MsBA Transporter-dependent Lipid A 1-Dephosphorylation on the Periplasmic Surface of the Inner Membrane

TOPOGRAPHY OF FRANCISELLA NOVICIDA LpxE EXPRESSED IN ESCHERICHIA COLI

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The lipid A anchor of Francisella tularensis lipopolysaccharide (LPS) lacks both phosphate groups present in Escherichia coli lipid A. Membranes of Francisella novicida (an environmental strain related to F. tularensis) contain enzymes that dephosphorylate lipid A and its precursors at the 1- and 4′-positions. We now report the cloning and characterization of a membrane-bound phosphatase of F. novicida that selectively dephosphorylates lipid A. By transferring an F. novicida genomic DNA library into E. coli and selecting for low level polymyxin resistance, we isolated FnlpxE as the structural gene for the 1-phosphatase, an inner membrane enzyme of 239 amino acid residues. Expression of FnlpxE in a heptose-deficient mutant of E. coli caused massive accumulation of a previously uncharacterized LPS molecule, identified by mass spectrometry as 1-dephospho-Kdo₂-lipid A. The predicted periplasmic orientation of the FnlpxE active site suggested that LPS export might be required for 1-dephosphorylation of lipid A. LPS and phospholipid export depend on the activity of MsbA, an essential inner membrane ABC transporter. Expression of FnlpxE in the msbA temperature-sensitive E. coli mutant WD2 resulted in 90% 1-dephosphorylation of lipid A at the permissive temperature (30 °C). However, the 1-phosphate group of newly synthesized lipid A was not cleaved at the nonpermissive temperature (44 °C). Our findings provide the first direct evidence that lipid A 1-dephosphorylation catalyzed by LpxE occurs on the periplasmic surface of the inner membrane.

Francisella tularensis is an intracellular Gram-negative bacterium that causes tularemia, a severe and often fatal infection of humans and animals (1). The structure of the lipid A anchor of lipopolysaccharide (LPS) in F. tularensis (Fig. 1B) is strikingly different from that of Escherichia coli (Fig. 1A). E. coli lipid A is a hexa-acetylated disaccharide of glucosamine, which is phosphorylated at the 1- and 4′-positions (2, 3). It is synthesized by nine constitutive enzymes in the cytoplasm or on the outer surface of the inner membrane (3). Despite having the structural genes for the nine enzymes of the E. coli lipid pathway, F. tularensis synthesizes lipid A lacking phosphate groups (4). Similar phosphate-deficient lipid A structures have been reported in the plant endosymbionts Rhizobium etli and leguminosarum (5–7). In the latter case, specific phosphatases remove the 1- and 4′-phosphate residues late in the pathway (8–10). The biological significance of phosphate-deficient lipid A molecules is uncertain. Mutants lacking the phosphatases have not been reported.

E. coli lipid A potently activates the TLR-4 receptor of the mammalian innate immune system (11, 12). The phosphate groups are crucial for this bioactivity (13). Lipid A with two phosphate moieties induces a cytokine profile that is lethal to animals (13). Lipid A with one phosphate group is much less toxic, whereas lipid A lacking both phosphates is inactive (13–15). Vaccine development has recently focused on monophosphoryl lipid A analogues, since these induce an altered cytokine profile that retains adjuvant activity without toxicity (15–17). Lipid A without phosphate groups might provide selective advantages for F. tularensis during infections, since a strong innate immune response via TLR-4 would not be induced.

We now describe an inner membrane phosphatase from Francisella novicida, a nonvirulent strain isolated from ground water (18), that selectively removes the 1-phosphate group of lipid A. The Francisella enzyme is distantly related to the R. leguminosarum lipid A 1-phosphatase LpxE (10), but its specific activity in membranes is several orders of magnitude higher. The FnlpxE gene was cloned based on its ability to confer low level polymyxin resistance in E. coli. Approximately 90% of the E. coli lipid A molecules are dephosphorylated at the 1-position in constructs expressing FnlpxE, converting most of the E. coli lipid A from the endotoxin to the adjuvant structure (15). In contrast to lipid A biosynthesis, 1-dephosphorylation of lipid A in living cells requires MsbA, an essential inner membrane ABC transporter and flippase needed for export of LPS and phospholipids to the outer membrane (19). In the temperature-sensitive msbA mutant WD2 (19), FnlpxE is unable to dephosphorylate newly synthesized lipid A in vivo at 44 °C (3, 19). Our results demonstrate for the first time that 1-dephos-
phorylation of lipid A is an extracytoplasmic event, occurring on the periplasmic surface of the inner membrane.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glass-beaded 0.25-mm Silica Gel 60 TLC plates were from Merck. Chlorform, ammonium acetate, and sodium acetate were obtained from EM Science, whereas pyridine, methanol, and formic acid were from Mallinckrodt. Trypsitace soy broth, yeast extract, and tryptic enzyme were purchased from Difco. PCR reagents were purchased from Stratagene, and restriction enzymes were from New England Biolabs. Easy DNA kits were from Invitrogen. Spin Miniprep kits and gel excitation kits were from Qiagen. Strain or plasmid

