Escherichia coli Mutants that Synthesize Dephosphorylated Lipid A Molecules

Brian O. Ingram[1], **Ali Masoudi**[1], and **Christian R. H. Raetz**[1,*]

[1] Department of Biochemistry, Duke University Medical Center, Durham, NC 27710

Abstract

The lipid A moiety of *Escherichia coli* lipopolysaccharide is a hexa-acylated disaccharide of glucosamine that is phosphorylated at the 1 and 4′ positions. Expression of the *Francisella novicida* lipid A 1-phosphatase FnLpxE in *E. coli* results in dephosphorylation of the lipid A proximal unit. Co-expression of FnLpxE and the *Rhizobium leguminosarum* lipid A oxidase RlLpxQ in *E. coli* converts much of the proximal glucosamine to 2-amino-2-deoxy-gluconate. Expression of the *F. novicida* lipid A 4′-phosphatase FnLpxF in wild-type *E. coli* has no effect because FnLpxF cannot dephosphorylate hexa-acylated lipid A. However, expression of FnLpxF in *E. coli lpxM* mutants, which synthesize penta-acylated lipid A lacking the secondary 3′-myristate chain, causes extensive 4′-dephosphorylation. Co-expression of FnLpxE and FnLpxF in *lpxM* mutants results in massive accumulation of lipid A species lacking both phosphate groups, and introduction of RlLpxQ generates phosphate-free lipid A variants containing 2-amino-2-deoxy-gluconate. The proposed lipid A structures were confirmed by electrospray ionization mass spectrometry. Strains with 4′-dephosphorylated lipid A display increased polymyxin resistance. Heptose-deficient mutants of *E. coli* lacking both the 1- and 4′-phosphate moieties are viable on plates but sensitive to $CaCl_2$. Our methods for re-engineering lipid A structure may be useful for generating novel vaccines and adjuvants.

The lipid A moiety of lipopolysaccharide (LPS) makes up the outer surface of the outer membrane of Gram-negative bacteria. In *Escherichia coli*, the most abundant lipid A species is a β,1′-6-linked disaccharide of glucosamine that is phosphorylated at the 1- and 4′-positions (Fig. 1A), and acylated at the 2-, 3-, 2′-, and 3′-positions with *R*-3-hydroxymyristate chains (*1*). Secondary lauroyl and myristoyl groups form the acyloxyacyl moieties at the 2′- and 3′-positions (Fig. 1A) (1). These features are required for full activation of the toll-like receptor 4 (TLR4)/MD-2 complex of the innate immune system (2). Lipid A derivatives that lack the phosphate group normally present at the 1-position can be prepared by chemical synthesis (3), acid hydrolysis of LPS (4,5), or LpxE phosphatase treatment (Fig. 1B, Product I) (6–8). Some 4′-monophosphoryl-lipid A (MPLA) variants have recently been approved as adjuvants for use in human vaccines (9). They appear to function as partial TLR4/MD-2 agonists that induce relatively small amounts of pro-inflammatory cytokines but normal levels of interferon-β (10,11). Consequently, 4′-MPLAs are 100–1000 fold less toxic and pyrogenic than intact *E. coli* or *Salmonella* lipid A species yet retain full adjuvant activity (12).

*Author to whom correspondence should be addressed: C. R. H. Raetz at (919) 684-3384; Fax (919) 684-8885; raetz@biochem.duke.edu.

E. coli lipid A is assembled on the cytosolic surface of the inner membrane by nine constitutive enzymes (1) to generate Kdo_2-lipid A (Fig. 1B), which is further glycosylated with core sugars in wild-type cells and then flipped to the outer surface of the inner membrane by the ABC transporter MsbA (1,13,14). Nascent LPS is transported across the periplasm and to the outer surface of the outer membrane by the Lpt proteins (15,16). In some bacteria, additional extracellular modification enzymes alter the structure of lipid A during its transit to the outer membrane (1). These modifications are usually not required for growth (1) but may confer selective advantages. For instance, *E. coli* and *Salmonella* can modify their lipid A phosphate groups with 4-amino-4-deoxy-L-arabinose (L-Ara4N) and/or phosphoethanolamine (pEtN) moieties (structures not shown) to evade cationic antimicrobial peptides (1). These modifications neutralize the negative charges of the phosphate groups, reducing the affinity for cationic peptides like polymyxin (17,18).

In contrast to *E. coli*, *Francisella novicida* and *Francisella tularensis*, the causative agents of tularemia in mice and humans, possess two inner membrane phosphatases, termed LpxE (8) and LpxF (19). These enzymes remove the 1- and 4′-phosphate groups of lipid A respectively on the outer surface of the inner membrane. Like L-Ara4N addition, lipid A dephosphorylation promotes resistance to cationic antimicrobial peptides by reducing the negative charge. *F. novicida* LpxF mutants retain their lipid A 4′-phosphate group. However, they are very sensitive to polymyxin and attenuated in a mouse infection model (20).

The lipid A moieties of the LPS of *Rhizobium etli* and *Rhizobium leguminosarum*, bacterial endosymbionts that form nitrogen-fixing nodules on certain bean plants, are completely dephosphorylated (Figs. 1C and 1D) (21–23). Homologues of the *Francisella* LpxE and LpxF phosphatases are present in *R. leguminosarum* and *R. etli*, and account for the absence of the phosphate groups (24). After its removal, the 4′-phosphate moiety is replaced with a galacturonosyl residue in many strains of *Rhizobium* (Figs. 1C and 1D) (21–23), but not in *Francisella* (not shown). The proximal dephosphorylated glucosamine unit of *R. etli* and *R. leguminosarum* may be present as a hemiacetal (Fig. 1C), or it may be further oxidized to 2-amino-2-deoxy-gluconate (aminogluconate) (Fig. 1D) (21–23). Oxidation of the proximal glucosamine moiety is catalyzed by the O_2-dependent outer membrane oxidase LpxQ (Fig. 1B) (25, 26). Wild-type *Rhizobium* lipid A is mostly penta-acylated (Figs. 1C and 1D) (22, 23).

In the present study we have expressed the *F. novicida* phosphatases LpxE and LpxF, and the *R. leguminosarum* lipid A oxidase LpxQ, in several strains of *E. coli* (Fig. 1B and Fig. 2). We show that the presence of these modification enzymes results in dramatic alterations of *E. coli* lipid A structure (Fig. 1B and Fig. 2). The novel lipid A species accumulating in these constructs are those expected based on the previously reported lipid A modification pathways found in *R. etli* and *R. leguminosarum* (1). Removal of the 4′-phosphate group (Fig. 2) greatly increases the resistance of *E. coli lpxM* mutants to polymyxin. Co-expression of LpxE and LpxQ in *E. coli* results in the efficient conversion of the proximal glucosamine unit to aminogluconate. Expression of these modification enzymes in *E. coli* provides access to large quantities of novel lipid A derivatives with possible utility as adjuvants or endotoxin antagonists (Fig. 1B and Fig. 2).

MATERIALS AND METHODS

Materials

$[\gamma\text{-}^{32}P]ATP$ was obtained from PerkinElmer Life and Analytical Sciences, Inc. (Waltham, MA). Acetic acid, chloroform, sodium acetate, and glass-backed 0.25-mm Silica Gel-60 thin layer chromatography (TLC) plates were from EMD Chemicals, Inc. (Gibbstown, NJ).

Formic acid, pyridine, and methanol were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Yeast extract, agar, and tryptone were from Becton, Dickinson (Franklin Lakes, NJ). Bicinchoninic protein assay reagents (27) and Triton X-100 were from Thermo Fisher Scientific (Waltham, MA). PCR reagents were purchased from EMD and Stratagene (La Jolla, CA). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was from Invitrogen (Carlsbad, CA). All other chemicals were reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or VWR International (West Chester, PA).

Bacterial strains and growth conditions

Table I summarizes the characteristics of bacterial strains and plasmids relevant to this study. *E. coli* strains were grown at 37 °C in LB broth (1% tryptone, 0.5% yeast extract, and 1% sodium chloride) (28), supplemented with the following antibiotics, as appropriate: ampicillin (100 μg/mL), kanamycin (50 μg/mL), tetracycline (12.5 μg/ml) or chloramphenicol (30 μg/mL).

Recombinant DNA Techniques

Handling of DNA and preparation of *E. coli* for electroporation were performed using standard protocols (29). Transformation-competent cells of *E. coli* were generated by the method of Inoue *et al* (30). Plasmids were isolated from cell cultures and purified using the QiAprep and QIAquick gel extraction kits from Qiagen (Valencia, CA). KOD Hot Start DNA polymerase (EMD), Pfu Turbo DNA polymerase (Stratagene), T4 DNA ligase (Invitrogen), restriction endonucleases (New England Biolabs, Ipswich, MA) and shrimp alkaline phosphatase (Affymetrix, Santa Clara, CA) were used according to the manufacturers' instructions. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from MWG Biotech (Huntsville, AL) and IDT Biosciences (Coralville, IA).

