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Escherichia coli Mutants That Synthesize Dephosphorylated Lipid A Molecules†

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ABSTRACT: The lipid A moiety of *Escherichia coli* lipopolysaccharide is a hexaacylated 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. Coexpression of FnLpxE and the *Rhizobium leguminosarum* lipid A oxidase RlLpxQ in *E. coli* converts much of the proximal glucosamine to 2-amino-2-deoxygluconate. Expression of the *F. novicida* lipid A 4′-phosphatase FnLpxF in wild-type *E. coli* has no effect because FnLpxF cannot dephosphorylate hexaacylated lipid A. However, expression of FnLpxF in *E. coli lpxM* mutants, which synthesize pentaacylated lipid A lacking the secondary 3′-myristate chain, causes extensive 4′-dephosphorylation. Coexpression 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-deoxygluconate. 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 reengineering lipid A structure may be useful for generating novel vaccines and adjuvants.

The lipid A moiety of lipopolysaccharide (LPS)[1] 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 (Figure 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 (Figure 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 (Figure 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 proinflammatory 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*).

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 (Figure 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 (Figure 1C,D) (*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* (Figure 1C,D) (*21*−*23*) but not in *Francisella*

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[1]Abbreviations: 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.

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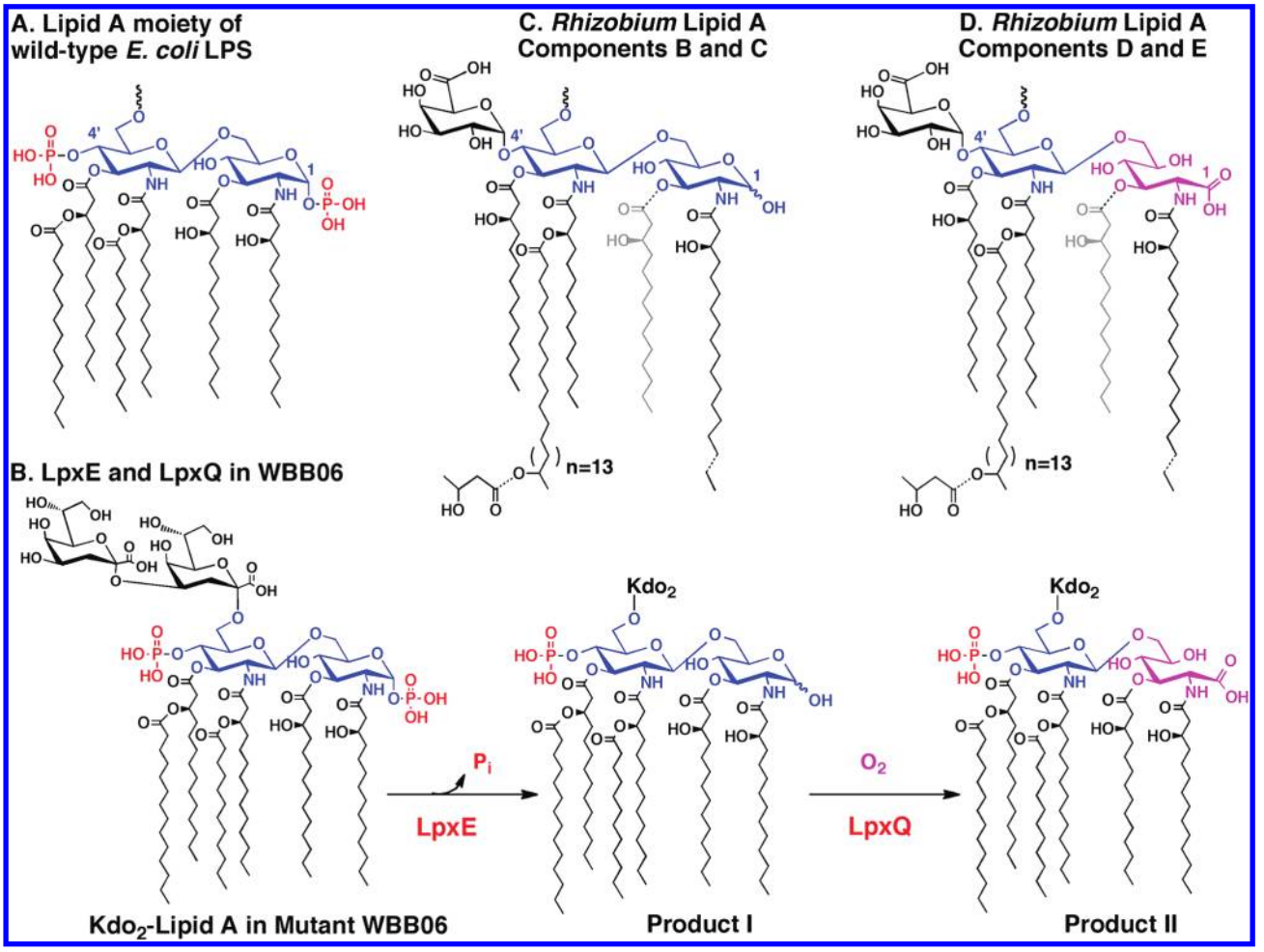