| Strains or plasmid | Genotype or description | Source or reference |
|--------------------|------------------------|---------------------|
| Strains            |                        |                     |
| U112               | Wild-type *F. novicida* | University of Victoria, Canada |
| W3110A             | Wild type *E. coli*, F−, λ−, ara−:Tn10 | Ref. 53 |
| XLI-Blue           | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac | Stratagene |
| XW-1               | XLI-Blue transformed by pACYC184-FnLpxE | This work |
| W2D                | W3110, ara−:Tn10 mshA(A270T) | Ref. 19 |
| WB606              | E. coli mutant with a deletion of *waaC* and *waaF* genes | Ref. 35 |
| Novablaue (DE3)    | E. coli host strain used for expression | Novagen |
| Plasmids           |                        |                     |
| pACYC184           | Low copy vector, Tetr' Cam' | New England Biolabs |
| pXYW-1             | pACYC184 carrying a 2.5-kb genomic DNA containing *FnLpxE* | This work |
| pET28b             | Expression vector, T7lac promoter, Kan' | Novagen |
| pET28b-FnLpxE      | pET28b harboring *FnLpxE* | This work |
| pWSK29             | Low copy vector, Amp' | Ref. 39 |
| pWSK29-FnLpxE      | pWSK29 harboring *FnLpxE* | This work |

**Bacterial Growth Conditions and Membrane Preparation**—*F. novicida* U112 (18) was grown at 37 °C in TSB-C (3% trypticase soy broth, 10 mM K2HPO4, 2 mM KH2PO4, 0.1% yeast extract, 0.1 mM MgSO4, 0.5% NaCl) with 1% AroA (18) was grown at 37 °C in TSB-H11003 selective medium. All of the pools were active, but the level was variable. The glycerol stock from the pool with the highest activity was used to repurify eight polymyxin-resistant colonies by streaking on LB plates, supplemented with 30 μg/ml chloramphenicol and 1 μg/ml polymyxin. This amount of polymyxin causes several logs of killing of wild-type *E. coli*, although much less than 10 μg/ml polymyxin was used to select classical polymyxin-resistant mutants (2, 3). The plates were incubated at 37 °C overnight. The colonies from 15 plates were transferred into 15 separate tubes containing 5 ml of LB broth, 1 ml portions of which were stored as glycerol stocks. Membranes were then prepared from the remaining 4 ml and assayed for 1-phosphatase activity. All of the pools were active, but the level was variable. The glycerol stock from the pool with the highest activity was used to repurify eight polymyxin-resistant colonies by streaking on LB plates, supplemented with 30 μg/ml chloramphenicol and 1 μg/ml polymyxin. Cultures of the repurified colonies were then grown to late log phase and centrifuged. Only two of eight colonies grew under selective conditions in liquid medium. The cell pellets of these two strains were resuspended in 5 ml of 50 mM HEPES, pH 7.5, and disrupted with a French pressure cell. Unbroken cells were removed by centrifugation at 13,000 × g for 20 min. The membranes were collected by centrifugation at 100,000 × g for 1 h, washed once by suspension in 10 ml of 50 mM HEPES, pH 7.5, and then resuspended in the same buffer at a protein concentration of about 10 mg/ml. The protein concentrations were determined by the bicinchoninic acid assay with bovine serum albumin as the standard (20).