Construction of plasmids expressing R. leguminosarum lpxQ, F. novicida lpxE, and/or F. novicida lpxF

Plasmids expressing combinations of LpxQ, LpxE, and LpxF were created from the lactose-inducible, low-copy expression vector, pWSK29 (31). Plasmids expressing multiple genes were designed so that the coding region of each gene would be preceded by a ribosome binding site. The oligonucleotide primers are shown in Supporting Table I. The oligonucleotide primers RlegQfor and RlegQrev were used to amplify the *R. leguminosarum lpxQ* gene from plasmid pQN233 (25), resulting in PCR product 1. The PCR product contained the coding region of LpxQ. The oligonucleotide primers FnovEfor and FnovEHind were used to amplify the *F. novicida lpxE* gene from pWSK29-LpxE (8), resulting in PCR product 2. PCR product 2 contained the coding region of *lpxE*. The sequence upstream of the *lpxE*-coding region in the primer FnovEfor was complementary to the sequence downstream of the *lpxQ*-coding region in the primer RlegQrev. PCR product 3, which contained the coding regions of *lpxQ* and *lpxE*, each with their own ribosome binding sites, was created by amplifying PCR products 1 and 2 with the primers RlegQfor and FnovEHind. PCR reaction product 3 was digested using *XbaI* and *HindIII* and analyzed on a 1% agarose gel. The desired band was excised and gel-purified. The purified PCR product was ligated into pWSK29 which had been similarly digested and treated with shrimp alkaline phosphatase. The ligation mixture was transformed into XL1-Blue cells, and several ampicillin-resistant colonies were selected on LB plates. The plasmid pWSK29-LpxQE was isolated from this pool and transformed into W3110A and WBB06 cells.

Plasmid pWSK29-LpxQEF, encoding the *RllpxQ*, *FnlpxE*, and *FnlpxF* genes, was created in a second series of PCR reactions. The oligonucleotide primers RlegQfor and FnovErev were used to amplify the *lpxQ* and *lpxE* genes from plasmid pWSK29-LpxQE, resulting in PCR

product 4. Primers FnovFfor and FnovFrev were used to amplify the *lpxF* gene from pWSK29-LpxF (19) to create PCR product 5. The sequence upstream of the *lpxF*-coding region in the primer FnovFfor also coded for a ribosome binding site and was complementary to the sequence downstream of the *lpxE*-coding region in the primer FnovErev. PCR product 6, containing the coding regions of *RllpxQ*, *FnlpxE*, and *FnlpxF* each with their own ribosome binding sites, was created by amplifying the genes from PCR products 4 and 5 using the RlegQfor and FnovFrev primers. PCR product 6 was digested using *XbaI* and *KpnI*, analyzed on a 1% agarose gel, and gel-purified. PCR product 6 was ligated into pWSK29 which had been similarly digested and treated with shrimp alkaline phosphatase. The ligation mixture was transformed into XL1-Blue cells, and several ampicillin-resistant colonies were selected on LB plates. The plasmid pWSK29-LpxQEF was isolated from this pool and transformed into *E. coli* strains MLK1067 and SR8209.

Plasmid pWSK29-LpxEF which encodes both *lpxE* and *lpxF*, was constructed in another set of PCR reactions. The oligonucleotide primers FnovEXbaI and FnovFrev were used to amplify the *lpxE* and *lpxF* genes from plasmid pWSK29-LpxQEF to create PCR product 7. PCR product 7 was digested with *XbaI* and *KpnI*, and analyzed on a 1% agarose gel. The desired band was excised and gel-purified. The purified PCR product was ligated into pWSK29 which had been similarly digested and treated with shrimp alkaline phosphatase. The ligation mixture was transformed into XL1-Blue cells, and several ampicillin-resistant colonies were selected on LB plates. The plasmid pWSK29-LpxEF was isolated from this pool and transformed into *E. coli* strains MLK1067and SR8209.

Preparation of Washed Membranes

All enzyme preparations were carried out at 0–4 °C. Cell-free extracts and washed membranes were prepared as described previously (32). Typically 200-mL cultures were grown to $A_{600} = 1.0$. The cells were harvested by centrifugation at $4000 \times g$ for 20 min. The cell pellets were washed with 40 mL of 50 mM HEPES, pH 7.5 and stored at −80 °C. The frozen pellets were later thawed and resuspended in 10 mL of 50 mM HEPES, pH 7.5. The cells were disrupted by passage through a French pressure cell at 18,000 p.s.i., and unbroken cells were removed by centrifugation at $10,000 \times g$ for 20 min. The membranes were collected by centrifugation at $100,000 \times g$ for 1 h and washed by suspension in 10 mL of 50 mM HEPES, pH 7.5. The membranes were resuspended in the same buffer to a protein concentration of 5–10 mg/mL and stored in aliquots at −80 °C. The bicinchoninic acid method (27) was used to determine protein concentrations using bovine serum albumin as the standard.

Preparation of Substrates

Preparation of the Kdo_2-lipid IV_A and Kdo_2-[4′-^{32}P]-lipid IV_A substrates were described previously (33,34). The substrates were stored frozen in 25 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1mM EGTA, and 0.1% Triton X-100. Prior to use, the lipid substrates were dispersed by sonic irradiation for 1 min in a bath appartus.

In vitro assays of LpxE and LpxQ

LpxE and LpxQ activities were assayed in 10–30 μL reaction mixtures at 30 °C. Standard assay conditions contained 50 mM MES, pH 6.5, 0.1% Triton X-100, 1 mM $MgCl_2$, and 10 μM Kdo_2-[4′-^{32}P]lipid IV_A (45,000 cpm/nmol). Reactions were initiated with the enzyme and incubated for the indicated times. Reactions were terminated by spotting 3-μL samples onto a TLC plate, which was developed in the solvent chloroform/methanol/acetic acid/water (25:15:4:4, v/v). After drying and overnight exposure of the plate to a PhosphorImager screen (GE Healthcare, Waukesha, WI), product formation was detected and quantified with

a Storm 840 PhosphorImager equipped with ImageQuant software (GE Healthcare, Waukesha, WI).

Lipid A extraction

Single colonies of cells harboring pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxQE, pWSK29-LpxEF, or pWSK29-LpxQEF were used to inoculate 5 mL cultures of LB medium supplemented with 100 μg/mL ampicillin and grown overnight with shaking at 37 °C. The overnight cultures were used to inoculate 200 mL cultures of LB supplemented with 100 μg/mL ampicillin and 1mM IPTG, starting at $A_{600} = 0.01$. The cells were shaken (220 rpm) at 37 °C and grown to $A_{600} = 1.0$. Cells were harvested by centrifugation at $4000 \times g$ for 20 min at 4 °C, washed once with phosphate-buffered saline (PBS) and stored at −80 °C. The washed W3110A and MLK1067 pellets were later thawed and extracted for 1 h at room temperature with a single phase Bligh-Dyer mixture consisting of 47.5 mL chloroform/methanol/water (1:2:0.8, v/v) (35). The cell debris was collected by centrifugation at $4000 \times g$ for 20 min, and the insoluble material was washed once more with 47.5 mL a single-phase Bligh-Dyer mixture. The insoluble material was again recovered by centrifugation. The washed pellets were then resuspended in 35 mL 12.5 mM sodium acetate, pH 4.5, and subjected to probe sonic irradiation. The suspensions were placed in a boiling water bath for 30 min, after which they were cooled to room temperature. The suspensions were converted to two-phase Bligh-Dyer mixtures (35) (chloroform:methanol:water, 2:2:1.8, v/v) by adding appropriate amounts of chloroform and methanol. The phases were mixed and separated by centrifugation at $4000 \times g$ for 20 min. The lower phases were removed, and the upper water-methanol phases were extracted a second time by the addition of fresh pre-equilibrated lower phase. The lower phases were pooled and dried with a rotary evaporator. The lipids were stored at −80 °C.

The lipid A extraction procedure for the WBB06 and SR8209 cells differed slightly because these harbor deletions in the *waaC* and *waaF* genes, which encode the heptosyl transferases of LPS biosynthesis. These strains accumulate a truncated LPS, consisting of Kdo_2-lipid A, which is soluble in a single-phase Bligh Dyer mixture and therefore recovered with the glycerophospholipids. The extraction procedure was the same as that used for the other strains except the hydrolysis step was omitted. The supernatants from the single-phase Bligh Dyer extractions of WBB06 and SR8209 cells were directly converted to two-phase Bligh Dyer solutions, and further extracted as described above.

