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Lipid A as a Drug Target and Therapeutic Molecule

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Abstract

In this review, lipid A, from its discovery to recent findings, is presented as a drug target and therapeutic molecule. First, the biosynthetic pathway for lipid A, the Raetz pathway, serves as a good drug target for antibiotic development. Several assay methods used to screen for inhibitors of lipid A synthesis will be presented, and some of the promising lead compounds will be described. Second, utilization of lipid A biosynthetic pathways by various bacterial species can generate modified lipid A molecules with therapeutic value.

Key Words: Lipid A, Endotoxin, Drug target, Antibacterial, Adjuvant

INTRODUCTION

Lipids have not been a very popular research topic for some time, because their study imposes more technical challenges than other biological molecules, such as proteins, nucleic acids, and carbohydrates. While a lot of questions remain to be answered, it is true that remarkable advances have been recently achieved in the lipid field. In this review, lipid A, from its discovery to recent findings, is presented as a drug target with therapeutic value.

Discovery of lipid A

Lipid A is present mainly in Gram-negative bacteria; however, a recent study indicates it might exist in plants. It functions as a hydrophobic anchor for lipopolysaccharide (LPS) attached to the outer membrane, and it appears to be essential for the growth of cells and in LPS-free mutants. The term 'lipid A' was first coined by Westphal and Luderitz as the lipid rich hydrolytic fragment of LPS, and lipid B was defined as the other, more readily separated portion of phospholipids in *Escherichia coli* (*E. coli*) (Brade, 1999). The molecular identities of both lipid species are now well known, and lipid B is better known as phosphatidylethanolamine. When lipid A was first separated from the bacterial cell envelope, neither its exact molecular identity nor its biosynthetic pathway were known. In the late 1970s, when the structure of lipid A was not yet known, lipid X, a biosynthetic intermediate of lipid A, was identified from an *E. coli* mutant lacking phosphatidylglycerol (Nishijima and Raetz, 1979). While it was determined that lipid X contains acylated glucosamine (GlcN) and a phosphoryl group, the exact structure had yet to be defined. With the structure of lipid A not available, lipid X could not be shown to be related to lipid A. In 1983, the exact structure of lipid A was determined with the help of mass spectrometry and NMR spectroscopy (Imoto *et al*., 1983; Strain *et al*., 1983). Then, the structure of lipid X was revisited, and it was determined that lipid X is an intermediate in lipid A biosynthesis (Takayama *et al*., 1983). The starting compounds of lipid A biosynthesis are N-acetylglucosamine linked to uridine diphosphate (UDP-GlcNAc), and the acyl chain linked to acyl carrier protein (acyl-acyl carrier protein; acyl-ACP). After acylation of UDP-GlcNAc mediated by UDP-GlcNAc acyltransferase or lpxA (Crowell *et al*., 1986), eight reactions ensue to synthesize Kdo_2-lipid A (Raetz and Whitfield, 2002; Raetz *et al*., 2007). This pathway is called the Raetz pathway (Fig. 1) as explained below.

Function of lipid A in Gram-negative bacteria

Obviously, the function of lipid A in Gram-negative bacteria is to anchor LPSs to the outer membrane of the cell. Generally, lipid A is considered essential for survival. Some laboratory-grown bacterial strains lack LPS, but not lipid A. The outer membrane of *Francisella novicida* (*F. novicida*) contains a lot of lipid A not linked to LPS (Wang *et al*., 2004). However, there are some results reporting the absence of lipid A in viable Gram-negative bacteria. Moffatt and colleagues reported a mutant of *Acinetobacter baumannii* in which lipid A biosyn-

