. The connection of units in O-antigen may be linear or branched. The O-antigen repeats can be homopolymers or heteropolymers.

1.2.4 Crossing the Inner Membrane

After synthesized at the cytoplasmic surface of the inner membrane, the core-lipid A and the O-antigen are flipped separately to the periplasmic surface of the inner membrane (Doerrler, 2006).

The flip of core-lipid A is carried out by a membrane protein MsbA (Doerrler et al., 2004; Doerrler and Raetz, 2002). MsbA is highly conserved in Gram-negative bacteria and shares homology with the multidrug resistance proteins of eukaryotes. It works as a homodimer and each monomer contains six transmembrane helices and a cytosolic ATP-binding domain (Ward et al., 2007). The flip efficiency of MsbA depends on the exact structure of LPS. In wild type *E. coli*, the best substrate for MsbA is core-lipid A, precursors of core-lipid A usually are not flipped. When core-lipid A is not available, MsbA could flip precursors of core-lipid A, such as Kdo_2-lipid A produced in a heptose-deficient mutant of *E. coli*

(Raetz et al., 2006). Recently, Klein et al. (2009) constructed a *kdtA* mutant of *E. coli* that can grow at low temperature and synthesize lipid IV_A, the minimal LPS structure that can be synthesized so far in vivo. The slow growth appears to be due to defects in the export of lipid IV_A by MsbA. The fast synthesis of lipid IV_A and slow flip of lipid IV_A by MsbA at high temperatures could cause the accumulation of lipid IV_A in the cytosol, leading to cell death. Lowering the growth temperature or overexpressing MsbA help the survival of *kdtA* mutant. The *kdtA* mutants grown at 21°C can synthesize lipid IV_A as well as its pentaacylated and hexaacylated derivatives, indicating in vivo late acylation of lipid A can occur without Kdo (Klein et al., 2009). Further study by Reynolds and Raetz (2009) showed that the optical substrates for MsbA are penta- and hexaacylated lipid A. Once flipped by MsbA, any structures of LPS can always end up on the surface of bacteria.

The O antigen is translocated across the membrane mostly by the Wzy-dependent pathways (Raetz and Whitfield, 2002). The Wzy-dependent pathway requires the membrane proteins Wzx, Wzy and Wzz. Wzx protein is a flippase for the O-antigen across the inner membranes (Alaimo et al., 2006; Liu et al., 1996). Wzx proteins from different bacteria have similar hydropathy profiles (Macpherson et al., 1995) 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 (Feldman et al., 1999). Wzx proteins might function by recognizing the first sugar phosphate bound to the undecaprenyl-phosphate (Marolda et al., 2004). ATP-binding domains do not exist in the primary sequences of Wzx proteins (Marolda et al., 1999, 2004).

1.2.5 Assembling LPS at the Periplasmic Surface of the Inner Membrane

After flipped to the periplasmic face of the inner membranes, O-antigen is polymerized by Wzy and Wzz and ligated to terminal sugar residues of the core-lipid A in a reaction mediated by WaaL, resulting in a nascent LPS (Abeyrathne et al., 2005) (Fig. 1.2). Wzy is an O-antigen polymerase; Wzz is a membrane protein with two transmembrane helices that flank a large periplasmic domain. Wzz functions as a molecular ruler to determine the O-antigen chain length (Larue et al., 2009). WaaL proteins from different bacteria all have 12 transmembrane helices, but show significant divergence in their primary amino acid sequence (Raetz et al., 2007). They have conserved amino acids in two adjacent periplasmic loops which might interact with undecaprenyl-diphosphate, the common component in all WaaL substrates (Perez et al., 2008). Although WaaL catalyses the formation of a glycosidic bond, it bears no relationship with classical glycosyltransferases, which modify sugar nucleotide substrates. WaaL could be a good target for searching novel LPS biosynthesis inhibitors because O-antigens of pathogenic bacteria are usually required for resistance to complement-mediated killing (Joiner, 1988).