Electrospray Ionization-Mass Spectrometry (ESI-MS) of Lipid A Preparations

Lipid A or Kdo_2-lipid A molecules extracted from 200 mL cell cultures were re-dissolved in 300 μL chloroform:methanol (4:1, v/v), subjected to sonic irradiation in a bath apparatus, and subsequently diluted 10-fold into chloroform:methanol (2:1, v/v), supplemented 1% piperidine. The samples were immediately infused into the ion source at 5–10 μL/min and analyzed by ESI/MS in the negative ion mode, as described previously (36–38). All mass spectra were acquired on a QSTAR XL quadrupole time-of-flight mass spectrometer (ABI/MDS-Sciex, Toronto, Canada), equipped with an ESI source. Spectra were the accumulation of 60 scans from *m/z* 200 to *m/z* 2000 amu. Data acquisition and analysis were performed using Analyst QS software version 1.1.

Growth Phenotypes of SR8209 cells expressing *RlLpxQ*, *FnLpxE*, and *FnLpxF*

SR8209 cells (39) expressing pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF were grown at 37 °C overnight on LB broth containing 100 μg/mL ampicillin. The cultures were used to inoculate 25 mL of prewarmed LB broth (37 °C) containing 1 mM IPTG and 100 μg/mL ampicillin to $A_{600} = 0.02$. Whenever the A_{600}

reached 0.3–0.4, the cultures were diluted 10-fold into 25 mL of prewarmed LB broth containing 1 mM IPTG and 100 μg/mL ampicillin, and growth was continued as indicated.

Minimal inhibitory concentration (MIC) determinations

The MICs of polymyxin B sulfate, rifamycin, erythromycin, and vancomycin were determined for MLK1067 strains expressing combinations of FnLpxE, FnLpxF, and RlLpxQ, as described below. The MIC of polymyxin B sulfate was determined similarly for SR8209 cells expressing combinations of FnLpxE, FnLpxF, and RlLpxQ. Briefly, 100 μL LB medium supplemented with 100 μg/mL ampicillin and 1 mM IPTG was added to the wells of several 96-well plates (Corning Costar 3596, flat bottomed lid, polystyrene wells). Different concentrations of each tested antibiotic were prepared by making serial dilutions (1:2) of antibiotic stock solutions into the wells. MLK1067 or SR8209 cells expressing pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF were prepared by growing the strains overnight in LB broth containing 100 μg/mL ampicillin at 37 °C. The cultures were used to inoculate tubes containing 5 mL of LB broth supplemented with 1 mM IPTG and 100 μg/mL ampicillin to a starting $A_{600} = 0.02$. The cultures were grown to an $A_{600} = 0.6$ and then diluted 1:100 into LB supplemented with 1 mM IPTG and 100 μg/mL ampicillin. The diluted cultures were further diluted 1:2 into the wells containing 100 μL media supplemented with various concentrations of antibiotics. The plates were incubated at 37 °C for 20 hours. After the incubation period, 50 μL of a 1 mg/mL [4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide stock solution was added to each well. The plates were incubated for 3 additional hours at 37 °C. Cell viability and MIC were assessed visually by the absence of purple color, indicating complete cell death. Results are reported as the means of three replicates.

Ca²⁺ sensitivity assays

SR8209 cells harboring pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF were grown overnight starting from single colonies in LB broth supplemented with 100 μg/mL ampicillin. The cultures were used to inoculate tubes containing 5 mL of LB broth supplemented with 1 mM IPTG and 100 μg/mL ampicillin to $A_{600} = 0.02$. The cultures were grown to $A_{600} = 0.1$ (~10^8 CFU). Six ten-fold serial dilutions of each strain were made into LB broth. Ten μL of each dilution was then transferred to the individual grids of a 100×15 mm square LB agar plate, scored with 13 mm square grids. These plates were supplemented either with 100 μg/mL ampicillin, 1 mM IPTG, and 10 mM $CaCl_2$, or with 100 μg/mL ampicillin and 1 mM IPTG. Strains were grown at 37 °C for 16 h. The plates were incubated an additional 24 h at room temperature before being photographed. All strains were plated in duplicate.

RESULTS

Co-expression of FnLpxE and RlLpxQ in wild-type E. coli

Expression of the *F. novicida* lipid A 1-phosphatase FnLpxE behind the *lac* promoter on pWSK29 causes nearly complete lipid A 1-dephosphorylation in living wild-type *E. coli* cells (8). To determine if the 1-dephosphorylated lipid A could be further oxidized to aminogluconate (Fig. 1B), we constructed an inducible plasmid that co-expresses both LpxE and LpxQ by cloning the *RllpxQ* gene and the *FnlpxE* gene in tandem behind the *lac* promoter of pWSK29 (Supporting Scheme 1). We used *FnlpxE* instead of *R. leguminosarum lpxE* because the former is expressed at higher activity levels in *E. coli* (6,8). The *RllpxQ* gene was cloned upstream of *FnlpxE* in pWSK29-LpxQE to maximize its expression. Plasmid pWSK29-LpxQE was then transformed into wild-type *E. coli* K-12 W3110A (40) to assess its effects on lipid A structure and in vitro enzyme activity.

ESI-MS of lipid A from W3110A cells expressing LpxE and LpxQ

The negative ion ESI mass spectra of the lipid A species recovered after mild acid hydrolysis of LPS from wild-type W3110A expressing pWSK29, pWSK29-LpxE, or pWSK29-LpxQE are shown in Fig. 3. The predominant lipid A species in the vector control (Fig. 3A) appears at *m/z* 1796.24 (Fig. 1A), consistent with the [M-H]⁻ ion of *bis*-phosphorylated wild-type *E. coli* lipid A (predicted [M-H]⁻ ion at *m/z* 1796.21). As expected, expression of FnLpxE in W3110A generates a large amount of 1-dephosphoryl-lipid A (Fig. 3B). The major peak at *m/z* 1716.26 is interpreted as the [M-H]⁻ ion of 1-dephosphorylated lipid A (predicted [M-H]⁻ ion at *m/z* 1716.25). There is very little residual *bis*-phosphorylated lipid A.

W3110A cells expressing pWSK29-LpxQE accumulate both 1-dephosphorylated lipid A and a large quantity of a new species at *m/z* 1732.28. This peak is consistent with the [M-H]⁻ ion of a lipid A molecule in which the 1-dephosphorylated proximal unit has been oxidized to an aminogluconate residue (Fig. 3C) (predicted [M-H]⁻ ion at *m/z* 1732.24). Virtually no wild-type lipid A is detectable (Fig. 3C). The formation of the aminogluconate residue was dependent upon the presence of molecular oxygen, but no ¹⁸O is incorporated into the product (data not shown). These results show that FnLpxE and RlLpxQ can function together efficiently *in vivo* to modify the lipid A moiety of wild-type *E. coli* LPS (Fig. 1A).

Enzymatic modification of lipid A in vitro by membranes of W3110A cells expressing LpxE and LpxQ

The activity of recombinant RlLpxQ and FnLpxE was measured in vitro, using washed membranes of W3110A/pWSK29-LpxQE as the enzyme source, and tetra-acylated *E. coli* Kdo_2-[4′-³²P]lipid IV_A as the substrate (Fig. 4). The assay was performed under conditions optimized for LpxQ. The product of LpxE, 1-dephosphorylated Kdo_2-[4′-³²P]lipid IV_A, was formed rapidly in reactions initiated with membranes from cells harboring either pWSK29-LpxE or pWSK29-LpxQE (Fig. 4). However, membranes from cells containing pWSK29-LpxQE further metabolized the 1-dephosphorylated Kdo_2-[4′-³²P]lipid IV_A to a more slowly migrating product in a time-dependent fashion (Fig. 4), consistent with LpxQ catalyzed oxidation of the proximal glucosamine unit (Fig. 1B) (25,26). The small amounts of faster migrating products indicated by the asterisks (Fig. 4) are probably generated by the acyltransferase PagP, which is present in membranes of W3110A (41).

ESI-MS of hexa-acylated Kdo_2-lipid A derivatives from WBB06 cells expressing LpxE and LpxQ

A portion of the oxidized lipid A generated by LpxQ is degraded during the mild acid hydrolysis procedure used to cleave the Kdo-lipid A linkage of wild-type LPS prior to mass spectrometry (Supporting Fig. 1) (22, 23). This material, the [M-2H]²⁻ ion of which is observed at *m/z* 734.51 (Supporting Fig. 1, inset), might be generated by lactone formation of the aminogluconate residue under the mild acid hydrolysis conditions, followed by β-elimination of *R*-3-hydroxymyristic acid (22, 23).

To avoid mild acid hydrolysis of LPS altogether, WBB06 mutant cells, which are heptose-deficient because of a deletion spanning *waaC* and *waaF* (42), were grown harboring either pWSK29, pWSK29-LpxE, or pWSK29-LpxQE (Fig. 1B) under the same conditions used above for W3110A. WBB06 cells synthesize mainly hexa-acylated Kdo_2-lipid A (Fig. 1B and Fig. 5A, inset) as their LPS (43). This substance can be extracted without the hydrolysis step together with the glycerophospholipids by using chloroform/methanol/water mixtures in Bligh-Dyer proportions (43). The Kdo_2-lipid A can then be analyzed directly by ESI-MS (Fig. 5A). Kdo_2-lipid A is observed primarily as the doubly charged ion [M-2H]²⁻ at *m/z* 1117.66 (Fig. 5A), matching the predicted value for *E. coli* Kdo_2-lipid A (43).