**Assay for the 1-Phosphatase of *F. novicida***—The 1-phosphatase was assayed in a 10–50-μl reaction mixture at 30 °C, containing 50 mM potassium phosphate, pH 6, 0.1% Triton X-100, 0.1 mg/ml *E. coli* phospholipids (Avanti), and 10 μM Kdo2-[4-32]P lipid A (3000–6000 cpm/nmol). The reaction was terminated by spotting 5-μl samples onto a silica TLC plate, which was dried under a cold air stream for 30 min and then developed in the solvent chloroform/methanol/acetate acid/water (25:15:4:4, v/v/v/v). After drying and overnight exposure of the plate to a PhosphorImager screen, product formation was detected and quantified with an Amersham Biosciences Storm PhosphorImager equipped with ImageQuant software. 2–6 kb DNA fragments were then ligated into pACYC184 at 16 °C overnight in a 25-μl reaction mixture containing 200 ng of genomic DNA fragments, 200 ng of pACYC184 vector, and 2 units of T4 DNA ligase. *E. coli* XLI-Blue cells were transformed by electroporation with the ligation mixture, and chloramphenicol-resistant colonies were selected on LB plates. About 15,000 colonies were pooled using a cell scraper and transferred into 250 ml of fresh LB broth, containing 30 μg/ml of chloramphenicol. Cells were grown to saturation, and 10 2-ml 18% glycerol stocks were made and stored at −80 °C.

To select for the 1-phosphatase gene, a glycerol stock of the library was diluted to obtain about 500 colonies per LB plate, containing 30 μg/ml chloramphenicol and 1 μg/ml polymyxin. This amount of polymyxin causes several logs of killing of wild-type *E. coli*, although much less than 10 μg/ml polymyxin was used to select classical polymyxin-resistant mutants (2, 3). The plates were incubated at 37 °C overnight. The colonies from 15 plates were transferred into 15 separate tubes containing 5 ml of LB broth, 1 ml portions of which were stored as glycerol stocks. Membranes were then prepared from the remaining 4 ml and assayed for 1-phosphatase activity. All of the pools were active, but the level was variable. The glycerol stock from the pool with the highest activity was used to repurify eight polymyxin-resistant colonies by streaking on LB plates, supplemented with 30 μg/ml chloramphenicol and 1 μg/ml polymyxin. Cultures of the repurified colonies were then grown to late log phase and centrifuged. Only two of eight colonies grew under selective conditions in liquid medium. The cell pellets of these two strains were resuspended in 5 ml of 50 mM HEPES, pH 7.5, and disrupted with a French pressure cell. Unbroken cells were removed by centrifugation at 13,000 × g for 20 min. The membrane-free extracts of these 1-phosphatase-deficient colonies were used in this manner were positive for 1-phosphatase activity. A representative strain expressing the 1-phosphatase activity was designated XW-1.

The hybrid plasmid pXYW-1 was isolated from XW-1 using the QiaGen Spin Miniprep kit. The DNA insert was sequenced by using the Terminator Cycle system and the ABI Prism 377 instrument at the Duke University DNA Analysis Facility. According to the program ORF Finder (29), the 2.5-kb insert contains three open reading frames. BLASTp search analysis (24) showed that only one of these three open reading frames is a member of the lipid phosphatase family (25). We assumed that this 717-bp DNA fragment would be the *FnLpxE* gene, encoding the 1-phosphatase in *F. novicida*.

*FnLpxE* was amplified by PCR and cloned into the pET28b vector behind the T7lac promoter. The forward PCR primer (5′-CGCATGCA-TGGTCAAGACAGATTACACAAACACTTT-3′) was designed with a clamp region, a NcoI restriction site (underlined), and a match to the coding strand starting at the translation initiation site. The reverse primer (5′-CGCATGCTCCGACCTAAATACACCATCAGAACACAGGTTTTT-3′) was designed with a clamp region, an XhoI restriction site (underlined), and a match to the anti-coding strand that included the stop codon. The PCR was performed using *Pfu* polymerase and plasmid pXYW-1 as the template. Amplification was carried out in a 100-μl reaction mixture containing 100 ng of template, 250 ng of primers, and 2 units of *Pfu* polymerase. The reaction was started at 94 °C for 1 min, followed by 25 cycles of denaturation (30 s at 94 °C), annealing (30 s at 55 °C), and extension (45 s at 72 °C). After the 25th cycle, a 10-min extension time was used. The reaction product was analyzed on a 1% agarose gel. The desired band was excised and gel-purified. The PCR product was then digested using NcoI and XhoI and ligated into the MshA Transporter-dependent Lipid A 1-Dephosphorylation