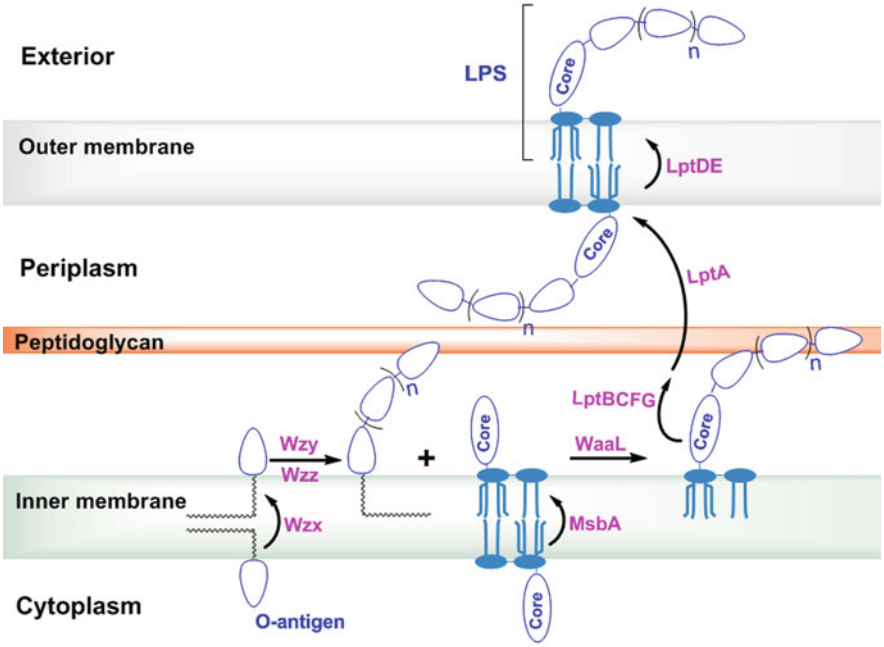

Fig. 1.2 Export of LPS and its precursors in *E. coli*. O-antigen oligosaccharides is assembled separately on undecaprenyl diphosphate, flipped from the cytoplasmic face to the periplasmic face of the inner membrane by the transporter Wzx, and polymerized on the periplasmic face of the inner membrane by Wzy and Wzz. The ABC transporter MsbA flips the core-lipid A from the inner surface to the outer surface of the inner membrane. Then the polymerized O-antigen is transferred to the core-lipid A in the periplasm 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 (Ma et al., 2008; Wu et al., 2006)

1.3 Export of LPS to the Surface of Bacteria

Nascent LPS molecules are synthesized in periplasm, and shuttled to the inner surface of the outer membrane by proteins LptA, LptB, LptC, LptF and LptG, where protein complex LptD and LptE assemble LPS into the outer surface of the outer membrane (Fig. 1.2). LptA is a periplasmic protein; LptB is a cytosolic protein with ATP binding cassette; LptC, LptF and LptG are inner membrane proteins; and LptD and LptE are outer membrane proteins (Ruiz et al., 2008; Sperandeo et al., 2007, 2008). Depletion of any of these proteins leads to abnormal membrane structures. For example, when LptA, LptB, or both were depleted, LPS was found to accumulate in the periplasm (Sperandeo et al., 2007). Some of these proteins may function as complexes (Bos et al., 2004a). The ABC transporter LptBFG, functioning with LptC and LptA, translocates LPS to the inner leaflet of the outer membrane

(Sperandeo et al., 2007, 2008). In the outer membrane, nascent LPS is exported to the outer leaflet by complex LptD and LptE (Bos et al., 2004b; Ma et al., 2008; Wu et al., 2006).

1.4 Structural Modification of LPS

After synthesized, the structure of LPS can be modified. The LPS modification study is focused on its most conserved part lipid A. Various structures of lipid A have been found in different bacteria (Fig. 1.3). The most conserved part of lipid A is its backbone, disaccharide of glucosamine. The groups connecting to the backbone of lipid A could be modified. Table 1.2 lists the known enzymes that modify the structure of LPS and their genes. The modification of LPS can occue in the hydrophilic polysaccharide region as well as the hydrophobic acyl chain domain. 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 LPS usually occur at the periplasmic face of the inner membrane or in the outer membrane. The structure modification of LPS might help the bacteria to resist the cationic antimicrobial peptides (CAMPs) released by the host immune system, or to evade recognition by the innate immune receptor TLR4.