WBB06 cells harboring pWSK29-LpxE generated mainly 1-dephosphorylated Kdo_2-lipid A (Fig. 1B, Product I), as reported previously (8) (data not shown). WBB06 cells harboring pWSK29-LpxQE (Fig. 5B) generated equal amounts of the same 1-dephosphorylated Kdo_2-lipid A, the $[M-2H]^{2-}$ ion of which is observed at m/z 1077.68 (Fig. 1B, Product I), and an oxidized product, the $[M-2H]^{2-}$ ion of which is observed at m/z 1085.68 (Fig. 1B, Product II). There is very little residual Kdo_2-lipid A. The β-elimination product of the aminogluconate derivative that accumulates in W3110A cells (Supporting Fig. 1) is not observed with this extraction procedure. However, a small peak corresponding to a putative lactone form of the aminogluconate moiety (Fig. 5B, black asterisk) is seen at m/z 1076.68, and its possible methanolysis product (Fig. 5B, red asterisk) is observed at m/z 1092.68 (44).

ESI-MS of penta-acylated Kdo_2-lipid A species in SR8209 expressing LpxE, LpxF, and LpxQ

To determine if *E. coli* cells can grow without phosphate groups at the 1- and/or the 4′-positions of lipid A, we utilized deletion mutants of the *E. coli* secondary acyltransferase LpxM (45–48), which are viable but synthesize only penta-acylated lipid A (Fig. 2). The plasmids pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-EF, and pWSK29-QEF were transformed into *E. coli* SR8209 (39), which lacks both LpxM and the heptosyl transferase WaaC. SR8209 generates penta-acylated Kdo_2-lipid A as its major LPS (Fig. 2 and Fig. 6A, inset) (39). This material can be extracted without acid hydrolysis and analyzed directly by ESI-MS in the negative ion mode. Like the hexa-acylated lipid A species (Fig. 5), the penta-acylated Kdo_2-lipid A derivatives are detected mainly as doubly-charged species (Fig. 6). The spectrum of the lipid A species from the SR8209 cells harboring the vector control pWSK29 shows a prominent peak at m/z 1012.55 (Fig. 6A), consistent with the predicted $[M-2H]^{2-}$ of 1012.56. The small peaks at m/z 1023.54 and m/z 1031.53 are attributed to $[M-3H+Na]^{2-}$ and $[M-3H+K]^{2-}$ adducts, respectively (Fig. 6A, asterisks).

FnLpxF can dephosphorylate penta-acylated but not hexa-acylated lipid A (19), whereas LpxE can dephosphorylate both. Expression of either pWSK29-LpxE or pWSK29-LpxF in SR8209 greatly reduces the peak attributed to the parental lipid A species at m/z 1012.55, but an intense new doubly charged peak is seen at m/z 972.57 or 972.58 (Fig. 6B and Fig. 6C, respectively). This observation is consistent with formation of the isomeric, mono-phosphorylated Kdo_2-lipid A derivatives, Products III or IV in Fig. 2, the predicted $[M-2H]^{2-}$ ions of which are both expected at m/z 972.58.

Remarkably, SR8209 cells expressing pWSK29-EF accumulate a large amount of a new doubly charged species at m/z 932.60 (Fig. 6D), consistent with the predicted $[M-2H]^{2-}$ ion at m/z 932.59 of Product V (Fig. 2). No residual peaks corresponding to either mono-phosphorylated or *bis*-phosphorylated lipid A are present, demonstrating the efficient combined action of FnLpxE and FnLpxF in this strain. SR8209 cells expressing pWSK29-LpxQEF (Fig. 6E) contain a mixture of the same fully dephosphorylated species and its aminogluconate derivative at m/z 940.61 (predicted $[M-2H]^{2-}$ ion at m/z 940.59), corresponding to Product VI in Fig. 2. The small peak indicated by the green asterisk in Fig. 6E may reflect the presence of the methyl ester of VI, possibly formed from the lactone form of Product VI during lipid extraction.

The same plasmids shown in Fig. 6 were introduced into *E. coli* MLK1067, a mutant that lacks LpxM but synthesizes a full-length core oligosaccharide (48). The same pattern of lipid A modifications was observed in this background (data not shown).

Phenotypes of cells expressing LpxE, LpxF, and LpxQ

The phosphate moieties of lipid A are thought to play a role in maintaining the integrity of the outer membrane of Gram-negative bacteria (49). The acidic phosphate groups can bind divalent cations like Mg^{2+}, effectively cross-linking the LPS (49). We assessed the importance of these ionic interactions in maintaining outer membrane integrity by examining the growth phenotypes of MLK1067 and SR8209 cells expressing combinations of LpxE, LpxF, and LpxQ.

The outer membrane permeability of MLK1067 cells has been analyzed previously (50). Loss of the secondary 3′-myristoyl chain in this mutant does not impact resistance to hydrophobic compounds and antibiotics (50). Expression of pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF in MLK1067 did not greatly alter this strain's resistance to vancomycin, rifamycin SV, or erythromycin (Table II). These results suggest that the phosphate groups of lipid A are not very important for the normal function of the outer membrane permeability barrier towards these three antibiotics. We did not measure the sensitivity of SR8209 expressing pWSK29-LpxEF and pWSK29-LpxQEF to these drugs, because it is well established that the outer membrane permeability barrier is greatly impaired in heptose-deficient deep-rough mutants of this kind (49,50).

SR8209 cells expressing pWSK29-LpxEF and pWSK29-LpxQEF displayed several interesting phenotypes. These constructs grew more slowly than cells expressing either LpxE or LpxF alone (Fig. 7). Expression of pWSK29-LpxEF inhibited growth of SR8209 after several generations in shaking liquid culture, but these cells could still form small colonies on plates (Fig. 8). However, SR8209 cells expressing pWSK29-LpxEF and pWSK29-LpxQEF displayed sensitivity to 10 mM calcium chloride on LB agar plates (Fig 8). SR8209 cells expressing pWSK29-LpxE displayed partial calcium chloride sensitivity, but were not as impaired as SR8209 cells harboring pWSK29-LpxEF or pWSK29-LpxQEF (Fig. 8).

Polymyxin resistance of E. coli mutants expressing LpxF

Deletion of LpxF in *F. novicida* results in extreme hypersensitivity to polymyxin B and attenuation of virulence (20). We determined the MICs for polymyxin B in *E. coli* MLK1067 and SR8209 harboring pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, and pWSK29-LpxQEF. Strains expressing pWSK29-LpxEF and pWSK29-LpxQEF were much more resistant to polymyxin compared to the strains expressing the vector control, pWSK29 (Table III). Removal of the 4′ phosphate group by LpxF alone caused the most dramatic reduction in polymyxin sensitivity (Table III). The MICs of strains expressing LpxF are comparable to those observed in *pmrA* constitutive mutants of *E. coli* or *S. typhimurium* (17,51). These results support the idea that a strong binding interaction between the lipid A phosphate groups and polymyxin B is necessary as a prelude to cell killing.

DISCUSSION

F. novicida and *R. leguminosarum* encode two polytopic inner membrane phosphatases, designated LpxE and LpxF, that remove the 1- and 4′-phosphate groups of lipid A, respectively (8,19). The active sites of both enzymes are located on the periplasmic surface of the inner membrane (8,19). Genetic studies (24) have shown that LpxE and LpxF are solely responsible for the absence of the lipid A phosphate groups in *R. leguminosarum*. LpxE is Kdo-dependent, whereas LpxF is not (8,19). Available LpxF orthologues cannot dephosphorylate hexa-acylated lipid A species, but only penta- or tetra-acylated variants (19).

Expression of FnLpxE in wild-type cells, or of FnLpxF in *lpxM* mutants of *E. coli*, results in 1- or 4′-dephosphorylation of lipid A, respectively (8,19). We have now coupled the over-expression of FnLpxE, FnLpxF, and the *R. leguminosarum* lipid A oxidase RlLpxQ to generate entirely novel lipid A hybrid structures in *E. coli*. When co-expressed in *E. coli*, FnLpxE and RlLpxQ generate lipid A species that are more than 90% dephosphorylated at the 1-positon, and at least half of this material is converted to the aminogluconate form (Figs. 3 and 5). This result demonstrates that lipid A modification enzymes from diverse sources can be combined to facilitate efficient re-engineering of lipid A structure. LpxQ has not yet been purified to homogeneity, and its orientation within the outer membrane is unknown. The fact that LpxQ can generate the aminogluconate unit efficiently in *E. coli* suggests that it does not require a second protein component.