FIGURE 1: Structures of *E. coli* and *R. etli* lipid A and the origin of aminogluconate. Panel A: The most abundant lipid A moiety of LPS in wild-type *E. coli* consists of a hexaacylated disaccharide of glucosamine, substituted with monophosphate groups at the 1 and 4′ positions (*1*). The squiggles indicate the sites of Kdo attachment. Panel B: Proposed reactions catalyzed by LpxE and LpxQ in the heptose-deficient *E. coli* mutant WBB06, yielding hexaacylated 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 microheterogeneity of *R. etli* or *R. leguminosarum* lipid A with respect to its acyl chains.

(not shown). The proximal dephosphorylated glucosamine unit of *R. etli* and *R. leguminosarum* may be present as a hemiacetal (Figure 1C), or it may be further oxidized to 2-amino-2-deoxygluconate (aminogluconate) (Figure 1D) (*21*−*23*). Oxidation of the proximal glucosamine moiety is catalyzed by the O_2-dependent outer membrane oxidase LpxQ (Figure 1B) (*25*, *26*). Wild-type *Rhizobium* lipid A is mostly pentaacylated (Figure 1C,D) (*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* (Figures 1B and 2). We show that the presence of these modification enzymes results in dramatic alterations of *E. coli* lipid A structure (Figures 1B and 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 (Figure 2) greatly increases the resistance of *E. coli lpxM* mutants to polymyxin. Coexpression 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 (Figures 1B and 2).

MATERIALS AND METHODS

Materials. [γ-^{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 of reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or VWR International (West Chester, PA).