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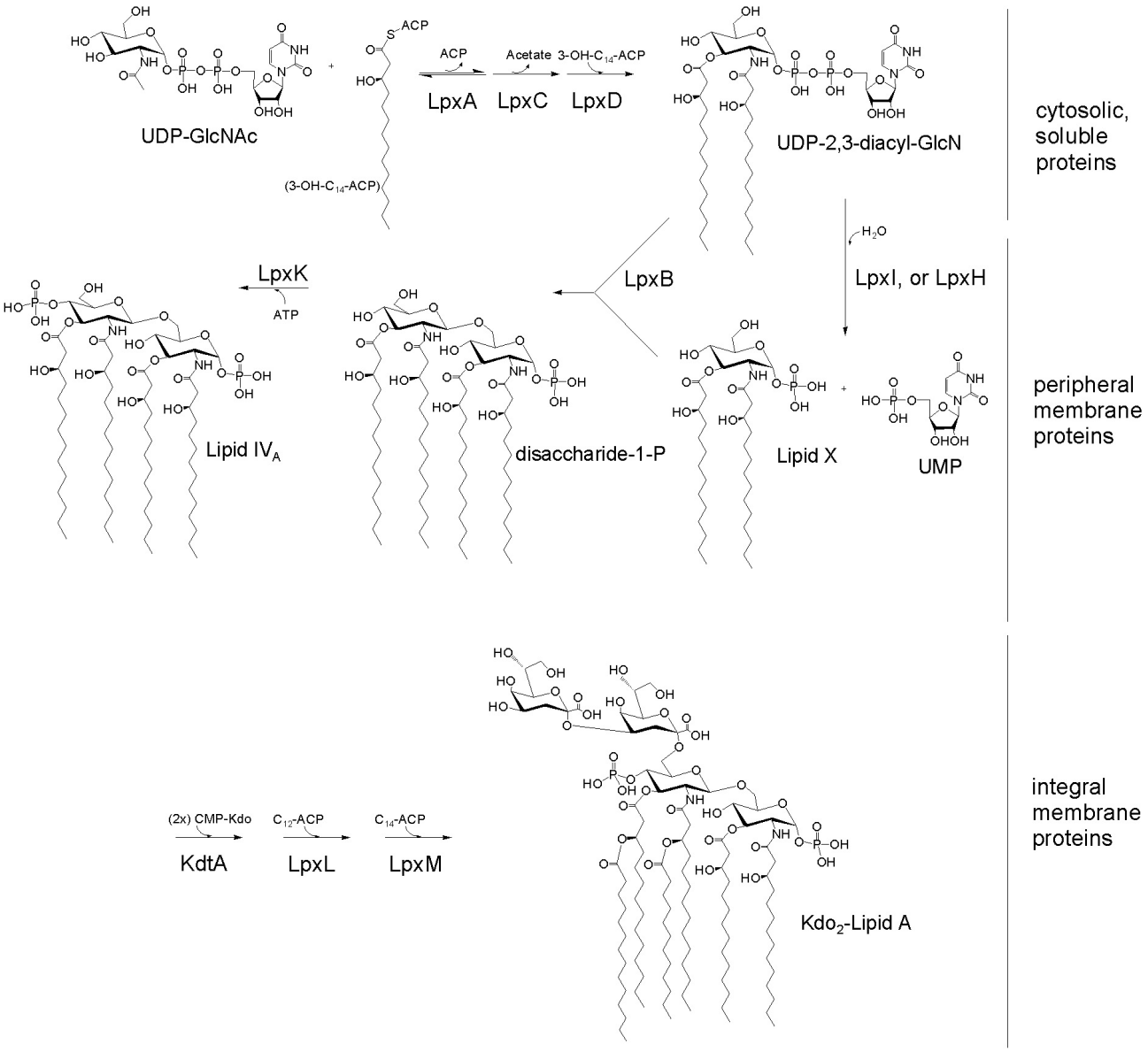


Fig. 1. The Raetz pathway. The biosynthetic pathway for Kdo_2-lipid A (*E. coli*) is shown. The pathway is redrawn from (Raetz *et al*., 2007) for simplicity, and to indicate the location of each reaction.

thesis is absent. These mutants were isolated from colistin-containing media used to induce colistin resistance. All the isolated mutants showed mutations in the first two enzymes of the Raetz pathway, and LPS was not present (Moffatt *et al*., 2011). In the case of *Neisseria meningitidis*, cells can grow slowly without lipid A (Steeghs *et al*., 1998).With the exception of the above, lipid A appears to be essential for the survival of Gram-negative bacteria. In laboratory experiments, inhibition of lipid A in a LPS-deficient mutant results in the inhibition of growth, implying that lipid A functions as more than a lipid anchor for LPS. Other biological roles of lipid A and the identities of its biosynthetic intermediates are still unclear.