The dephosphorylated lipid A species in our constructs resemble those of *Rhizobium* (Figs. 1 and 2) (22,23). Lipid A variants of this kind are potentially interesting, because synthetic lipid A derivatives based on the lipid A of *Rhizobium* Sin-1 (52) are endotoxin antagonist (53,54). The aminogluconate moiety, in particular, may be important for this pharmacology (44,55). Derivatives lacking the 4′-phosphate group may function as novel endotoxin antagonists or partial agonists. Furthermore, Kdo_2-lipid A derivatives that retain the 4′-phosphate unit but lack the 1-phosphate group may be very useful as adjuvants (12). The existing 4′-MPLAs approved for clinical use are prepared by acid hydrolysis of LPS and therefore lack the Kdo disaccharide (12), which is retained when LpxE is used to dephosphorylate the 1-position. The Kdo disaccharide of LPS interacts with the TLR-4 protein in the crystal structure of the LPS/TLR-4/MD2 complex (56), and its presence might influence biological activity.

The interchangeability and synergy of lipid A modification enzymes was further demonstrated by coupling the expression of FnLpxF with FnLpxE and RlLpxQ in *E. coli* *lpxM* mutants (Fig. 6). Expression of both phosphatases resulted in nearly quantitative dephosphorylation of lipid A (Fig. 6). Expression of RlLpxQ together with the two phosphatases led to the further generation of oxidized lipid A species (Fig. 6), containing aminogluconate. Although the heptose-deficient mutant SR8209 grew poorly in liquid medium when both phosphate groups were missing (Fig. 7), the *E. coli lpxM* mutant MLK1067, which synthesizes a complete core, grew almost normally when expressing LpxE and LpxF (data not shown). Its membrane integrity was not greatly compromised, as judged by antibiotic sensitivity (Table II). The core oligosaccharide of LPS apparently can maintain outer membrane stability, even when the ionic interactions between neighboring lipid A phosphate groups mediated by divalent cations are disrupted.

The viability of strains with fully dephosphorylated lipid A species was unexpected. The phosphate moieties of lipid A are incorporated in the early stages of biosynthesis (1). Our results show that they are not essential for cell viability and outer membrane integrity, if removed by LpxE and LpxF after the MsbA step of lipid A transport (1). Hence, the conservation of the lipid A biosynthetic pathway and the *bis*-phosphorylated structure of lipid A in Gram-negative bacteria may be more important for LPS transport across the inner membrane than for outer membrane function.

The heptose deficient mutant SR8209 (39), harboring pWSK29-LpxEF or pWSK29-LpxQEF, accumulates the fully dephosphorylated lipid A derivatives, Products V and VI (Fig. 6). These constructs not only displayed impaired growth in liquid medium (Fig. 7) but also failed to grow on plates in the presence of 10 mM $CaCl_2$ (Fig. 8). The significance of this phenotype will require further study. The normal intracellular concentration of Ca^{2+} in *E. coli* cells is 0.1 μM or less (57) and too much may be toxic. Sensitivity to extracellular

Ca^{2+} is also seen in heptose-deficient strains of *E. coli* lacking EptB, a Ca^{2+}-inducible enzyme that transfers a phosphoethanolamine residue to the outer Kdo unit (33).

Lipid A phosphatases have been described not only in *R. leguminosarum* and *F. novicida* but also in *Helicobacter pylori* and *Porphyromonas gingivalis* (58,59). Chromosomal deletion of the phosphatases in many of these organisms leads to increased sensitivity to cationic antimicrobial peptides and attenuation of pathogenesis (20,60). We therefore expected to see an increase in polymyxin B resistance in *E. coli* strains expressing LpxE and LpxF (Table III). However, removal of the 4′-phosphate moiety increased polymyxin resistance much more than did the removal of the 1-phosphate group, both in MLK1067 and SR8209 (Table III). The 4′-phosphate group is the predominant site of aminoarabinose attachment in polymyxin-resistant mutants of *E. coli* and *S. typhimurium* (61). These observations suggest that polymyxin interacts preferentially with the 4′-phosphate group of lipid A.

Lipid A modification enzymes not only provide access to new lipid A derivatives that may be useful as adjuvants or endotoxin antagonists but also may be useful for generating novel live bacterial vaccines. Heterologous expression of lipid A modification enzymes like LpxE, LpxF, LpxR, or PagL in pathogens such as *Salmonella* might attenuate these bacteria by altering lipid A structural elements recognized by the TLR-4/MD2 complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The abbreviations used are

EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
IPTG	isopropyl β-D-1-thiogalactopyranoside
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
L-Ara4N	4-amino-4-deoxy-L-arabinose
LPS	lipopolysaccharide
MES	2-(N-morpholino)-ethanesulfonic acid
MIC	minimal inhibitory concentration
MPLA	monophosphoryl lipid A
MS	mass spectrometry
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
pEtN	phosphoethanolamine

| TLC | thin layer chromatography |
| TLR-4 | Toll-like receptor-4 |

References

1. Raetz CRH, Reynolds CM, Trent MS, Bishop RE. Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem. 2007; 76:295–329. [PubMed: 17362200]

2. Rietschel ET, Kirikae T, Schade FU, Mamat U, Schmidt G, Loppnow H, Ulmer AJ, Zähringer U, Seydel U, Di Padova F, Schreier M, Brade H. Bacterial endotoxin: molecular relationships of structure to activity and function. FASEB Journal. 1994; 8:217–225. [PubMed: 8119492]

3. Loppnow H, Brade H, Dürrbaum I, Dinarello CA, Kusumoto S, Rietschel ET, Flad HD. IL-1 induction capacity of defined lipopolysaccharide partial structures. J Immunol. 1989; 142:3229–3238. [PubMed: 2651523]

4. Qureshi N, Mascagni P, Ribi E, Takayama K. Monophosphoryl lipid A obtained from lipopolysaccharides of Salmonella minnesota R595. Purification of the dimethyl derivative by high performance liquid chromatography and complete structural determination. J Biol Chem. 1985; 260:5271–5278. [PubMed: 3988753]

5. Qureshi N, Takayama K, Ribi E. Purification and structural determination of non-toxic lipid A obtained from the lipopolysaccharide of *Salmonella typhimurium*. J Biol Chem. 1982; 257:11808–11815. [PubMed: 6749846]

6. Karbarz MJ, Kalb SR, Cotter RJ, Raetz CRH. Expression cloning and biochemical characterization of a *Rhizobium leguminosarum* lipid A 1-phosphatase. J Biol Chem. 2003; 278:39269–39279. [PubMed: 12869541]

7. Karbarz MJ, Six DA, Raetz CRH. Purification and characterization of the lipid A 1-phosphatase LpxE of *Rhizobium leguminosarum*. J Biol Chem. 2009; 284:414–425. [PubMed: 18984595]

8. Wang X, Karbarz MJ, McGrath SC, Cotter RJ, Raetz CRH. MsbA transporter-dependent lipid A 1-dephosphorylation on the periplasmic surface of the inner membrane: topography of *Francisella novicida* LpxE expressed in *Escherichia coli*. J Biol Chem. 2004; 279:49470–49478. [PubMed: 15339914]

9. Kundi M. New hepatitis B vaccine formulated with an improved adjuvant system. Expert Rev Vaccines. 2007; 6:133–140. [PubMed: 17408363]

10. Fitzgerald KA, Golenbock DT. Immunology. The shape of things to come. Science. 2007; 316:1574–1576. [PubMed: 17569850]

11. Mata-Haro V, Cekic C, Martin M, Chilton PM, Casella CR, Mitchell TC. The vaccine adjuvant monophosphoryl lipid A as a TRIF-biased agonist of TLR4. Science. 2007; 316:1628–1632. [PubMed: 17569868]

12. Baldridge JR, McGowan P, Evans JT, Cluff C, Mossman S, Johnson D, Persing D. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin Biol Ther. 2004; 4:1129–1138. [PubMed: 15268679]

13. Doerrler WT, Reedy MC, Raetz CRH. An *Escherichia coli* mutant defective in lipid export. J Biol Chem. 2001; 276:11461–11464. [PubMed: 11278265]

14. Ward A, Reyes CL, Yu J, Roth CB, Chang G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. Proc Natl Acad Sci U S A. 2007; 104:19005–19010. [PubMed: 18024585]

15. Sperandeo P, Deho G, Polissi A. The lipopolysaccharide transport system of Gram-negative bacteria. Biochim Biophys Acta. 2009; 1791:594–602. [PubMed: 19416651]

16. Ruiz N, Kahne D, Silhavy TJ. Transport of lipopolysaccharide across the cell envelope: the long road of discovery. Nat Rev Microbiol. 2009; 7:677–683. [PubMed: 19633680]

17. Gunn JS, Lim KB, Krueger J, Kim K, Guo L, Hackett M, Miller SI. PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol Microbiol. 1998; 27:1171–1182. [PubMed: 9570402]