**TABLE I**

**Bacterial strains and plasmids used in this study**
expression vector pET28b that had been similarly digested and treated with shrimp alkaline phosphatase. The ligation mixture was transformed into XL1-Blue cells and screened for positive inserts on LB plates containing kanamycin (20 μg/ml). Plasmid pWSK29-FnLpxE was then purified from E. coli strain Novablue expressing the FnLpxE domain, the lipid A is covalently attached to the LPS in the insoluble membranes when hexa-acylated Kdo2-[4-32P]lipid A (Fig. 1) is employed (Fig. 3A, 3B). Neither the 1- nor 4'-phosphatase activities that might account for the 4'-phosphatase activity in E. coli harboring the vector control (Fig. 3A, 3B) are present in F. novicida. We therefore searched for lipid A 1- and 4'-phosphatase activities in F. novicida membranes. Although the lipid A of F. tularensis lacks phosphate groups, and its LPS contains only one Kdo residue (Fig. 1B) (4), orthologs of the nine E. coli enzymes that synthesize E. coli Kdo-2-lipid A (Fig. 1A) (3) are present in F. tularensis (see, on the World Wide Web, artedi.ebc.uu.se/Projects/Francisella/). We then purified Kdo2-[4-32P]lipid IVα (Fig. 1D) was initially employed as the probe substrate. As shown in Fig. 2, lanes 2–5, F. novicida membranes catalyzed the rapid release of the 4'-phosphate group from Kdo2-[4-32P]lipid IVα, as judged by the formation of [32P]P. In addition, a more rapidly migrating lipid was formed (Fig. 2, lanes 2–5). This material was tentatively identified as 1-dephospho-Kdo2-[4-32P]lipid IVα, given its migration with a standard generated by membranes of E. coli Novablue expressing the R. leguminosarum 1-phosphatase, LpxE (Fig. 2, lanes 6 and 7). Neither the 1- nor the 4'-phosphatase activities were present in membranes of E. coli harboring the vector control (Fig. 3A, lane 6). An Alternative Assay for the 1-Phosphatase and Evidence for a Kdo Trimming Activity—Robust 1-phosphatase activity was detected after 30 min at 30 °C with only 0.2 mg/ml F. novicida membranes when hexa-acylated Kdo2-[4-32P]lipid A (Fig. 1C) was used as the substrate (Fig. 3A, lane 2 versus lanes 4 and 6). An additional product, migrating more rapidly than 1-dephospho-Kdo2-[4-32P]lipid A (Fig. 3A, lane 2), was also observed. This material was tentatively identified as 1-dephospho-Kdo2-[4-32P]lipid A by mass spectrometry and enzymatic treatments, as described in the legend to Fig. 3. Its appearance was dependent upon the formation of 1-dephospho-Kdo2-[4-32P]lipid A (Fig. 3B), suggesting that the Kdo-trimming enzyme of F. novicida prefers a substrate lacking the 1-phosphatase group. The 4'-phosphatase is greatly suppressed when hexa-acylated Kdo2-[4-32P]lipid A (Fig. 1C) is employed (Fig. 3A, lane 2, versus Fig. 2, lanes 2–5). The 4'-phosphatase appears to be
selective for lipid A molecules containing four or five fatty acyl chains. Accordingly, only hexa-acylated Kdo₂-[4'-32P]lipid A was used in subsequent assays of the 1-phosphatase, since interference by the 4'-phosphatase was eliminated.

Selection for the F. novicida 1-Phosphatase Gene Based on Low Level Polymyxin Resistance—A genomic DNA library of F. novicida U112 was constructed in E. coli XL1-Blue. The library was grown on LB plates containing 30 µg/ml chloramphenicol and 1 µg/ml polymyxin. Wild-type E. coli growth is inhibited by 1 µg/ml polymyxin, causing several logs of cell killing. Removal of the 1-phosphate group reduces the net negative charge of lipid A (10), decreasing its affinity for polymyxin. Therefore, a clone expressing the F. novicida 1-phosphatase might be somewhat resistant to polymyxin. Membranes of strain XW-1, a transformant of E. coli XL1-Blue harboring plasmid pXYW-1, which was selected as polymyxin-resistant at 1 µg/ml, showed strong lipid A 1-phosphatase activity (Fig. 3A, lane 3). The 1-phosphatase was absent in the vector control (Fig. 3A, lane 4), as in all strains of wild-type E. coli.

The 2.5-kb F. novicida DNA insert in plasmid pXYW-1 was sequenced and found to contain a 717-bp open reading frame homologous to members of the lipid phosphate phosphatase superfamily (25). This gene was amplified by PCR, ligated into the expression vector pET28b, and transformed into E. coli Novablue cells. Membranes from Novablue/pET28b-FnLpxE were prepared and assayed with Kdo₂-[4'-32P]lipid A for 1-phosphatase activity. Massive expression of 1-phosphatase activity was observed in membranes of Novablue/pET28b-FnLpxE (Fig. 3A, lane 5) but not in the vector control Novablue/pET28b (Fig. 3A, lane 6). The Kdo trimming activity present in F. novicida membranes (Fig. 3A, lane 2) was not present with the recombinant lipid A 1-phosphatase expressed in E. coli (Fig. 3A, lanes 3 and 5), indicating that it is probably catalyzed by a different enzyme.