1.4.1 Regulation of LPS Modification

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 (Gibbons et al., 2005; Guo et al., 1998; Soncini et al., 1996). It consists of an inner membrane sensor PhoQ and a cytoplasmic regulator PhoP. 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 (Bader et al., 2005; Cho et al., 2006). The PhoP–PhoQ system can also be activated when the bacterium is exposed to CAMPs (Bader et al., 2003, 2005; Martin-Orozco et al., 2006). The activation of the PhoP–PhoQ system can lead to the activation or repression of over 40 genes (Alpuche Aranda et al., 1992; Gooderham and Hancock, 2009). PmrA-PmrB two-component system is also required for *S. enterica* virulence in mice (Gunn et al., 2000). It is usually induced by high Fe^{3+}, the specific signal recognized by the sensor PmrB (Wosten et al., 2000). It can also be induced by low Mg^{2+}, which is detected by the sensor PhoQ of the PhoP-PhoQ system (Garcia Vescovi et al., 1996). The activation by low Mg^{2+}

Fig. 1.3 (continued) the 2′-positon but no phosphate. Additional sugars were also found connecting to the backbone of lipid A in *F. tularensis* (**B**), *S. typhimuriu* (**C**) and *R. etli* (**D**). The carbon position and the carbon number of fatty acid chains in lipid A are labeled