18. Breazeale SD, Ribeiro AA, McClerren AL, Raetz CRH. A formyltransferase required for polymyxin resistance in *Escherichia coli* and the modification of lipid A with 4-amino-4-deoxy-L-arabinose. Identification and function of UDP-4-deoxy-4-formamido-L-arabinose. J Biol Chem. 2005; 280:14154–14167. [PubMed: 15695810]

19. Wang X, McGrath SC, Cotter RJ, Raetz CRH. Expression cloning and periplasmic orientation of the *Francisella novicida* lipid A 4′-phosphatase LpxF. J Biol Chem. 2006; 281:9321–9330. [PubMed: 16467300]

20. Wang X, Ribeiro AA, Guan Z, Abraham SN, Raetz CRH. Attenuated virulence of a *Francisella* mutant lacking the lipid A 4′-phosphatase. Proc Natl Acad Sci U S A. 2007; 104:4136–4141. [PubMed: 17360489]

21. Bhat UR, Forsberg LS, Carlson RW. The structure of the lipid A component of *Rhizobium leguminosarum* bv. *phaseoli* lipopolysaccharide A unique, non-phosphorylated lipid A containing 2-amino-2-deoxy-gluconate, galacturonate, and glucosamine. J Biol Chem. 1994; 269:14402–14410. [PubMed: 8182046]

22. Que NLS, Lin S, Cotter RJ, Raetz CRH. Purification and mass spectrometry of six lipid A species from the bacterial endosymbiont *Rhizobium etli*; demonstration of a conserved distal and a variable proximal portion. J Biol Chem. 2000; 275:28006–28016. [PubMed: 10856303]

23. Que NLS, Ribeiro AA, Raetz CRH. Two-dimensional NMR spectroscopy and structures of six lipid A species from *Rhizobium etli* CE3; detection of an acyloxyacyl residue in each component and origin of the aminogluconate moiety. J Biol Chem. 2000; 275:28017–28027. [PubMed: 10856304]

24. Ingram BO, Sohlenkamp C, Geiger O, Raetz CRH. Altered lipid A structures and polymyxin hypersensitivity of *Rhizobium etli* mutants lacking the LpxE and LpxF phosphatases. Biochim Biophys Acta. 2010; 1801:593–604. [PubMed: 20153447]

25. Que-Gewirth NLS, Karbarz MJ, Kalb SR, Cotter RJ, Raetz CRH. Origin of the 2-amino-2-deoxy-gluconate unit in *Rhizobium leguminosarum* lipid A. Expression cloning of the outer membrane oxidase LpxQ. J Biol Chem. 2003; 278:12120–12129. [PubMed: 12531908]

26. Que-Gewirth NLS, Lin S, Cotter RJ, Raetz CRH. An outer membrane enzyme that generates the 2-aminogluconate moiety of *Rhizobium leguminosarum* lipid A. J Biol Chem. 2003; 278:12109–12119. [PubMed: 12531907]

27. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150:76–85. [PubMed: 3843705]

28. Miller, JR. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory; Cold Spring Harbor, NY: 1972.

29. Sambrook, JG.; Russell, DW. Molecular Cloning: A Laboratory Manual. 3. Cold Spring Harbor; Cold Spring Harbor, NY: 2001.

30. Inoue H, Nojima H, Okayama H. High efficiency transformation of *Escherichia coli* with plasmids. Gene. 1990; 96:23–28. [PubMed: 2265755]

31. Wang RF, Kushner SR. Construction of versatile low-copy-number vectors for cloning, sequencing and gene expression in *Escherichia coli*. Gene. 1991; 100:195–199. [PubMed: 2055470]

32. Basu SS, Karbarz MJ, Raetz CRH. Expression cloning and characterization of the C28 acyltransferase of lipid A biosynthesis in *Rhizobium leguminosarum*. J Biol Chem. 2002; 277:28959–28971. [PubMed: 12019272]

33. Reynolds CM, Kalb SR, Cotter RJ, Raetz CRH. A phosphoethanolamine transferase specific for the outer 3-deoxy-D-*manno*-octulosonic acid residue of *Escherichia coli* lipopolysaccharide. Identification of the *eptB* gene and Ca^{2+} hypersensitivity of an *eptB* deletion mutant. J Biol Chem. 2005; 280:21202–21211. [PubMed: 15795227]

34. Six DA, Carty SM, Guan Z, Raetz CRH. Purification and mutagenesis of LpxL, the Lauroyltransferase of *Escherichia coli* lipid A biosynthesis. Biochemistry. 2008; 47:8623–8637. [PubMed: 18656959]

35. Bligh EG, Dyer JJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol. 1959; 37:911–917. [PubMed: 13671378]

36. Kanjilal-Kolar S, Basu SS, Kanipes MI, Guan Z, Garrett TA, Raetz CRH. Expression cloning of three *Rhizobium leguminosarum* lipopolysaccharide core galacturonosyltransferases. J Biol Chem. 2006; 281:12865–12878. [PubMed: 16497674]

37. Kanjilal-Kolar S, Raetz CRH. Dodecaprenyl phosphate-galacturonic acid as a donor substrate for lipopolysaccharide core glycosylation in *Rhizobium leguminosarum*. J Biol Chem. 2006; 281:12879–12887. [PubMed: 16497671]

38. Wang X, Ribeiro AA, Guan Z, McGrath S, Cotter R, Raetz CRH. Structure and biosynthesis of free lipid A molecules that replace lipopolysaccharide in *Francisella tularensis* subsp. *novicida*. Biochemistry. 2006; 45:14427–14440. [PubMed: 17128982]

39. Klein G, Lindner B, Brabetz W, Brade H, Raina S. *Escherichia coli* K-12 suppressor-free mutants lacking early glycosyltransferases and late acyltransferases: minimal lipopolysaccharide structure and induction of envelope stress response. J Biol Chem. 2009; 284:15369–15389. [PubMed: 19346244]

40. Doerrler WT, Gibbons HS, Raetz CRH. MsbA-dependent translocation of lipids across the inner membrane of *Escherichia coli*. J Biol Chem. 2004; 279:45102–45109. [PubMed: 15304478]

41. Bishop RE, Gibbons HS, Guina T, Trent MS, Miller SI, Raetz CRH. Transfer of palmitate from phospholipids to lipid A in outer membranes of Gram-negative bacteria. Embo J. 2000; 19:5071–5080. [PubMed: 11013210]

42. Brabetz W, Muller-Loennies S, Holst O, Brade H. Deletion of the heptosyltransferase genes *rfaC* and *rfaF* in *Escherichia coli* K-12 results in an Re-type lipopolysaccharide with a high degree of 2-aminoethanol phosphate substitution. Eur J Biochem. 1997; 247:716–724. [PubMed: 9266718]

43. Raetz CRH, Garrett TA, Reynolds CM, Shaw WA, Moore JD, Smith DC Jr, Ribeiro AA, Murphy RC, Ulevitch RJ, Fearns C, Reichart D, Glass CK, Benner C, Subramaniam S, Harkewicz R, Bowers-Gentry RC, Buczynski MW, Cooper JA, Deems RA, Dennis EA. (Kdo)$_2$-lipid A of *Escherichia coli*, a defined endotoxin that activates macrophages via TLR-4. J Lipid Res. 2006; 47:1097–1111. [PubMed: 16479018]

44. Lee HS, Wolfert MA, Zhang Y, Boons GJ. The 2-aminogluconate isomer of *Rhizobium* sin-1 lipid A can antagonize TNF-alpha production induced by enteric LPS. Chembiochem. 2006; 7:140–148. [PubMed: 16317789]

45. Karow M, Georgopoulos C. Isolation and characterization of the *Escherichia coli msbB* gene, a multicopy suppressor of null mutations in the high-temperature requirement gene *htrB*. J Bacteriol. 1992; 174:702–710. [PubMed: 1732206]

46. Somerville JE Jr, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP. A novel *Escherichia coli* lipid A mutant that produces an anti-inflammatory lipopolysaccharide. J Clin Invest. 1996; 97:359–365. [PubMed: 8567955]

47. Clementz T, Zhou Z, Raetz CRH. Function of the *Escherichia coli msbB* gene, a multicopy suppressor of *htrB* knockouts, in the acylation of lipid A; acylation by MsbB follows laurate incorporation by HtrB. J Biol Chem. 1997; 272:10353–10360. [PubMed: 9099672]

48. Vorachek-Warren MK, Ramirez S, Cotter RJ, Raetz CRH. A triple mutant of *Escherichia coli* lacking secondary acyl chains on lipid A. J Biol Chem. 2002; 277:14194–14205. [PubMed: 11830595]

49. Nikaido H. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev. 2003; 67:593–656. [PubMed: 14665678]

50. Vaara M, Nurminen M. Outer membrane permeability barrier in *Escherichia coli* mutants that are defective in the late acyltransferases of lipid A biosynthesis. Antimicrob Agents Chemother. 1999; 43:1459–1462. [PubMed: 10348770]