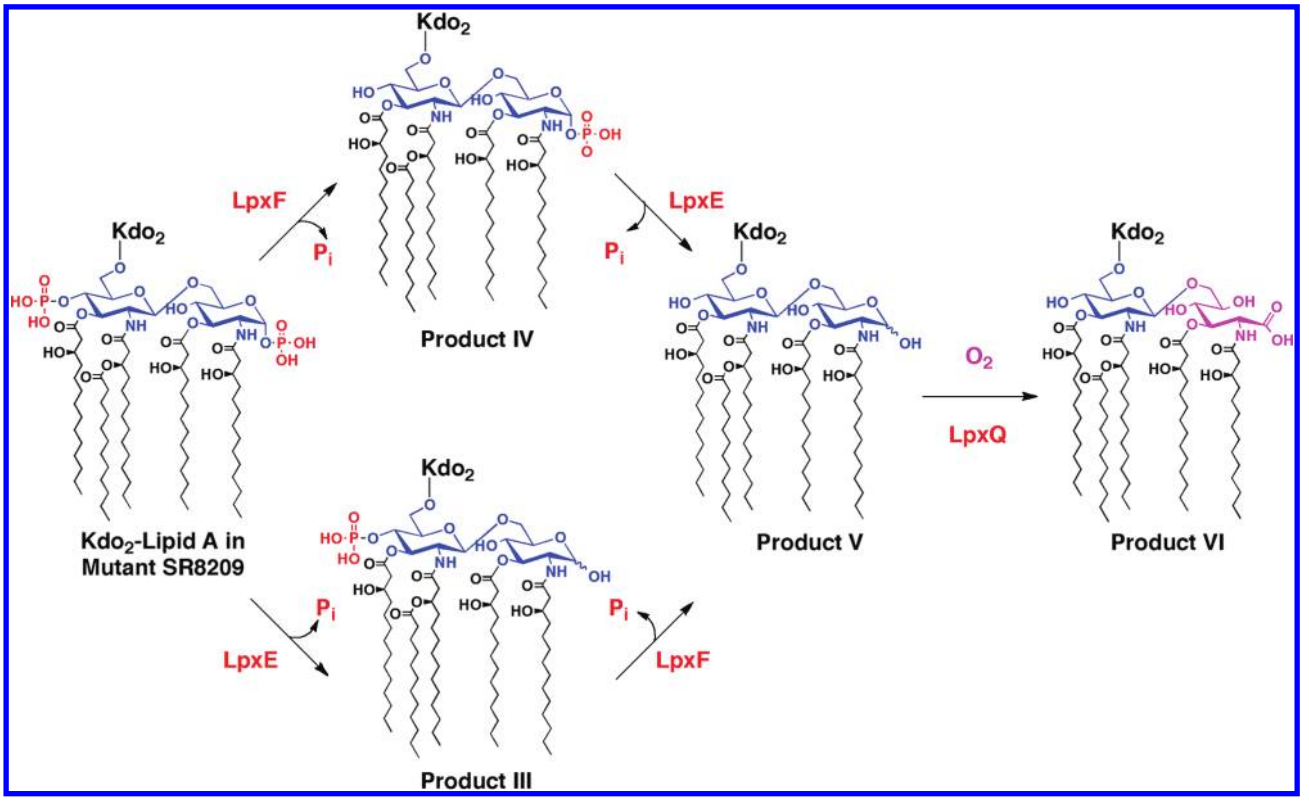

FIGURE 2: Novel Kdo_2-lipid A derivatives in SR8209 cells harboring FnLpxE, FnLpxF, and RlLpxQ. Expression of LpxE or LpxF alone in the heptose-deficient *lpxM* mutant SR8209 yields the isomeric monophosphoryl-Kdo_2-lipid A derivatives, products III and IV, respectively. Coexpression of the 1-phosphatase LpxE and the 4′-phosphatase LpxF in SR8209 yields fully dephosphorylated lipid A (product V). Coexpression of LpxE, LpxF, and LpxQ additionally generates the oxidized derivative, product VI.

Bacterial Strains and Growth Conditions. Table 1 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 Information Table 1. 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 *Xba*I and *Hin*dIII 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*,

Table 1: Bacterial Strains and Plasmids Used in This Work[a]

	genotype or description	source or ref
strains		
W3110A	wild-type *E. coli*, F$^-$,λ_-, aroA::Tn10	*40*
WBB06	W3110 *mtl*, Δ(*waaC-waaF*)::*tet*6	*42*
MLK1067	W3110 *lpxM::*Ω*cam*	*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

[a]Abbreviations: tet, tetracyline; cam, chloramphenicol; amp, ampicillin.

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 *Xba*I and *Kpn*I, 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 *Xba*I and *Kpn*I 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*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 18000 psi, and unbroken cells were removed by centrifugation at 10000*g* for 20 min. The membranes were collected by centrifugation at 100000*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 was described previously (*33*, *34*). The substrates were stored frozen in 25 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1 mM 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 (45000 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 1 mM 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*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*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 of 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*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 preequilibrated 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 redissolved in 300 μL of 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 of 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 of media supplemented with various concentrations of antibiotics. The plates were incubated at 37 °C for 20 h. 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 an additional 3 h 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.

Ca^{2+} 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 ($\sim 10^8$ CFU). Six 10-fold serial dilutions of each strain were made into LB broth. Ten microliters 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

Coexpression 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 (Figure 1B), we constructed an inducible plasmid that coexpresses both LpxE and LpxQ by cloning the *RllpxQ* gene and the *FnlpxE* gene in tandem behind

