A *Leptospira interrogans* Enzyme with Similarity to Yeast Ste14p That Methylates the 1-Phosphate Group of Lipid A

Distinct from other spirochetes, cells of *Leptospira interrogans* contain orthologues of all the *Escherichia coli* lpx genes required for lipid A biosynthesis, but they synthesize a modified form of lipopolysaccharide that supposedly activates toll-like receptor 2 (TLR2) instead of TLR4. The recent detection of the L. *interrogans* lipid A structure revealed an unprecedented O-methylation of its 1-phosphate group (Que-Gewirth, N. L. S., Ribeiro, A. A., Kalh, S. R., Cotter, R. J., Bulach, D. M., Adler, B., Saint Girons, I., Werts, C., and Raetz, C. R. H. (2004) *J. Biol. Chem.* 279, 25420–25429). The enzymatic activity responsible for selective 1-phosphate methylation has not been previously explored. A membrane enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to the 1-phosphate moiety of *E. coli* Kdo₂-[1⁴-3²P]lipid A has now been discovered. The gene encoding this enzyme was identified based on the hypothesis that methylation of a phosphate group is chemically analogous to methylation of a carboxylate moiety at a membrane-water interface. Database searching revealed a candidate gene (renamed *lmtA*) in *L. interrogans* showing distant homology to the yeast isoprenylcysteine carboxyl methyltransferase, encoded by *STE14*, which methylates the a-type mating factor. Orthologues of *lmtA* were not present in *E. coli*, but the lipid A of which normally lacks the 1-phosphomethyl group, or in other spirochetes, which do not synthesize lipid A. Expression of the *lmtA* gene behind the lac promoter on a low copy plasmid resulted in the appearance of SAM-dependent methyltransferase activity in *E. coli* inner membranes and methylation of about 30% of the endogenous *E. coli* lipid A. Inactivation of the ABC transporter MsbA did not inhibit methylation of newly synthesized lipid A. Methylated *E. coli* lipid A was analyzed by mass spectrometry and NMR spectroscopy to confirm the location of the phosphomethyl group at the 1-position. In human cells, engineered to express the individual TLR subtypes, 1-phosphomethyl-lipid A purified from *lmtA*-expressing *E. coli* potently activated TLR4 but not TLR2.

The outer membrane of Gram-negative bacteria is an asymmetric lipid bilayer. The inner monolayer consists of glycerophospholipids, whereas the outer monolayer consists of lipopolysaccharide (LPS). ¹ LPS is composed of a saccharolipid anchor (1) termed lipid A, a nonrepeating oligosaccharide core, and a distal polysaccharide (O-antigen). The minimal LPS required for growth in *Escherichia coli* consists of lipid A and two 3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) residues, designated Kdo₂-lipid A. Lipid A is a potent immunostimulant in animals that activates the toll-like receptor 4 (TLR4) (2–4), and it is implicated in Gram-negative septic shock (5). The Lpx enzymes responsible for the assembly of Kdo₂-lipid A have been fully characterized in *E. coli* (5). Several of these enzymes are attractive targets for the design of new antibiotics (6, 7). The *lpx* genes are well conserved among Gram-negative bacteria, despite some variations in lipid A structure. Additional modifying enzymes, not present in *E. coli*, are responsible for generating most of the structural diversity, and they generally function late in the biosynthetic pathway (8–13).

One example of a bacterium with an unusual, modified lipid A is *Leptospira interrogans*, a spirochete responsible for causing leptospirosis in humans. This disease is a problem in highly populated, tropical urban centers, and its clinical presentations range from flu-like symptoms to fatal kidney, liver, or pulmonary damage (14, 15). Typical O-antigen gene clusters and orthologues of nearly all of the *E. coli* lpx genes are present in the genomes of various *Leptospira* (16, 17). The presence of LPS is a major distinguishing feature that sets *L. interrogans* apart from the other spirochetes (such as *Treponema pallidum, Treponema denticola*, and *Borrelia burgdorferi*), perhaps explaining why *L. interrogans* is easily cultivated outside of its host.