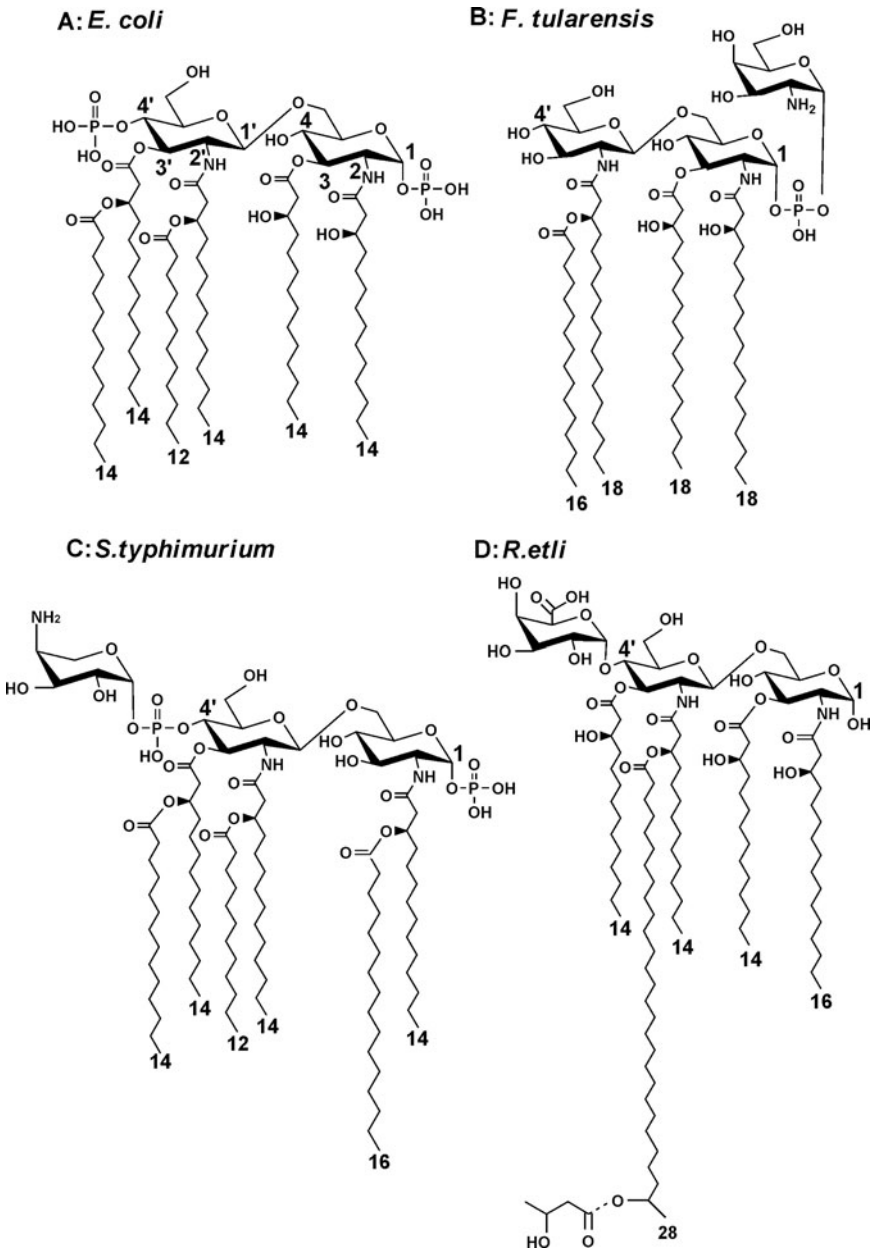

Fig. 1.3 Various structures of lipid A in different bacteria. The most conserved part of lipid A is its backbone, disaccharide of glucosamine. The groups connecting to the backbone of lipid A could be different from one bacterium to another. (**A**) In *E. coli* lipid A there are two phosphates and six fatty acid chains connecting to the backbone. (**B**) In *F. tularensis* lipid A there are only one phosphate and four fatty acid chains. (**C**) In the lipid A of *S. typhimurium* there is an additional second fatty acid chain at 2-position. (**D**) In *R. etli* lipid A there is a very long fatty acid chain at

Table 1.2 Enzymes involved in the structural modification of LPS in Gram-negative bacteria

Enzyme	Gene	Function
LpxE	*lpxE*	Remove the phosphate group from the 1-position of lipid A (Wang et al., 2004).
LpxF	*lpxF*	Remove the phosphate group from the 4′-position of lipid A (Wang et al., 2006a).
LpxO	*lpxO*	Add an OH group to the $\alpha_{\beta 3'}$-position of lipid A (Gibbons et al., 2000, 2008).
ArnT	*arnT*	Transfer the L-Ara4N unit to lipid A (Trent et al., 2001b).
LpxR	*lpxR*	Remove the 3′-acyloxyacyl moiety of lipid A (Reynolds et al., 2006).
PagL	*pagL*	Remove the 3-O-linked acyl chain of lipid A (Rutten et al., 2006).
PagP	*pagP*	Transfer a palmitate to the β2-position of lipid A (Bishop, 2008; Hwang et al., 2004).
LpxXL	*lpxXL*	Add a very long fatty acid chain to the $\beta_{2'}$-position of lipid A (Haag et al., 2009).
LpxT	*lpxT*	Transfer a phosphate group to the 1-phosphate of lipid A (Touze et al., 2008).
LpxQ	*lpxQ*	Oxidize the proximal glucosamine of lipid A to form an aminogluconate unit (Que-Gewirth et al., 2003).
LmtA	*lmtA*	Catalyze the methylation of 1-phosphate of lipid A (Boon Hinckley et al., 2005).
RgtA	*rgtA*	Add a GalA moiety to the distal unit of Kdo (Kanjilal-Kolar et al., 2006).
RgtB	*rgtB*	Add a GalA moiety to the distal unit of Kdo (Kanjilal-Kolar et al., 2006).
RgtC	*rgtC*	Add a GalA moiety to the mannose residue of core oligosaccharide of LPS (Kanjilal-Kolar et al., 2006).
EptA	*eptA*	Add a phosphoethanolamine to 1-position of lipid A (Lee et al., 2004).
EptB	*eptB*	Add a pEtN moiety to the distal unit of Kdo (Reynolds et al., 2005).

requires PhoP, PhoQ, PmrA and PmrB proteins (Soncini et al., 1996) as well as the PhoP-activated PmrD protein (Kox et al., 2000). In *E. coli*, the PmrA-PmrB pathway cannot be triggered by the PhoP-PhoQ system because the PmrD is not functional (Winfield and Groisman, 2004).

Some modifications of LPS are under control of the PhoP-PhoQ system and/or PmrA-PmrB system (Guo et al., 1997). The best example of the regulation of PmrA is the *arn* operon (Breazeale et al., 2002, 2003; Gunn et al., 1998; Trent et al., 2001a) and *ugd* gene (Groisman et al., 1997). 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 part of LPS (Breazeale et al., 2003; Gunn et al., 1998; Zhou et al., 2001). This modification can assist the bacteria resist the antibiotic polymyxin B (Roland et al., 1993). 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 (Breazeale et al., 2005). 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 (Yan et al., 2007), where ArnT transfers the L-Ara4N unit to the core-lipid A (Fig. 1.3c).