The gene encoding the F. novicida 1-phosphatase was designated FnLpxE. The FnLpxE protein (Fig. 4) consists of 239 amino acid residues and is related to LpxE of R. leguminosarum with an E value of ~6 × 10⁻³⁰ in a two-sequence comparison without low complexity filtering (10). Both LpxEs possess six putative transmembrane helices and the conserved lipid phosphate motifs KXXRP--------PSGH--------SRX₅HX₅X₅D (25), which are predicted to face the outer surface of the inner membrane in both proteins (38). The FnLpxE 1-phosphatase sequence has been deposited under GenBank™ accession number AY713119.

Optimizing the Expression of FnLpxE in E. coli—The formation of 1-dephosphorylated lipid A was linear with respect to time and protein concentration in all membrane preparations. However, the specific activity of the 1-phosphatase under optimized conditions was orders of magnitude higher in F. novicida membranes (27.4 nmol/min/mg) than in R. leguminosarum membranes (0.014 nmol/min/mg) (10). Recombinant Francisella LpxE was likewise more active than recombinant Rhi zobium LpxE. When FnLpxE was expressed behind the T7 promoter on pET28b-FnLpxE in E. coli Novablue (Fig. 3A, lane 5), the 1-phosphatase specific activity in membranes was 6.4 nmol/min/mg. Production of active FnLpxE in E. coli was improved further when the FnLpxE gene was expressed behind the lac promoter on pWSK29 (39). The 1-phosphatase specific activity of XL1-Blue/pWSK29-FnLpxE membranes was 62.3 nmol/min/mg. The true specific activities of the two phosphatases will have to be reevaluated with homogeneous enzyme preparations.

Membrane proteins from XL1-Blue/pWSK29-FnLpxE were separated by SDS-PAGE, followed by staining with Coomassie...
Lipid A and its 1-diphosphate derivative (Fig. 1) were isolated and separated by TLC. The presumed 1-dephospho-Kdo2-lipid A in WBB06 (lane 2) was more rapidly migrating than a major new lipid A species accumulated in XL1-Blue/pWSK29 (lane 3), demonstrating that the Kdo-trimming enzyme prefers a substrate lacking the 1-phosphate group. The 1-dephospho-Kdo2-lipid A was identified by mass spectrometry based on its [M – H]− at m/z 1939.9 and by its conversion to 1-dephospho-Kdo2-lipid A in the presence of E. coli KdtA (65).

FIG. 1A. Solvent Front.

FIG. 2. Detection of lipid A 1-phosphatase and 4’-phosphatase activities in F. novicida U112 membranes. Membranes (0.5 mg/ml) of F. novicida U112 (lanes 2–5) or E. coli Novablue/pLpxE (10), which expresses the 1-phosphatase of R. leguminosarum (lanes 6 and 7), were assayed for the indicated times at 30 °C with 5 μM Kdo2-([4’-32P]liphid IVa (20,000 cpm/nmol) as the substrate (structure in Fig. 1D). The products were separated by TLC in the solvent chloroform, pyridine, 88% formic acid/water (30:70:16:10, v/v/v/v) and were detected with a PhosphorImager. The Rf of the 1-dephospho-Kdo2-([4’-32P]liphid IVa was confirmed by comparison with the product generated with the previously characterized LpxE 1-phosphatase of R. leguminosarum, expressed in E. coli Novablue (lanes 6 and 7).

Blue (Fig. 5A). When compared with vector control, an additional protein was observed at 27.5 kDa, the size expected for FnLpxE. To determine whether FnLpxE functions to dephosphorylate lipid A at its 1-position when expressed in living cells of E. coli, strains XL1-Blue/pWSK29 and XL1-Blue/pWSK29-FnLpxE were labeled for several generations with 32Pi. The 32P-labeled lipid A species were isolated and separated by TLC. Lipid A and its 1-diphosphate derivative (Fig. 1A) were recovered from the vector XL1-Blue/pWSK29 (Fig. 5B, lane 1), whereas a major new lipid A species accumulated in XL1-Blue/pWSK29-FnLpxE (Fig. 5B, lane 2). The rapid migration of this substance is consistent with 1-dephosphorylation of lipid A in wild-type E. coli cells expressing recombinant FnLpxE. The levels of both lipid A and its diphosphate derivative (Fig. 1A) are greatly reduced (Fig. 5B, lane 2). The transfer of pWSK29-FnLpxE into Salmonella typhimurium also resulted in extensive 1-dephosphorylation of endogenous lipid A (data not shown).