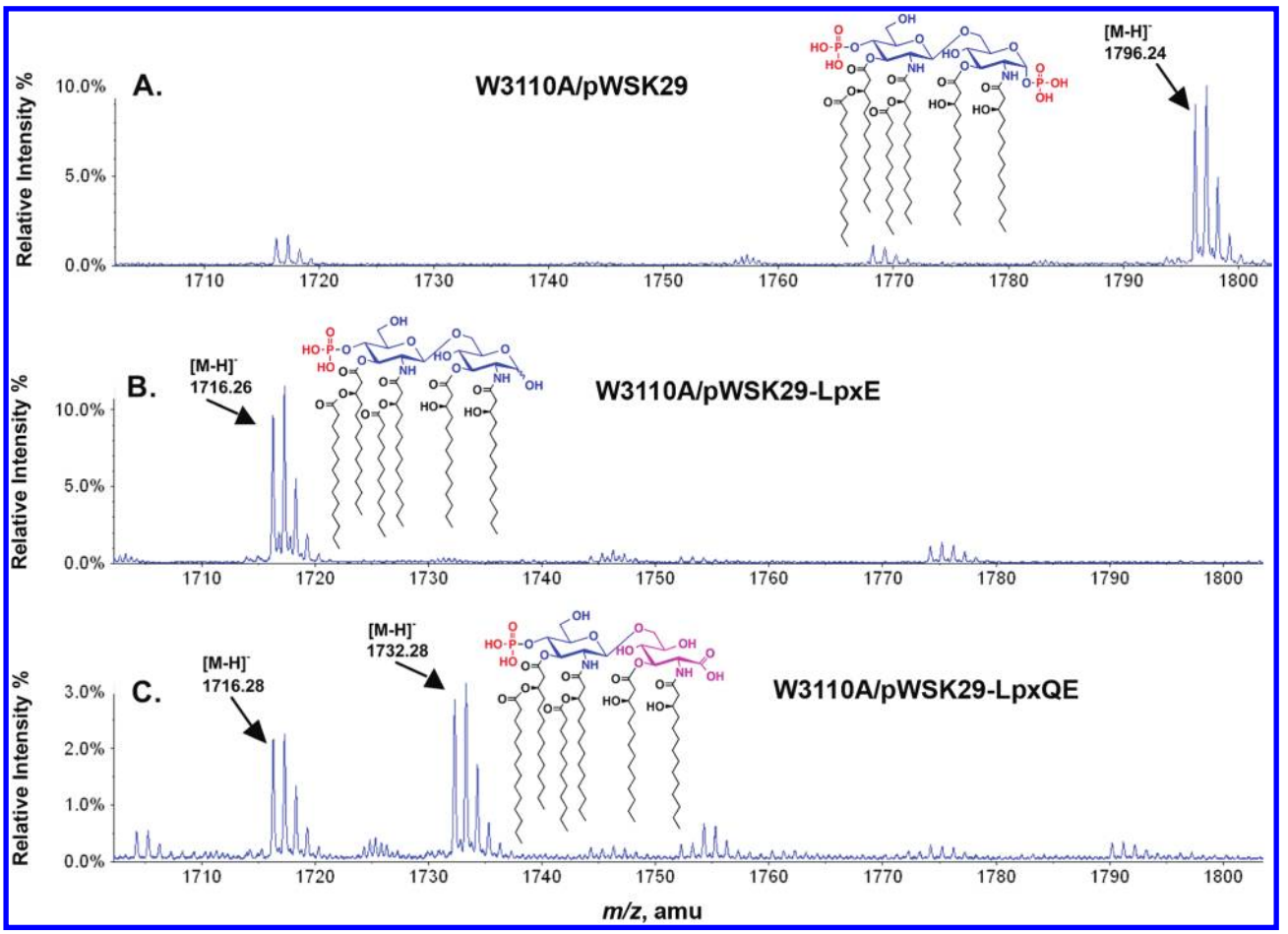

FIGURE 3: ESI-MS of lipid A species from W3110A expressing FnLpxE and RlLpxQ. W3110A cells were grown at 37 °C with ampicillin and IPTG and harvested in late log phase. Lipid A species were extracted following mild acid hydrolysis of the cell residue. Panel A: W3110A harboring pWSK29 contain bisphosphorylated 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.

the *lac* promoter of pWSK29 (Supporting Information 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 Figure 3. The predominant lipid A species in the vector control (Figure 3A) appears at m/z 1796.24 (Figure 1A), consistent with the $[M - H]^-$ ion of bisphosphorylated 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 (Figure 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 bisphosphorylated 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 (Figure 3C) (predicted $[M - H]^-$ ion at m/z 1732.24). Virtually no wild-type lipid A is detectable (Figure 3C). The formation of the aminogluconate residue was dependent upon the presence of molecular oxygen, but no ^{18}O from $^{18}O_2$ 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 (Figure 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 tetraacylated *E. coli* Kdo_2-[4′-^{32}P]lipid IV_A as the substrate (Figure 4). The assay was performed under conditions optimized for LpxQ. The product of LpxE, 1-dephosphorylated Kdo_2-[4′-^{32}P]lipid IV_A, was formed rapidly in reactions initiated with membranes from cells harboring either pWSK29-LpxE or pWSK29-LpxQE (Figure 4). However, membranes from cells containing pWSK29-LpxQE further metabolized the 1-dephosphorylated Kdo_2-[4′-^{32}P]lipid IV_A to a more slowly migrating product in a time-dependent fashion (Figure 4), consistent with LpxQ catalyzed oxidation of the proximal glucosamine unit (Figure 1B) (*25*, *26*). The small amounts of faster migrating products indicated by the asterisks (Figure 4) are