Lipid A as an endotoxin

As picomolar levels of lipid A are sufficient to induce inflammation in the mammalian immune system by triggering TLR4/MD activation (Beutler and Cerami, 1988), lipid A itself can be considered an endotoxin. We can separate the immunogenicity of LPS from its toxicity; its immunogenicity depends on polysaccharide components and its toxicity is due to the lipid A moiety. While inflammation is necessary to clear up infection, overreaction of the immune system can be fatal, and Gram-negative septic shock can occur. The structure of the LPS bound to the TLR4/MD2 complex (Fig. 2) explains the formation of receptor multimers upon binding of LPS (Park *et al*., 2009; Ohto *et al*., 2012). TLR4 and MD2 exist as dimers (TLR4/MD2), and LPS binding to MD2 results in the formation of the TLR4/MD2/LPS complex (2:2:2 complex). The lipid chains in LPS are located in the hydrophobic pocket of MD2, and two phosphate groups in lipid A engage in favorable in-

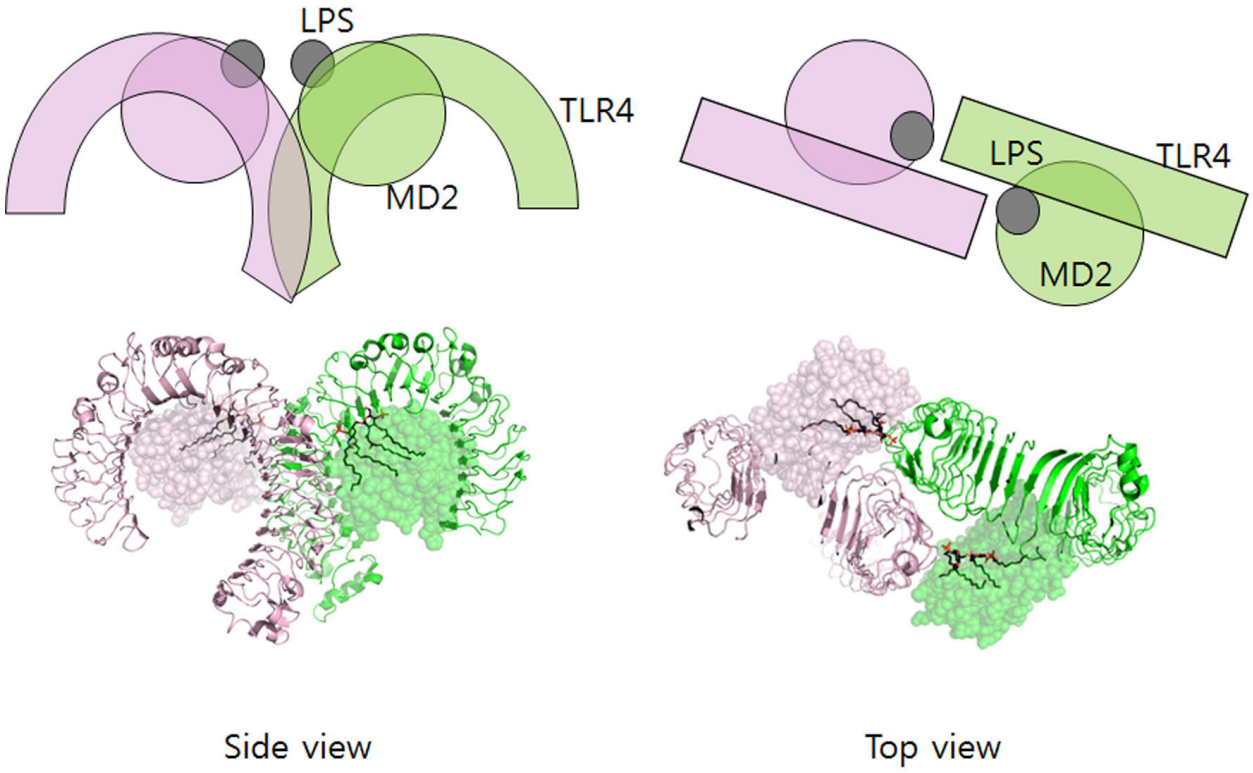


Fig. 2. LPS binding induces multimerization of TLR4/MD2 complexes. Model and structure (PDB ID: 3VQ2) based on (Ohto *et al*., 2012).

teractions with positively-charged residues in TLR4 and MD2, inducing complex formation. In human cells, the presence of two phosphate and acyloxyacyl groups is necessary to activate fully TLR4-MD-2 (Rietschel *et al*., 1994). Species-specificities exist among lipid A and its analogs. For example, lipid IV_A, a tetra-acylated precursor of lipid A, is a weak agonist of TLR-4-MD-2 activity in mouse, while it shows antagonistic activity in human (Montminy *et al*., 2006). It may be possible to utilize the different lipid A analogs as therapeutics molecules. This will be discussed later.