Mass Spectrometry of LPS from Hep-tose-deficient E. coli Expressing FnLpxE—To show conclusively that FnLpxE is functional and absolutely specific for the 1-position of lipid A in living cells of E. coli, the 1-phosphatase-expressing pWSK29-FnLpxE plasmid was transformed into E. coli WBB06 (35), a heptose-deficient mutant with a deletion spanning the waaC and waaF genes. When grown in the absence of calcium ions, WBB06 synthesizes a truncated LPS consisting mainly of Kdo2-lipid A and some of the 1-diphosphate variant (Fig. 1A) (30). However, it cannot add any additional core sugars. WBB06/pWSK29-FnLpxE and the vector control strain WBB06/pWSK29 were grown in parallel for several generations in the presence of 32Pi. LPS and phospholipids were extracted directly with chloroform/methanol without the need for a hydrolysis step, resolved by TLC, and detected with a PhosphorImager. Both Kdo2-lipid A and the Kdo2-lipid A 1-diphosphate (Fig. 1A) were present in WBB06/pWSK29 (Fig. 6, lane 2) (30). The levels of both compounds were greatly reduced in WBB06/pWSK29-FnLpxE (Fig. 6, lane 3) and replaced by a more rapidly migrating substance, presumed to be 1-dephospho-Kdo2-lipid A (Fig. 6, lane 3). Phosphatidylglycerol and phosphatidylethanolamine were present at normal levels (Fig. 6, lanes 2 and 3), suggesting that FnLpxE does not hydrolyze phospholipid precursors, such as phosphatidic acid (40), which would inhibit phospholipid synthesis. The selectivity of FnLpxE for lipid A was confirmed independently by demonstrating that it does not catalyze significant phosphatidic acid or phosphatidylglycerophosphate dephosphorylation in vitro compared with vector controls (data not shown). Furthermore, it does not dephosphorylate the 4’-position of tetaacylated lipid A precursors.

The presumed 1-dephospho-Kdo2-lipid A in WBB06/pWSK29-FnLpxE was isolated in mg amounts and analyzed by MALDI-TOF mass spectrometry. The negative mode (Fig. 7A) demonstrates a major peak at m/z 2156.5, which is consistent with [M – H]− for 1-dephospho-Kdo2-lipid A (Fig. 7A, structure inset). The peak at m/z 1716.5 probably arises by loss of the Kdo disaccharide, which is labile under these conditions. In the positive mode (Fig. 7B), the peak at m/z 2180.3 is interpreted as [M + Na]+ for 1-dephospho-Kdo2-lipid A, whereas the peak at m/z 1740.0 arises by loss of the Kdo disaccharide. The peak at m/z 1087.2 is the B1+ ion (41) arising from the distal glucosamine unit, which is the same as the B2+ ion seen in the positive ion spectrum of Kdo2-lipid A or lipid A.
This finding demonstrates unequivocally that the distal glucosamine unit is intact in the novel LPS derivative that accumulates in WBB06/pWSK29-FnLpxE. FnLpxE dephosphorylates only the 1-position of lipid A \textit{in vivo} and not the 4/\textit{H}11032-position.

Additional validation for the structure shown in Fig. 7 was obtained by examining the $^{31}$P and $^{1}$H NMR spectra (42) of the 1-dephospho-Kdo2-lipid A obtained from WBB06/pWSK29-FnLpxE (data not shown).

FnLpxE-catalyzed 1-Dephosphorylation of Lipid A Is MsbA-dependent—FnLpxE is predicted to have six transmembrane helices with its conserved lipid phosphatase motifs facing the periplasmic surface of the inner membrane (38). Because Kdo$_2$-lipid A is synthesized on the cytosolic face of the inner membrane, it would need to be flipped to the periplasmic surface to be 1-dephosphorylated. MsbA is an essential ABC transporter and LPS flippase in \textit{E. coli} (19). Therefore, blocking MsbA in living cells might prevent 1-dephosphorylation of newly synthesized lipid A. We previously demonstrated that a temperature-sensitive point mutation in \textit{msbA} can lead to rapid cessation of phospholipid and lipid A export (19). Since there is no cessation of phospholipid and lipid A biosynthesis when MsbA is inactivated, newly made lipids accumulate in the inner membrane (19), presumably on its inner surface. MsbA is a homodimer that is closely related to the mammalian Mdr proteins (43, 44).