1.4.2 Modifications in the Hydrophobic Region of LPS

Membrane proteins PgaP, PagL, LpxR and LpxO have been reported to modify the fatty acyl chain region of LPS. PagP is a palmitoyl transferase which locates in the outer membrane; it transfers a palmitate from glycerophospholipids to the β2-position of lipid A (Fig. 1.3c), resulting in a hepta-acylated structure (Ahn et al., 2004). 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 its structure has been determined by both NMR spectroscopy and X-ray crystallography (Bishop, 2008; Hwang et al., 2004). PagL is a lipase that removes the 3-O-linked acyl chain of lipid A (Fig. 1.3c) but plays no role in antimicrobial peptide resistance (Kawasaki et al., 2004). Like PagP, PagL is also located in the outer membrane. The *pagL* mutant of *S. 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 (Rutten et al., 2006). 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. 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 (Kawasaki et al., 2004). LpxR is another outer membrane protein that removes the 3′-acyloxyacyl moiety of *Salmonella* lipid A (Reynolds et al., 2006). Orthologs of *Salmonella* LpxR can be found in various Gram-negative bacteria such as *H. pylori*, *Y. enterocolitica*, *E. coli* O157:H7, and *V. 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 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 (Rutten et al., 2009). LpxO is an inner membrane protein that can generate a 2-OH at the $\alpha_{\beta 3'}$-position of *Salmonella* lipid A (Gibbons et al., 2000). 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.

Different Gram-negative bacteria could make LPS containing different length of the fatty acid chains. For example, the fatty acyl chains of *E. coli* lipid A are 12 or 14 carbons long, while that of *F. novicida* lipid A are 16–18 carbons long (Wang et al., 2006b; Shaffer et al., 2007). The lipid A molecules of *Rhizobium etli*, a legume symbiont and *Brucella abortus*, a phylogenetically related mammalian pathogen, are unusually modified with a very-long-chain fatty acid which has 28 carbons and is attached to lipid A (Fig. 1.3d) by acyltransferase LpxXL (Basu et al., 2002). LpxXL plays an important role in bacterial development (Haag et al., 2009). This unusual lipid A modification could be crucial for the chronic infection of both *S. meliloti* and *B. abortus*. The fatty acid chains in LPS 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 (Montminy et al., 2006). 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 (Golenbock et al., 1991). Therefore, *Y. pestis* can escape attack by the immune system because of its unique molecular structure of lipid A.

1.4.3 Modifications in the Hydrophilic Region of LPS

Except for the fatty acid region of LPS, the hydrophilic region of LPS can also be modified. For example, lipid A usually contains two phosphate groups which impart net negative charges to the molecule. The negative charges of lipid A allow the binding of positively charged CAMPs produced by the immune system. To evade the attack by the immune system some bacterial pathogens have evolved less negatively-charged variations of lipid A by removing or decorating the phosphate groups at the 1- and 4′-positions. The decoration includes the addition of amine-containing residues such as α-L-Ara4N and phosphoethanolamine. These modifications result in resistance to CAMPs and are controlled by the PmrA-PmrB two-component system.

Two genes *lpxE* and *lpxF* encoding the lipid A phosphatases have been identified in *F. novicida* (Wang et al., 2004, 2006a). LpxE selectively removes the phosphate group at the 1-position of lipid A, while LpxF selectively removes the phosphate group at the 4′-position (Fig. 1.3b). Orthlogs of LpxE also exist in *R. etli* and in *H. pylori* (Karbarz et al., 2003; Tran et al., 2004). 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. The *lpxF* mutant of *F. novicida* is avirulent in a mouse infection model and is hypersensitive to CAMPs. Following short-term intraperitoneal injection, the *lpxF* mutant bacteria triggers the production of a subset of cytokines, whereas wild-type cells do not (Wang et al., 2007). The lipid A of *lpxF* mutant does not activate TLR4, and *lpxF* mutant cells do not trigger the production of TNF. The *lpxF* mutant of *F. novicida* no longer infects host mice (Wang et al., 2007), suggesting that the phosphate group on lipid A is closely related to the infectivity of bacteria. 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 (Que et al., 2000b), while *F. tularensis* lipid A contains only one phosphate group (Wang et al., 2006b). The absence of a phosphate group would greatly decrease the surface negative charge of these bacteria.