BIOSYNTHETIC PATHWAY OF LIPID A AS A DRUG TARGET

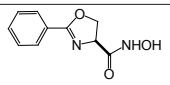
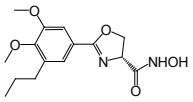
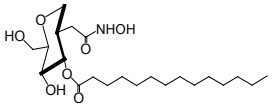
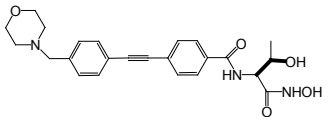
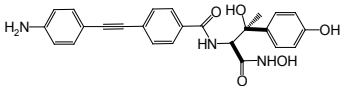
The raetz pathway

As described above, UDP-GlcNAc and acyl-ACP, in the case of *E. coli*, are substrates for the first step of lipid A biosynthesis. LpxA, the first enzyme in the Raetz pathway, is an acyltransferase mediating the acylation of UDP-GlcNAc and yielding UDP-3-(*O*)-acyl-GlcNAc. This reaction occurs in the cytoplasm, and is followed by the deacetylation of the GlcNAc moiety, mediated by lpxC (Jackman *et al*., 1999). This reaction yields UDP-3-(*O*)-acyl-GlcN as a product. As the equilibrium in the lpxA reaction is toward the substrate, the lpxC reaction is the actual step of commitment to the pathway. Once UDP-3-(*O*)-acyl-GlcN is formed, another acylation reaction occurs, mediated by lpxD (Vuorio and Vaara, 1995), to yield UDP-2,3-(*O*)-diacyl-GlcN. These three reactions occur in the cytoplasm, and the enzymes responsible are soluble proteins.

One molecule of UDP-2,3-(*O*)-diacyl-GlcN, and one molecule of lipid X, formed by the removal of UMP moiety from UDP-2,3-(*O*)-diacyl-GlcN, undergo lipid A disaccharide formation. The formation of lipid X is mediated by either lpxH (Babinski *et al*., 2002a; Babinski *et al*., 2002b) or lpxI (Metzger and Raetz, 2010), depending on the bacterial species, and lipid A disaccharide formation is mediated by lpxB (Crowell *et al*., 1986). These three enzymes are peripheral membrane proteins, and it appears that the reactions occur in the vicinity of the membrane.

Lipid A disaccharide undergoes further modifications. First, the phosphorylation of lipid A disaccharide results in the formation of lipid IV_A, a tetra-acylated lipid A species. This reaction is mediated by lpxK (Garrett *et al*., 1997), a phosphorylase, or a kinase. Second, two Kdo residues are attached to lipid IV_A, yielding Kdo_2-IV_A, and the reaction is mediated by KdtA (White *et al*., 1997), a Kdo transferase. The second and third reactions, mediated by lpxL (Carty *et al*., 1999) and lpxM (Clementz *et al*., 1997), are acylation reactions. Tetra-acylated Kdo_2-IV_A is transformed into Kdo_2-lipid A, the final product in the Raetz pathway. LpxL transfers lauroyl residues while lpxM transfers a myristoyl residue to Kdo_2-IV_A. Two reactions occur in a sequential manner: the lpxL reaction occurs before the lpxM reaction, and the lpxM reaction does not occur in the *lpxL* mutant (Vorachek-Warren *et al*., 2002). The three enzymes that modify lipid A disaccharide are integral membrane proteins, and the reactions probably occur on the inner surface of the inner membrane.

Table 1. Structures of selected hydroxamic acids with LpxC inhibitory activity

Compound	Structure	Ki value against *E. coli* LpxC	Reference
L-573,655		24 μM	Onishi *et al*., 1996
L-161,240		~50 nM	Onishi *et al*., 1996
TU-514		~650 nM	Jackman *et al*., 2000
CHIR-090		~2 nM	McClerren *et al*., 2005
LPC-051		~0.024 nM	Liang *et al*., 2013