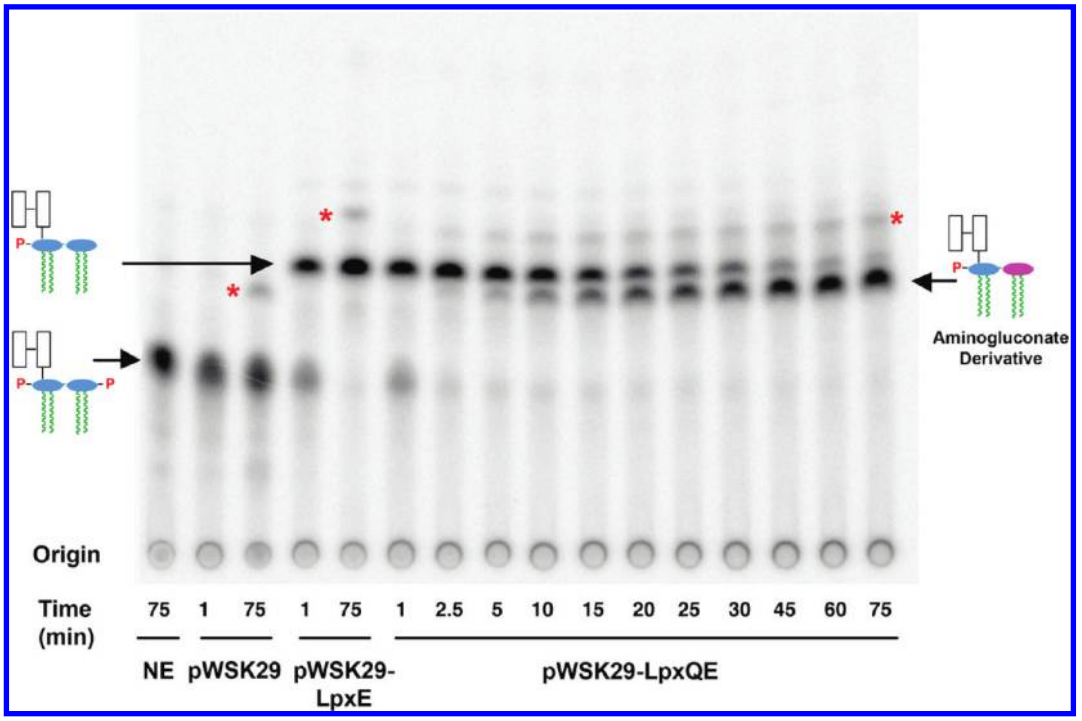

FIGURE 4: Lipid A phosphatase and oxidase activities in membranes of W3110A cells expressing FnLpxE and RlLpxQ. Membranes (1 mg/mL) were assayed for the indicated times at 30 °C with 10 μM [4′-^{32}P]-Kdo$_2$-lipid IV$_A$ as the substrate (schematic representation: blue ovals, glucosamine units; white 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. Key: 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.

probably generated by the acyltransferase PagP, which is present in membranes of W3110A (*41*).

ESI-MS of Hexaacylated 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 Information Figure 1) (*22*, *23*). This material, the $[M - 2H]^{2-}$ ion of which is observed at *m*/*z* 734.51 (Supporting Information Figure 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 (Figure 1B) under the same conditions used above for W3110A. WBB06 cells synthesize mainly hexaacylated Kdo$_2$-lipid A (Figures 1B and 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 (Figure 5A). Kdo$_2$-lipid A is observed primarily as the doubly charged ion $[M - 2H]^{2-}$ at *m*/*z* 1117.66 (Figure 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 (Figure 1B, product I), as reported previously (*8*) (data not shown). WBB06 cells harboring pWSK29-LpxQE (Figure 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 (Figure 1B, product I), and an oxidized product, the $[M - 2H]^{2-}$ ion of which is observed at *m*/*z* 1085.68 (Figure 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 Information Figure 1) is not observed with this extraction procedure. However, a small peak corresponding to a putative lactone form of the aminogluconate moiety (Figure 5B, black asterisk) is seen at *m*/*z* 1076.68, and its possible methanolysis product (Figure 5B, red asterisk) is observed at *m*/*z* 1092.68 (*44*).

ESI-MS of Pentaacylated 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 pentaacylated lipid A (Figure 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 pentaacylated Kdo$_2$-lipid A as its major LPS (Figures 2 and 6A, inset) (*39*). This material can be extracted without acid hydrolysis and analyzed directly by ESI-MS in the negative ion mode. Like the hexaacylated lipid A species (Figure 5), the pentaacylated Kdo$_2$-lipid A derivatives are detected mainly as doubly charged species (Figure 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 (Figure 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 (Figure 6A, asterisks).

FnLpxF can dephosphorylate pentaacylated but not hexaacylated lipid A (*19*), whereas LpxE can dephosphorylate both. Expression of either pWSK29-LpxE or pWSK29-LpxF in