Another strategy that bacteria employ to decrease the surface negative charge is the addition of amino groups at 1- or 4′-phosphates of lipid A. EptA encodes a protein necessary for addition of phosphoethanolamine to the 1-phosphate of lipid A (Lee et al., 2004). ArnT is an amino-arabinose transferase found in *S. typhimurium* and transfers L-Arn4N to the 4′-phosphate of lipid A (Trent et al., 2001b). Under some conditions, the positions of phosphoethanolamine and L-Ara4N substituents can be reversed, and lipid A species with two phosphoethanolamine units or two L-Ara4N moieties may be present. The expression of ArnT and EptA is controlled by PmrA. Another example is the galactosamine attached to the 1-phosphate of *F. novicida* lipid A, it is added by an enzyme encoded by an ortholog gene of *arnT* (Wang et al., 2006b). The pathway for the synthesis and incorporation of the galactosamine to lipid A has been characterized in *F. novicida* (Wang et al., 2009; Song et al., 2009). The 1-position of lipid A can also be modified by enzymes LpxT, LmtA and LpxQ. LpxT adds a second phosphate group at 1-phosphate of lipid A, using undecaprenyl pyrophosphate as the substrate donor, therefore one-third of the lipid A in *E. coli* contains a diphosphate unit at 1-position (Touze et al., 2008). LmtA is a membrane enzyme in *Leptospira interrogans* that transfers a methyl group from *S*-adenosylmethionine to the 1-phosphate of lipid A (Boon Hinckley et al., 2005). LpxQ can oxidize the proximal glucosamine of *Rhizobium* lipid A in the presence of O2 to form an aminogluconate unit (Que-Gewirth et al., 2003).

Several proteins have been found to modify the core region of LPS. For example, the inner core of *R. leguminosarum* LPS is modified with three galacturonic acid (GalA) moieties, two on the distal Kdo unit and one on the mannose residue. The three GalA transferases RgtA, RgtB, and RgtC have been characterized (Kanjilal-Kolar et al., 2006). Reconstitution experiments with the individual genes demonstrated that the activity of RgtA precedes and is necessary for the subsequent activity of RgtB, which is followed by the activity of RgtC. Another protein that could modify the Kdo unit is a pEtN transferase EptB (Reynolds et al., 2005). EptB could only be detected in membranes of *E. coli* grown in the presence of 5–50 mM Ca^{2+} (Kanipes et al., 2001), due to the PhoP-PhoQ dependent activation of sRNA MgrR production (Overgaard et al., 2009). MgrR could promote degradation of the *eptB* mRNA, resulting in decreased EptB-dependent LPS modification.

In the presence of high Ca^{2+} concentrations, however, phosphatase activity of PhoQ is activated, leading to dephosphorylation of PhoP and, in turn, to silencing of *mgrR* transcription.

1.5 Conclusion

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 (Wilkinson, 1996). Some modifications to the lipid A structure are regulated by two-component regulatory systems in response to specific environmental stimuli (Guo et al., 1997) while other bacteria appear to modify their lipid A constitutively (Wang et al., 2006b). 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 LPS biosynthetic pathway have been purified, and their structures have been characterised by X-ray diffraction and NMR methods (Buetow et al., 2007; Coggins et al., 2003; Williams and Raetz, 2007). Based on the structural information from these proteins, research into developing new antibiotics has been initiated (Barb et al., 2007, 2009).

LPS can cause diseases such as septic shock, multiple organ dysfunction and failure. Understanding the biochemistry of LPS modifications and their impact on pathogenesis could lead to novel treatment options for these diseases. By modifying the LPS structures, we could develop new LPS immune adjuvant or antagonists (Hawkins et al., 2004; Persing et al., 2002; Stover et al., 2004), or improve the traditional Gram-negative bacterial live vaccines.

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