In the above paragraphs, nine constitutive enzyme reactions in the Raetz pathway were described succinctly, using mainly *E. coli* as a model system. However, variations exist between bacterial species. First, lpxAs from different bacteria species show different selectivities for acyl chains and sugars. Acyl chain length can be different, either short (C10, *Pseudomonas aeruginosa*; *P. aeruginosa* (Dotson *et al*., 1998; Wyckoff *et al*., 1998), or long (*Helicobacter pylori* (Lee and Suh, 2003); C18, *F. novicida* (Wang *et al*., 2006b), and the lack of the 3-hydroxyl group makes a difference, as we observe myristoyl, not the 3-hydroxymyristoyl group, in the *Chlamydia trachomatis* lpxA reaction (Sweet *et al*., 2001). Sugar selectivity is observed in the case of *Leptospira interrogans* lpxA, as an amino sugar, UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose, is used, resulting in the formation of amide bonds between the acyl chain and the sugar (Robins *et al*., 2009). Second, the formation of lipid X from UDP-diacyl-GlcN is mediated by either lpxH or lpxI, depending on the bacterial species. LpxH exists in about two-thirds of Gram-negative bacteria, while lpxI is found in the other one-third (Metzger and Raetz, 2010). LpxH and lpxI are unrelated to each other in their DNA sequences, and the enzyme mechanisms are different. These differences are important considerations in the development of inhibitors against lipid A biosynthesis.

Lipid A biosynthesis inhibitors

The biosynthetic pathway for lipid A can serve as good drug target for antibiotic development, as lipid A is essential in most Gram-negative bacteria. There have been attempts to screen for inhibitors of lipid A biosynthetic enzymes. One way is to measure the incorporation of radioactive galactose into LPS (Austin *et al*., 1990) to screen for an inhibitor of LPS biosynthesis. One compound, L-573,655 (Table 1), was selected during a screen of compounds by a research group at Merck Research Laboratories (Onishi *et al*., 1996). After selection, all nine enzymes of lipid A biosynthesis were assayed individually for sensitivity to inhibition by L-573,655, which turned out to inhibit lpxC, the second enzyme of the Raetz pathway. The inhibition constant for L-573,655 was determined to be 24 μM, and 200 analogs were synthesized to bring the inhibition constant up to ~50 nM by L-161,240 (Table 1). While L-161,240 was an inhibitor of *E. coli* lpxC, the inhibition was not universal: L-161,240 could not inhibit lpxC from other species, such as *P. aeruginosa* or *Aquifex aeolicus* (*A. aeolicus*). Later on, hydroxamate-containing inhibitors, including TU-514 (Table 1), were developed, which turned out to be effective against both *E. coli* and *A. aeolicus* enzymes (Jackman *et al*., 2000). More hydroxamate-containing compounds were developed yielding several inhibitors, including CHIR-090 (Table 1) with antibacterial activity comparable to that of ciprofloxacin. CHIR-090 and other potent inhibitors have threonyl-hydroxamate in their head groups (McClerren *et al*., 2005). Based on knowledge of the interactions between LpxC and threonyl-hydroxamate-containing inhibitor, interactions of binding pocket and inhibitor were further increased by attaching bulky groups to threonyl-hydroxamate, as seen in the case of LPC-051 (Liang *et al*., 2013).

As stated above, the first three enzymes of the Raetz pathway are soluble proteins, while others are non-soluble. The non-soluble ones are either peripheral membrane proteins or integral membrane proteins associated with the membrane. The development of enzyme assay systems is challenging for non-soluble proteins. Even for soluble proteins, the enzyme reactions in the Raetz pathway are hard to reconstitute in test tubes. Assays usually involve the use of radioactive isotope-labeled substrates to measure the enzyme activities, and most of the materials are not commercially available. For example, the lpxA enzyme assay requires lpxA, acylated ACPs, and radio-labeled UDP-GlcNAc, but none of these are commercially available. Therefore enzyme activity assays have not been

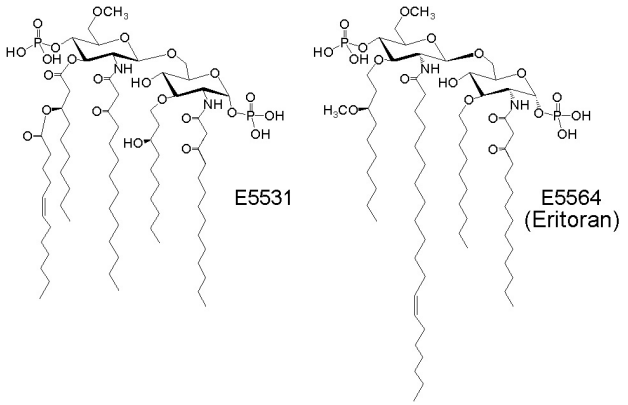


Fig. 3. Structures of E5531 and E5564 as lipid IV_A analogs.

used in high-throughput screening for inhibitors against lipid A biosynthesis. When Pirrung and colleagues developed lpxC inhibitors, they used a disk diffusion assay to test 70 oxazoline hydroxamate compounds (Pirrung *et al*., 2003).