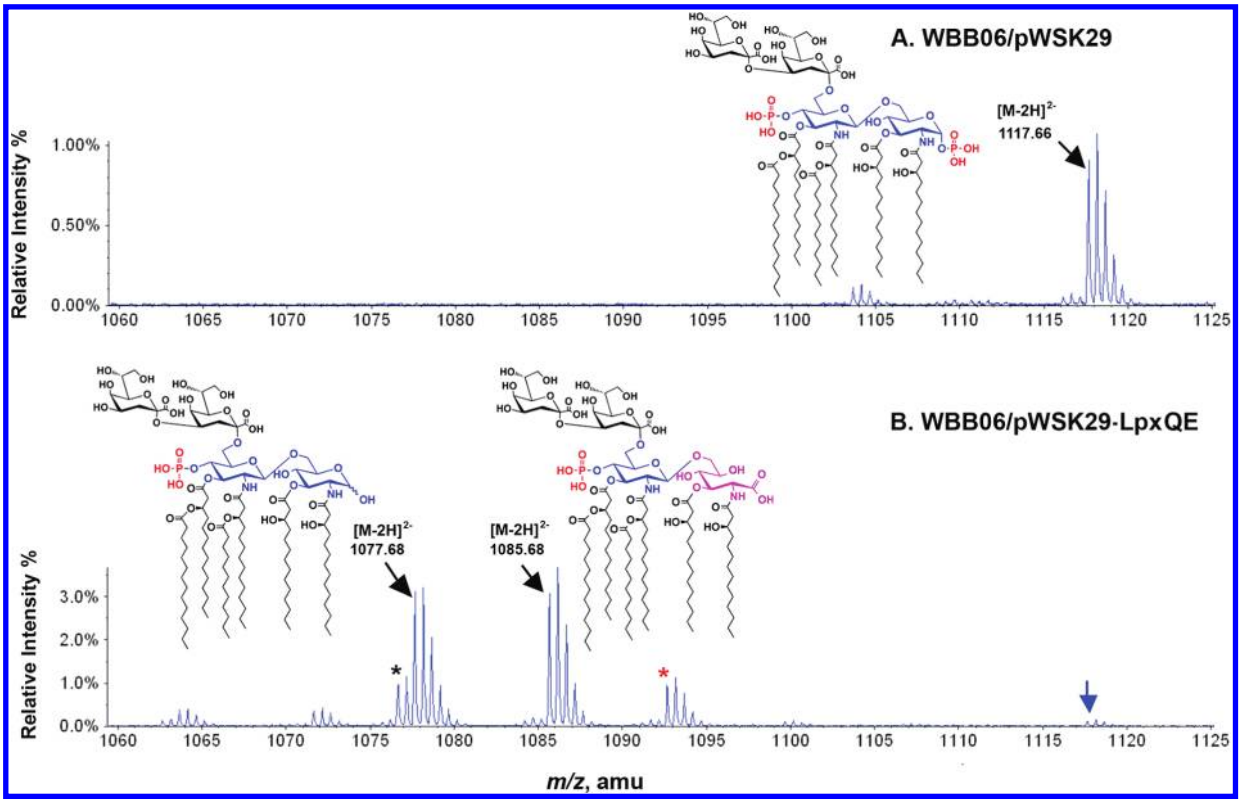

FIGURE 5: ESI-MS of Kdo_2-lipid A derivatives in *E. coli* WBB06 expressing FnLpxE and RlLpxQ. The heptosyl transferase-deficient mutant WBB06, which synthesizes hexaacylated Kdo_2-lipid A as its sole LPS (*43*), was grown at 37 °C with ampicillin and IPTG and harvested in late log phase. Lipids were extracted without mild acid hydrolysis, as described in the Materials and Methods section. Panel A: WBB06 cells, harboring the vector pWSK29, synthesize mainly hexaacylated 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 Figure 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 bisphosphorylated wild-type lipid A.

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 (panels B and C of Figure 6, respectively). This observation is consistent with formation of the isomeric, monophosphorylated Kdo_2-lipid A derivatives, products III or IV in Figure 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 (Figure 6D), consistent with the predicted $[M - 2H]^{2-}$ ion at m/z 932.59 of product V (Figure 2). No residual peaks corresponding to either monophosphorylated or bisphosphorylated lipid A are present, demonstrating the efficient combined action of FnLpxE and FnLpxF in this strain. SR8209 cells expressing pWSK29-LpxQEF (Figure 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 Figure 2. The small peak indicated by the green asterisk in Figure 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 Figure 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 2). These results suggest that the phosphate groups of lipid A are not very important for the normal function of the outer membrane permeability barrier toward 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 (Figure 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 (Figure 8). However, SR8209 cells expressing pWSK29-LpxEF and pWSK29-LpxQEF displayed sensitivity to 10 mM calcium