51. Trent MS, Ribeiro AA, Doerrler WT, Lin S, Cotter RJ, Raetz CRH. Accumulation of a polyisoprene-linked amino sugar in polymyxin resistant mutants in *Salmonella typhimurium* and *Escherichia coli*. Structural characterization and possible transfer to lipid A in the periplasm. J Biol Chem. 2001; 276:43132–43144. [PubMed: 11535605]

52. Jeyaretnam B, Glushka J, Kolli VS, Carlson RW. Characterization of a novel lipid-A from *Rhizobium* species Sin-1. A unique lipid-A structure that is devoid of phosphate and has a glycosyl backbone consisting of glucosamine and 2-aminogluconic acid. J Biol Chem. 2002; 277:41802–41810. [PubMed: 12193590]

53. Vandenplas ML, Carlson RW, Jeyaretnam BS, McNeill B, Barton MH, Norton N, Murray TF, Moore JN. *Rhizobium* sin-1 lipopolysaccharide (LPS) prevents enteric LPS-induced cytokine production. J Biol Chem. 2002; 277:41811–41816. [PubMed: 12193596]

54. Demchenko AV, Wolfert MA, Santhanam B, Moore JN, Boons GJ. Synthesis and biological evaluation of *Rhizobium* sin-1 lipid A derivatives. J Am Chem Soc. 2003; 125:6103–6112. [PubMed: 12785841]

55. Santhanam B, Wolfert MA, Moore JN, Boons GJ. Synthesis and biological evaluation of a lipid A derivative that contains an aminogluconate moiety. Chemistry. 2004; 10:4798–4807. [PubMed: 15372693]

56. Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature. 2009; 458:1191–1195. [PubMed: 19252480]

57. Gangola P, Rosen BP. Maintenance of intracellular calcium in Escherichia coli. J Biol Chem. 1987; 262:12570–12574. [PubMed: 2442165]

58. Tran AX, Karbarz MJ, Wang X, Raetz CRH, McGrath SC, Cotter RJ, Trent MS. Periplasmic cleavage and modification of the 1-phosphate group of *Helicobacter pylori* lipid A. J Biol Chem. 2004; 279:55780–55791. [PubMed: 15489235]

59. Coats SR, Jones JW, Do CT, Braham PH, Bainbridge BW, To TT, Goodlett DR, Ernst RK, Darveau RP. Human Toll-like receptor 4 responses to P. gingivalis are regulated by lipid A 1- and 4′-phosphatase activities. Cell Microbiol. 2009; 11:1587–1599. [PubMed: 19552698]

60. Coats SR, To TT, Jain S, Braham PH, Darveau RP. *Porphyromonas gingivalis* resistance to polymyxin B is determined by the lipid A 4′-phosphatase, PGN_0524. International Journal of Oral Science. 2009; 1:126–135. [PubMed: 20657724]

61. Zhou Z, Ribeiro AA, Lin S, Cotter RJ, Miller SI, Raetz CRH. Lipid A modifications in polymyxin resistant *Salmonella typhimurium*. PmrA dependent 4-amino-4-deoxy-L-arabinose and phosphoethanolamine incorporation. J Biol Chem. 2001; 276:43111–43121. [PubMed: 11535603]
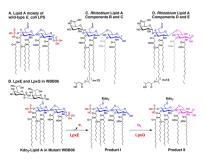
Figure 1. Structures of *E. coli* and *R. etli* lipid A and the origin of aminogluconate

Panel A. The most abundant lipid A moiety of LPS in wild-type *E. coli* consists of a hexa-acylated disaccharide of glucosamine, substituted with monophosphate groups at the 1 and 4′ positions (1). The squiggles indicate the sites of Kdo attachment. **Panel B**. Proposed reactions catalyzed by LpxE and LpxQ in the heptose deficient *E. coli* mutant WBB06, yielding hexa-acylated Products I and II. **Panels C and D**. Predominant lipid A moieties of *R. etli* or *R. leguminosarum* LPS (22,23). The major forms lack phosphate groups and differ from each other in their sugar composition and acylation status. Components B and C contain the typical glucosamine disaccharide found in the lipid A of other organisms (22,23). Components D and E feature an aminogluconate unit in place of the proximal glucosamine and are derived by LpxQ-catalyzed oxidation of B and C, respectively (25,26). Components C and E differ from B and D by the absence of a hydroxyacyl chain at position 3, which is removed by PagL (1). Other features include a long secondary acyl chain at position 2′ and a galacturonic acid unit in place of phosphate at position-4′ (21). Dashed bonds highlight the micro-heterogeneity of *R. etli* or *R. leguminosarum* lipid A with respect to its acyl chains.
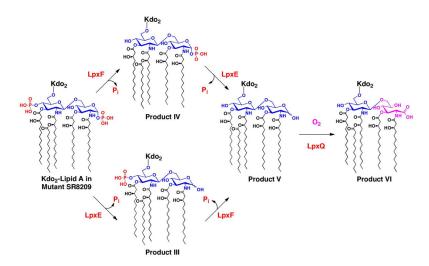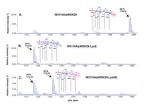
Figure 2. Novel Kdo$_2$-lipid A derivatives in SR8209 cells harboring FnLpxE, FnLpxF, and RlLpxQ

Expression of LpxE or LpxF alone in the heptose-deficient *lpxM* mutant SR8209 yields the isomeric monophosphoryl-Kdo$_2$-lipid A derivatives, Products III and IV, respectively. Co-expression of the 1-phosphatase LpxE and the 4′-phosphatase LpxF in SR8209 yields fully dephosphorylated lipid A (Product V). Co-expression of LpxE, LpxF, and LpxQ additionally generates the oxidized derivative, Product VI.

Figure 3. ESI-MS of lipid A species from W3110A expressing FnLpxE and RlLpxQ
W3110A cells were grown at 37 °C with ampicillin and IPTG, and harvested in late log
phase. Lipid A species were extracted following mild acid hydrolysis of the cell residue.
Panel A. W3110A harboring pWSK29 contain *bis*-phosphorylated lipid A species ([M-H]⁻
at *m/z* 1796.24), typical of wild-type cells. A small amount of 1-dephosphorylated lipid A is
generated as a byproduct of the acid hydrolysis procedure used to cleave the Kdo-lipid A
linkage. **Panel B.** W3110A cells expressing FnLpxE accumulate mostly 1-dephosphorylated
lipid A ([M-H]⁻ at *m/z* 1716.26). **Panel C.** The W3110A cells expressing FnLpxE and
RllpxQ accumulate 1-dephosphorylated lipid A and an additional oxidized derivative ([M-
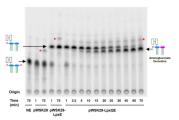H]⁻ at *m/z* at 1732.28), with the proposed structure shown in the inset.

Figure 4. Lipid A phosphatase and oxidase activities in membranes of W3110A cells expressing FnLpxE and RlLpxQ

Membranes (1 mg/mL) were assayed for the indicated times at 30 °C with 10 μM [4′-^{32}P] Kdo$_2$-lipid IV$_A$ as the substrate (Schematic representation: blue ovals, glucosamine units; white rectangles, Kdo residues; magenta oval, aminogluconate unit; green squiggles, R-3-hydroxylmyristoyl groups; red P, phosphate groups). Products were separated by TLC and analyzed with a PhosphorImager. NE: no enzyme control; pWSK29: membranes of the vector control; pWSK29-LpxE: membranes of W3110A/pWSK29-LpxE; pWSK29-LpxQE: membranes of W3110/pWSK29-LpxQE. The red asterisks indicate small amounts of an additional product that is probably generated by the endogenous acyltransferase PagP.
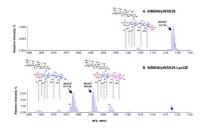
Figure 5. ESI-MS of Kdo$_2$-lipid A derivatives in *E. Coli* WBB06 expressing FnLpxE and RlLpxQ
The heptosyl transferase-deficient mutant WBB06, which synthesizes hexa-acylated Kdo$_2$-lipid A as its sole LPS (43), was grown at 37 °C with ampicillin and IPTG, and harvested in late log phase. Lipids were extracted without mild acid hydrolysis, as described in the methods section. **Panel A.** WBB06 cells, harboring the vector pWSK29, synthesize mainly hexa-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 1117.66). **Panel B.** WBB06 expressing both FnLpxE and RlLpxQ accumulate 1-dephosphorylated and oxidized lipid A species (Products I and II in Fig. 1). The doubly charged peak at *m/z* 1085.68 corresponds to [M-2H]$^{2-}$ of Product II, and the doubly charged peak at *m/z* 1077.68 corresponds to [M-2H]$^{2-}$ of product I. The small peak at *m/z* 1092.68 (red asterisk) may correspond to the methanol hydrolysis product of the lactone form of the aminogluconate unit, seen at *m/z* 1076.68 (black asterisk). The blue arrow indicates residual *bis*-phosphorylated wild-type lipid A.
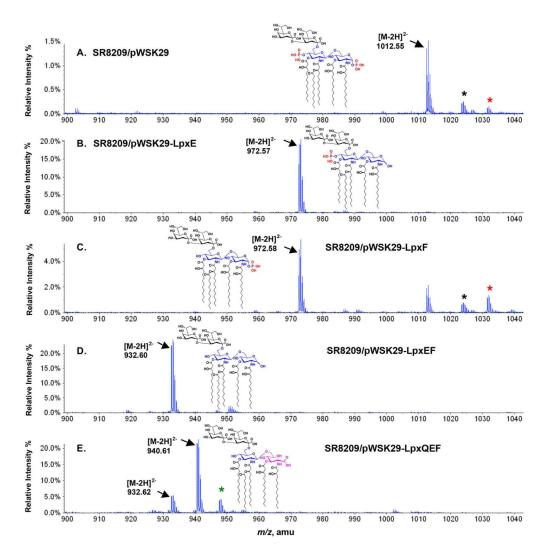
Figure 6. ESI-MS of novel Kdo$_2$-lipid A derivatives from SR8209 expressing FnLpxE, FnLpxF, and RlLpxQ