Recently, a fluorescence based enzyme assay was developed for the lpxA reaction. This assay is based on the formation of free thiol from ACPs (Jenkins and Dotson, 2012): free thiols are exposed after the acyl group is transferred from acyl-ACP to UDP-GlcNAc, and this can be monitored by linking thiol exposition to other enzymatic reactions. While a previous method used a discontinuous radioassay based on thin layer chromatography (Galloway and Raetz, 1990), the new assay is a continuous assay that monitors multiple enzyme reactions on a single plate. The new method would allow higher throughput screening of large compound libraries. While most inhibitors of lipid A biosynthesis target lpxC, it does not mean lpxC is the only target. It was shown, using a phage display screening assay, that lpxA is a good antibacterial target (Benson *et al*., 2003), and all the enzymes in the Raetz pathway are potential targets for antibacterial agents against Gram-negative bacteria.

LIPID A ANALOGS WITH THERAPEUTIC VALUE

Lipid A itself is an endotoxin, and thus has no therapeutic value. However, biosynthetic intermediates and modified lipid A could have clinical applications. Lipid IV_A is an antagonist of the TLR4 receptor in human cells, while it is an agonist in murine cells. It was initially hoped that lipid IV_A might be useful as a therapeutic agent for the treatment of Gram-negative septicemia (Golenbock *et al*., 1991). However, because of the lack of stability of lipid IV_A upon storage, the development of synthetic analogs was pursued instead, among which E5531 and E5564 were the most notable outcomes (Christ *et al*., 1995) (Fig. 3). While it is less encouraging that a recent clinical trial of E5564 (eritoran) did not reduce the mortality of septic patients (Opal *et al*., 2013), there are other possible clinical uses for this compound. For example, E5564 protected mice from lethal influenza infection (Shirey *et al*., 2013), presenting Toll-like receptors as a new drug target for antiviral therapy (Patel *et al*., 2014). Lipid X was once considered as an antagonist (Danner *et al*., 1987); however, later it was shown not to have affinity for the TLR4 receptor (Pohlman *et al*., 1988).

In the above paragraph, synthetic analogs of lipid A were shown to have therapeutic value. It is also possible to prepare lipid A analogs by combining different biosynthetic enzymes from different bacterial species. As seen in the Kdo_2-lipid A molecule from *E. coli* (Fig. 1), there are two phosphate groups, and the existence of these two phosphate groups is very important for TLR4/MD2 activation (Rietschel *et al*., 1994). While lipid A itself is too toxic when there are two phosphate groups present, monophospho-lipid A (MPLA) (Persing *et al*., 2002) can be prepared and used as an adjuvant, as MPLA partially activates TLR4/MD2. Interestingly, there are some bacterial species, such as *Francisella tularensis*, which produce lipid A devoid of phosphate groups. These species contain extra enzymes, including LpxE (Wang *et al*., 2004) and LpxF (Wang *et al*., 2006a), that function as phosphatases to get rid of the phosphate groups in lipid A. The lpxE encoding gene was inserted into a *Salmonella* strain to make MPLA-producing bacteria, in the hope of developing live oral vaccines (Kong *et al*., 2011; Wang *et al*., 2013). These bacterial strains could be used to deliver the antigen of choice, and to elicit moderate immune responses via MPLA, which would function as both an antigen presenter and an adjuvant.

It should also be possible to utilize biosynthetic enzymes from different species to prepare lipid A analogs *in vitro*. For example, PagL (Trent *et al*., 2001) and LpxR (Reynolds *et al*., 2006) exist in *Salmonella* species and function as lipases. These enzymes function in *E. coli*, and modified lipid A species are produced by cultured bacterial cells (Gibbons *et al*., 2000; Reynolds *et al*., 2006). It is expected that the repertoire of lipid A modifying enzymes from various bacterial species will allow the production of lipid A analogs with beneficial activities.

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