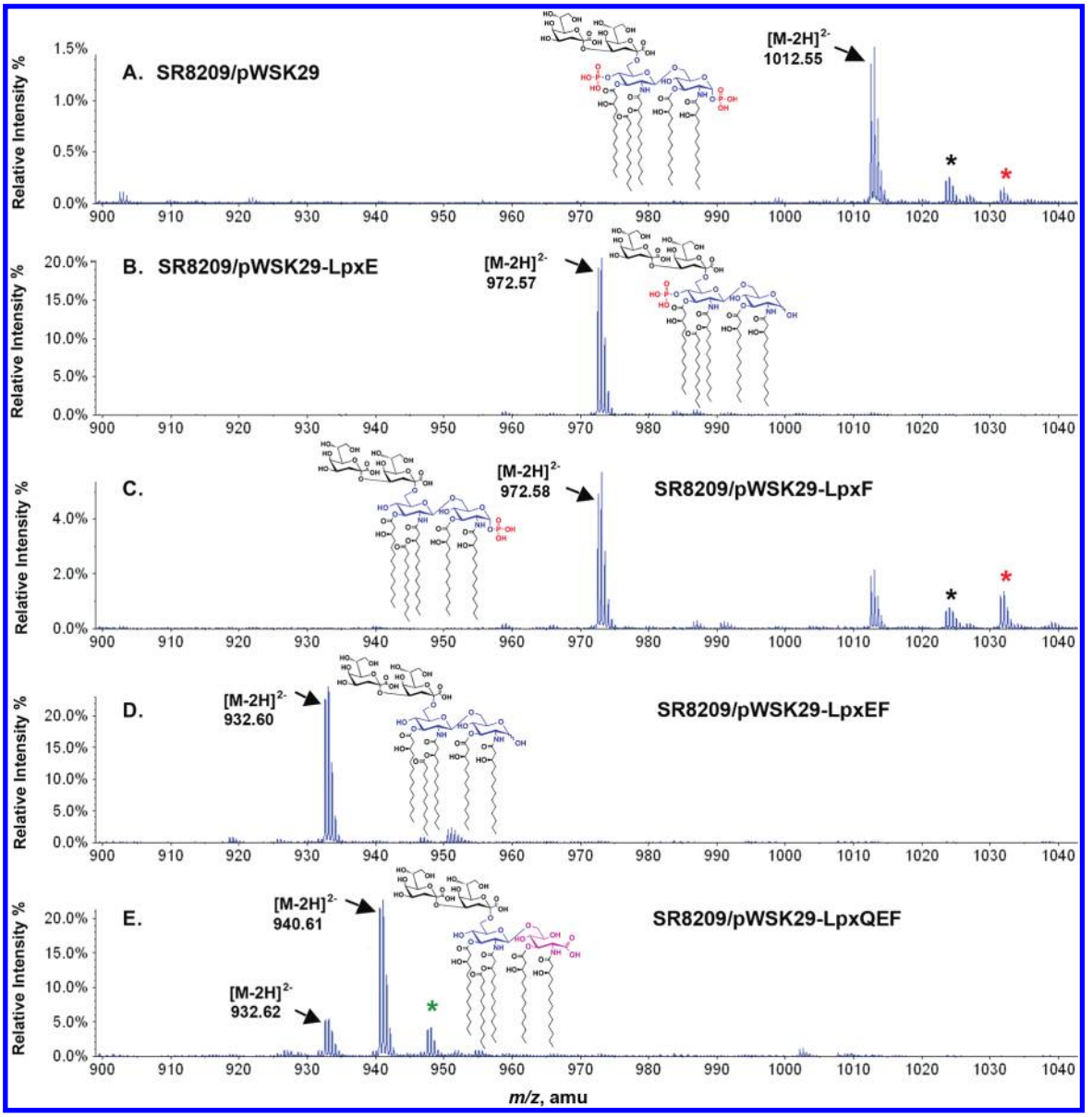

FIGURE 6: ESI-MS of novel Kdo_2-lipid A derivatives from SR8209 expressing FnLpxE, FnLpxF, and RlLpxQ. SR8209 cells, which synthesize pentaacylated Kdo_2-lipid A (Figure 2), were grown at 37 °C with ampicillin and IPTG and harvested in late log phase. Lipids were extracted without mild acid hydrolysis, as described in the Materials and Methods section. Panel A: SR8209 cells harboring pWSK29 contain bisphosphorylated, pentaacylated 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, pentaacylated Kdo_2-lipid A ($[M - 2H]^{2-}$ at m/z 972.57), product III in Figure 2. Panel C: SR8209 cells harboring pWSK29-LpxF accumulate the isomeric 4′-dephosphorylated, pentaacylated Kdo_2-lipid A ($[M - 2H]^{2-}$ at m/z 972.58), product IV in Figure 2. There is some residual wild-type lipid remaining in this construct. Panel D: SR8209 cells harboring pSK29-LpxEF accumulate fully dephosphorylated, pentaacylated Kdo_2-lipid A ($[M - 2H]^{2-}$ at m/z 932.60), product V in Figure 2. Panel E: SR8209 cells harboring pWSK29-LpxQEF accumulate fully dephosphorylated, pentaacylated 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 Figure 2. The putative methanolysis product of the lactone form of VI (structures not shown) may also be present (green asterisk).

chloride on LB agar plates (Figure 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 (Figure 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 3). Removal of the 4′-phosphate group by LpxF alone caused the most dramatic reduction in polymyxin sensitivity (Table 3). The MICs of strains expressing LpxF are comparable to those observed in *pmrA* constitutive mutants of *E. coli* or *Salmonella 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 hexaacylated lipid A species but only penta- or tetraacylated variants (*19*).