SR8209 cells, which synthesize penta-acylated Kdo$_2$-lipid A (Fig. 2), were grown at 37 °C with ampicillin and IPTG, and harvested in late log phase. Lipids were extracted without mild acid hydrolysis, as described in the methods section. **Panel A.** SR8209 cells harboring pWSK29 contain bis-phosphorylated, penta-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 1012.55) as their major LPS (inset structure). Sodium and potassium adducts are also present (black and red asterisks, respectively). **Panel B.** SR8209 cells harboring pWSK29-LpxE accumulate 1-dephosphorylated, penta-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 972.57), Product III in Fig. 2. **Panel C.** SR8209 cells harboring pWSK29-LpxF accumulate the isomeric 4′-dephosphorylated, penta-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 972.58), Product IV in Fig. 2. There is some residual wild-type lipid remaining in this construct. **Panel D.** SR8209 cells harboring pSK29-LpxEF accumulate fully dephosphorylated, penta-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 932.60), Product V in Fig. 2. **Panel E.** SR8209 cells harboring pWSK29-LpxQEF accumulate fully dephosphorylated, penta-acylated Kdo$_2$-lipid A ([M-2H]$^{2-}$ at *m/z* 932.62), and its oxidized derivative ([M-2H]$^{2-}$ at *m/z* 940.61), Product VI in Fig. 2. The putative methanolysis product of the lactone form of VI (structures not shown) may also be present (green asterisk).
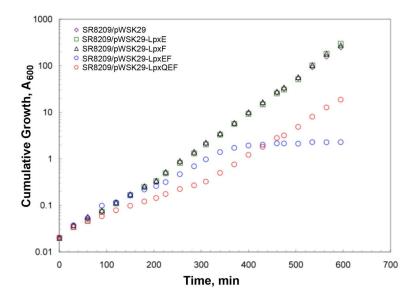
Figure 7. Growth phenotypes in shaking culture of SR8209 cells expressing FnLpxE, FnLpxF, and RlLpxQ

Cells were grown at 37 °C on LB broth supplemented with 100 μg/ml ampicillin and 1 mM IPTG. The cultures were started at $A_{600} = 0.02$. The cultures were diluted 10-fold whenever A_{600} reached 0.3–0.4. The cumulative A_{600} corrected for these dilutions is shown on the y-axis. Expression of LpxE or LpxF alone had no effect on the growth rate. Expression of LpxQ relieved the apparent toxicity of combined LpxE/LpxF expression in shaking culture.
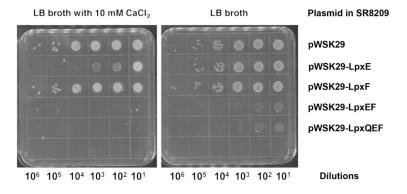
Figure 8. SR8209 cells expressing FnLpxE and FnLpxF are hypersensitive to CaCl$_2$
Serial dilutions ($\sim 10^7 - 10^2$ colony forming units) were prepared with SR8209 cells harboring either pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF. Ten μL of each dilution was plated onto LB agar supplemented with 100 μg/ml ampicillin, 1 mM IPTG, and 10 mM CaCl$_2$, or onto control plates containing only 100 μg/ml ampicillin and 1 mM IPTG.

Table 1

Bacterial strains and plasmids used in this work.

Strains	Genotype or Description	Source or reference
W3110A	Wild type *E. coli*, F⁻,λ₋, aroA::Tn10	(40)
WBB06	W3110 *mtl*, Δ(*waaC-waaF*)::*tet*6	(42)
MLK1067	W3110 *lpxM::Ω3cam*	(45)
SR8209	BW25113 *Φ* (p*cpxP-lacZ*) *waaCF*tet *lpxM* <> cam	(39)
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*	Stratagene
W3110A/pWSK29	W3110A transformed by pWSK29	this work
W3110A/pWSK29-LpxE	W3110A transformed by pWSK29-LpxE	this work
W3110A/pWSK29-LpxQE	W3110A transformed by pWSK29-LpxQE	this work
WBBO6/pWSK29	WBBO6 transformed by pWSK29	this work
WBBO6/pWSK29-LpxE	WBBO6 transformed by pWSK29-LpxE	this work
WBBO6/pWSK29-LpxQE	WBBO6 transformed by pWSK29-LpxQE	this work
MLK1067/pWSK29	MLK1067 transformed by pWSK29	this work
MLK1067/pWSK29-LpxE	MLK1067 transformed by pWSK29-LpxE	this work
MLK1067/pWSK29-LpxF	MLK1067 transformed by pWSK29-LpxF	this work
MLK1067/pWSK29-LpxQE	MLK1067 transformed by pWSK29-LpxQE	this work
MLK1067/pWSK29-LpxQEF	MLK1067 transformed by pWSK29-LpxQEF	this work
MLK1067pWSK29-LpxEF	MLK1067 transformed by pWSK29-LpxEF	this work
SR8209/pWSK29	SR8209 transformed by pWSK29	this work
SR8209/pWSK29-LpxE	SR8209 transformed by pWSK29-LpxE	this work
SR8209/pWSK29-LpxF	SR8209 transformed by pWSK29-LpxF	this work
SR8209/pWSK29-LpxQE	SR8209 transformed by pWSK29-LpxQE	this work
SR8209/pWSK29-LpxQEF	SR8209 transformed by pWSK29-QEF	this work
SR8209/pWSK29-LpxEF	SR8209 transformed by pWSK29-EF	this work
Plasmids		
pQN233	*lpxQ* in pET21a(+)	(25)
pWSK29	Low copy vector, Amp^r	(31)
pWSK29-LpxE	pWSK29 harboring *FnlpxE*	(8)
pWSK29-LpxF	pWSK29 harboring *FnlpxF*	(19)
pWSK29-LpxQE	pWSK29 harboring *RllpxQ and FnlpxE*	this work
pWSK29-LpxQEF	pWSK29 harboring *RllpxQ, FnlpxE, and Fn lpxF*	this work
pWSK29-LpxEF	pWSK29 harboring *FnlpxE* and *Fn lpxF*	this work

Abbreviations: tet-tetracycline, cam-chloramphenicol, amp-ampicillin.

Table II

Minimal effect of removing lipid A phosphate groups on sensitivity of *E. coli* to selected antibiotics.

Strain	Antibiotic	MIC*
MLK1067/pWSK29	Rifamycin SV	31.25
	Erythromycin	100
	Vancomycin	500
MLK1067/pWSK29-LpxEF	Rifamycin SV	15.6
	Erythromycin	25
	Vancomycin	500

MIC values were determined for the *lpxM* mutant MLK1067, which synthesizes penta-acylated lipid A, harboring the vector control pWSK29 or pWSK29-LpxEF, expressing both the lipid A 1- and 4′-phosphatases of *Francisella*.

Table III

Increased resistance to polymyxin of *E. coli* cells that synthesize lipid A lacking phosphate groups.

Strain	Vector	MIC
MLK1067	pWSK29	0.48
	pWSK29-LpxE	0.97
	pWSK29-LpxF	3.90
	pWSK29-LpxEF	3.90
	pWSK29-LpxQEF	3.90
SR8209	pWSK29	0.24
	pWSK29-LpxE	0.97
	pWSK29-LpxF	15.60
	pWSK29-LpxEF	15.60
	pWSK29-LpxQEF	15.60

Both MLK1067 and SR8209 harbor *lpxM* mutations and synthesize penta-acylated lipid A, which is the obligatory substrate for LpxF. MLK1067 generates a full core oligosaccharide, whereas SR8209 lacks the heptosyl transferases and therefore makes penta-acylated Kdo_2-lipid A (Fig. 2) as its LPS. Removal of the 4′-phosphate group accounts for most of the polymyxin resistance observed in this setting.