Accordingly, pWSK29-FnLpxE was transformed into WD2, a temperature-sensitive \textit{E. coli} mutant with an A270T substitution in the fifth transmembrane helix of MsbA (19). MsbA is inactivated in WD2 by shifting the cells from 30 to 44 °C for 30 min during midlog phase (19). To study the effects of MsbA inactivation on the function of the 1-phosphatase \textit{in vivo},...
W3110A/pWSK29, W3110A/pWSK29-FnLpxE, WD2/pWSK29-FnLpxE, and WD2/pWSK29 were grown at 30 °C until $A_{600}$ reached 0.6–0.8. After 30 min at 44 °C, the cells were labeled with $\mu$Ci/ml $^{32}$P for 20 min. The $^{32}$P-labeled lipid A species were extracted after hydrolysis at pH 4.5 to cleave off the Kdo and core sugars (36, 37) and then separated by TLC. At 44 °C, the control strain W3110A/pWSK29 synthesized the normal E. coli lipid A and its 1-diphosphate derivative (Fig. 8, lane 1), whereas W3110A/pWSK29-FnLpxE synthesized mainly the 1-dephosphorylated species (Fig. 8, lane 2). The same results were obtained at 30 °C (not shown). When MsbA was inactivated at 44 °C in WD2/pWSK29-FnLpxE, newly synthesized lipid A was not 1-dephosphorylated (Fig. 8, lane 3), demonstrating that FnLpxE is MsbA-dependent and confirming the periplasmic orientation of the 1-phosphatase active site.

At the permissive temperature of 30 °C, lipid A was 1-dephosphorylated by FnLpxE in WD2 (Fig. 8, lane 4), as in W3110A/pWSK29-FnLpxE. When the vector control strain WD2/pWSK29 was grown at 30 °C, both lipid A and the 1-diphosphate variant were present in normal amounts (Fig. 8, lane 5), as in W3110A/pWSK29. However, the lipid A 1-diphosphate did not accumulate together with lipid A in WD2/pWSK29-FnLpxE when MsbA was inactivated at 44 °C (Fig. 8, lane 3). This is consistent with previous data demonstrating that the lipid A 1-diphosphate species is produced in the periplasm or outer membrane (45). When MsbA is functioning properly, FnLpxE may prevent the formation of the lipid A 1-diphosphate by competing for newly synthesized lipid A molecules that appear on the periplasmic side of the inner membrane. Alternatively, FnLpxE might dephosphorylate the lipid A 1-diphosphate directly.

**DISCUSSION**

The biosynthesis of the lipid A and core domains of LPS in Gram-negative bacteria begins in the cytoplasm and on the inner surface of the inner membrane (2, 3). Additional enzymes located on the outer surface of the inner membrane and in the outer membrane complete the assembly of LPS. For example, when the O-antigen is present, it is attached to the LPS-core on the outer surface of the inner membrane (2, 3, 46). In the outer membrane, PagP (47, 48), which is induced by growth at low divalent cation concentrations (49, 50), adds a seventh acyl chain to lipid A in a transacylation reaction that requires a phospholipid as the donor substrate (47, 51). The present study demonstrates for the first time that lipid A 1-dephosphorylation, when it occurs, takes place on the outer surface of the inner membrane.

We previously reported that MsbA, an essential ABC transporter related to mammalian Mdr proteins (52), is required for the export of nascent LPS and phospholipids to the outer membrane in E. coli (19, 45). In addition, MsbA is a “flippase” that is necessary for the rapid translocation of newly synthesized LPS across the inner membrane (53). We have now demonstrated that recombinant FnLpxE, an enzyme that selectively dephosphorylates lipid A at the 1-position (Figs. 3 and 5),
Fig. 9. Proposed model for 1-dephosphorylation of lipid A in the inner membrane of Gram-negative bacteria. The lipid A and core portions of LPS are synthesized on the inner surface of the inner membrane (3). MsbA, an essential ABC transporter, exports the nascent LPS to the outer surface of the inner membrane (19, 43), where FnLpxE 1-dephosphorylates the lipid A portion of the molecule.

requires a functional MsbA protein for activity in living E. coli cells (Fig. 8). In contrast, the nine intracellular enzymes that assemble Kdo₂-lipid A are MsbA-independent (Fig. 8, lane 3). The active site of FnLpxE is predicted to face the periplasmic side of the inner membrane (38). The fact that FnLpxE requires MsbA in vitro (but not under in vitro assay conditions) provides compelling evidence to support the proposed topography of the 1-phosphatase active site (Fig. 9).