Table 2: Minimal Effect of Removing Lipid A Phosphate Groups on Sensitivity of *E. coli* to Selected Antibiotics

strain	antibiotic	MIC[a]
MLK1067/pWSK29	rifamycin SV	31.25
	erythromycin	100
	vancomycin	500
MLK1067/pWSK29-LpxEF	rifamycin SV	15.6
	erythromycin	25
	vancomycin	500

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

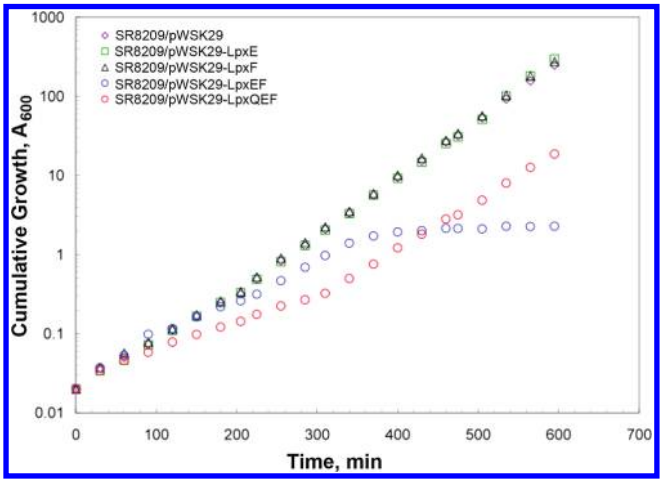

FIGURE 7: Growth phenotypes in shaking culture of SR8209 cells expressing FnLpxE, FnLpxF, and RlLpxQ. Cells were grown at 37 °C on LB broth supplemented with 100 μg/mL ampicillin and 1 mM IPTG. The cultures were started at A_{600} = 0.02. The cultures were diluted 10-fold whenever A_{600} reached 0.3−0.4. 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.

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 overexpression of FnLpxE, FnLpxF, and the *R. leguminosarum* lipid A oxidase RlLpxQ to generate entirely novel lipid A hybrid structures in *E. coli*. When coexpressed 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 (Figures 3 and 5). This result demonstrates that lipid A modification enzymes from diverse sources can be combined to facilitate efficient reengineering 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* (Figures 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*

Table 3: Increased Resistance to Polymyxin of *E. coli* Cells That Synthesize Lipid A Lacking Phosphate Groups[a]

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

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

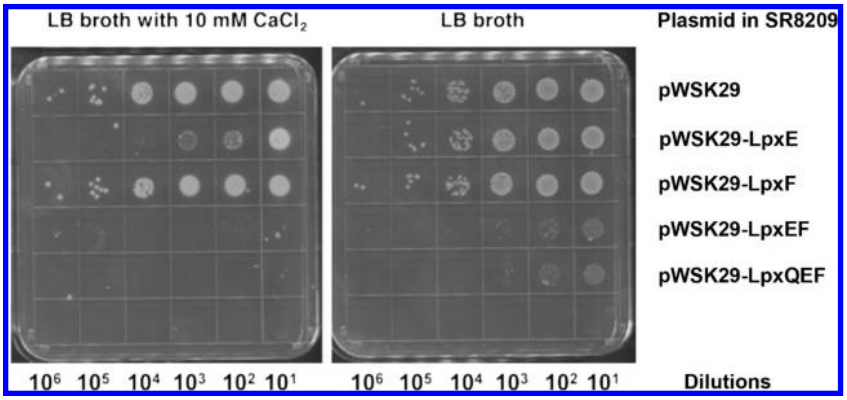

FIGURE 8: SR8209 cells expressing FnLpxE and FnLpxF are hypersensitive to $CaCl_2$. Serial dilutions (~10^7−10^2 colony forming units) were prepared with SR8209 cells harboring either pWSK29, pWSK29-LpxE, pWSK29-LpxF, pWSK29-LpxEF, or pWSK29-LpxQEF. Ten microliters of each dilution was plated onto LB 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.

Sin-1 (*52*) are endotoxin antagonists (*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 were further demonstrated by coupling the expression of FnLpxF with FnLpxE and RlLpxQ in *E. coli lpxM* mutants (Figure 6). Expression of both phosphatases resulted in nearly quantitative dephosphorylation of lipid A (Figure 6). Expression of RlLpxQ together with the two phosphatases led to the further generation of oxidized lipid A species (Figure 6) containing aminogluconate. Although the heptose-deficient mutant SR8209 grew poorly in liquid medium when both phosphate groups were missing (Figure 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 2). 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 bisphosphorylated 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 (Figure 6). These constructs not only displayed impaired growth in liquid medium (Figure 7) but also failed to grow on plates in the presence of 10 mM $CaCl_2$ (Figure 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 3). 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 in SR8209 (Table 3). 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.

ACKNOWLEDGMENT

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SUPPORTING INFORMATION AVAILABLE

The primers used to construct pWSK-LpxQE (Table 1), the PCR protocol used to construct this plasmid (Scheme 1), and the proposed structure and mass spectrum of the lipid A decomposition product formed from the aminogluconate unit of LpxE/LpxQ-modified *E. coli* lipid A during acid hydrolysis of LPS (Figure 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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