FnLpxE may facilitate the development of novel in vitro LPS flip-flop assays in oriented membrane vesicles, because water is the only co-substrate required. Previously reported periplasmic modifications of lipid A (53), such as the addition of phosphoethanolamine and aminoorabino-side groups in polymyxin-resistant mutants, require lipid donor co-substrates (phosphatidylethanolamine and aminoarabinose groups in polymyxin-resistant mutants) (54–56). These substrates would also have to be transported across the inner membrane to be available as substrates for periplasmic lipid A modification, complicating the interpretations of in vitro assays.

The LpxE orthologs of F. novicida and R. leguminosarum share sequence homology with each other, primarily in their lipid phosphate phosphatase motifs (Fig. 4). They are both members of the large superfamily of lipid phosphate phosphatases (25), the true substrates of which cannot be predicted without biochemical studies, such as those shown in Figs. 3 and 5. Site-directed mutagenesis has confirmed the absolute requirement of these active site motifs for 1-phosphatase activity. Functional FnLpxE is more readily expressed in E. coli and has a much higher apparent specific activity in washed membranes than the previously reported R. leguminosarum LpxE (10). The true specific activities of these phosphatases will have to be determined with homogeneous preparations. In addition, an as yet uncharacterized inhibitor of R. leguminosarum LpxE interferes with its full expression in E. coli membranes prior to solubilization and purification. Consequently, the kinds of experiments shown in Figs. 6–8 were not practical with the previously available LpxE ortholog.

The biological activities of the lipid A component of LPS are thought to involve the binding of the negatively charged phosphates to cationic amino acid side chains on LPS-binding proteins and receptors (13, 57, 58). Lipid A lacking phosphate groups might help bacteria evade the innate immune system, since they would not be recognized by TLR-4. Furthermore, dephosphorylated lipid A would be expected to have much lower affinity for polymyxin and other cationic antimicrobial peptides (59), conferring resistance to such substances in some organisms (10). This phenomenon allowed us to isolate the FnlpxE gene directly by heterologous expression in E. coli. The dephosphorylated lipid A species found in R. leguminosarum or R. etli (5, 6) similarly might help bacteroids evade the innate immune responses of plants during symbiosis

inside root cells. However, the plant can still defend itself against Gram-negative pathogens, which contain phosphorylated lipid A molecules. It will be of interest to isolate and characterize Francisella and Rhizobium mutants lacking the lipid A phosphatases to assess the effects on intracellular growth, symbiosis, and cationic antimicrobial peptide resistance.

The so-called “monophosphoryl” lipid A preparations (60, 61), which are used as adjuvants (15, 17), retain the immunostimulatory properties of lipid A with significantly reduced toxicity. They apparently still signal through TLR-4 but with altered kinetics and cytokine profiles (15). Current methods for preparing monophosphoryl lipid A from natural LPS involve acid hydrolysis at 100 °C (60, 61). This treatment not only removes the 1-phosphate substituent of lipid A but also cleaves the labile Kdo-lipid A linkage (60, 61). Recombinant FnLpxE specifically removes the phosphate from the 1-position of lipid A without disturbing the Kdo residues. As demonstrated in Fig. 7, large amounts of highly purified 1-dephospho-Kdo₂-lipid A can be obtained from E. coli expressing FnLpxE. This material, which was not previously available, may prove useful as a novel adjuvant. Synthetic lipid A analogues with a Kdo disaccharide have not been reported, since the Kdo moieties are difficult to incorporate by chemical methods (62).

FnlpxE can also be used to reengineer lipid A structure in living Gram-negative bacteria, such as E. coli (Figs. 6–8) or S. typhimurium (not shown). The effects of controlled 1-dephosphorylation on inflammation and pathogenesis should provide new insights into the interactions of lipid A and LPS with the innate immune system. It will be especially interesting to examine the utility of Salmonella constructs expressing FnLpxE as live oral vaccine candidates (63), possibly in conjunction with other mutations that attenuate virulence (64).

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MsbA Transporter-dependent Lipid A 1-Dephosphorylation on the Periplasmic Surface of the Inner Membrane: TOPOGRAPHY OF FRANCISELLA NOVICIDA LpxE EXPRESSED IN ESCHERICHIA COLI

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