The structure of *L. interrogans* lipid A has been elucidated...
Membrane Enzyme Methylating the 1-Phosphate Group of Lipid A

Fig. 1. Comparison of E. coli lipid A, L. interrogans lipid A, and 1-phosphomethyl-lipid A from E. coli expressing LmtA. Four key differences in the structures of E. coli (A) and L. interrogans (B) lipid A are the presence in the latter of four N-linked acyl chains instead of two, two unsaturated secondary acyl chains, a methylated 1-phosphate group, and the absence of the 4'-phosphate moiety. The structure of 1-phosphomethyl-lipid A from E. coli expressing LmtA is shown in C.

recently using mass spectrometry, NMR spectroscopy, and biochemical analysis (18). Fig. 1 illustrates the key structural differences between E. coli and L. interrogans lipid A. E. coli lipid A is a β,1'-6-linked disaccharide of glucosamine that is phosphorylated at the 1- and 4'-positions and is acylated with (R)-3-hydroxymyristate at the 2-, 3-, 2',- and 3'-positions (Fig. 1A) (5). The 2'- and 3'-linked fatty acyl chains are further esterified with secondary laurate and myristate chains, respectively. The structure of L. interrogans lipid A is a β,1'-6-linked disaccharide, consisting of the glucosamine analogue 2,3-diamino-2,3-dideoxy-α-D-glucopyranose (Fig. 1B) (18). L. interrogans lipid A is acylated with R-3-hydroxydecanoate at the 3- and 3'-positions and with R-3-hydroxypalmitate at the 2- and 2'-positions. The secondary acyl chains most likely are 12 or 14 carbons in length, and each contains one double bond. As in many strains of Rhizobium and Francisella, the 4'-phosphate group is missing in L. interrogans lipid A. However, the most unusual property of L. interrogans lipid A is the presence of a methylated 1-phosphate moiety.

The proposed biosynthetic pathway for the assembly of L. interrogans Kdo₂-lipid A is diagrammed in Fig. 2. For the most part, the pathway is catalyzed by orthologues of the E. coli lpx gene products. However, there are at least four additional genes that are required in the L. interrogans system. The first two, gnnA and gnnB, were originally discovered in Acidithiobacillus ferrooxidans because of their location between lpxA and lpxB (12). Together, these gene products function to synthesize the sugar nucleotide UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucose (UDP-GlcNAc3N). GnnA catalyzes the oxidation of the glucosamine 3-ΟH of UDP-GlcNAc, and GnnB catalyzes the subsequent transamination to form UDP-GlcNac3N. LpxA from L. interrogans is absolutely specific for UDP-GlcNac3N versus UDP-GlcNAc (19). E. coli LpxA uses both UDP-GlcNAc and UDP-GlcNac3N in vitro, but it cannot synthesize the latter, because it lacks the gnnA and gnnB genes. Consequently, L. interrogans lipid A contains four N-linked acyl chains, whereas E. coli has only two.

The chemical structure of L. interrogans A (Fig. 1B) implies that two additional lipid A-processing enzymes must be present in this organism. A 1-methyltransferase and a 4'-phosphatase are proposed to methylate the 1-phosphate group and dephosphorylate the 4'-position, respectively (Fig. 2). Methylated phosphate residues are relatively unknown in biology (20, 21). There is only one well characterized example of a methylated phospholipid, a methylated phosphatidyglycerophosphate analogue found in the halophile Halobacterium salinarium (22). In the field of lipid A biochemistry, a methylated phosphate moiety is without precedent (5).

The significance of the distinct lipid A structure seen in L. interrogans is unknown. A recent study suggested that leptospiral LPS might activate an alternative TLR as compared with the LPS from E. coli and most other Gram-negative bacteria (23). In the case of E. coli (2, 3), LPS first interacts with the LPS-binding protein, which delivers the LPS to the GPI-linked peripheral membrane protein, CD14. LPS is then brought into contact with the integral membrane protein, TLR4, and the accessory protein, MD-2. Upon activation, MyD88 is recruited to the cytoplasmic tail of TLR4, which in turn triggers a series of events that culminates in the translocation of NF-κB to the nucleus and the transcriptional activation of numerous cytokine genes. However, Werts et al. (23) reported that L. interrogans LPS instead activates TLR2.

It is tempting to speculate that the apparent differences in TLR activation between L. interrogans and E. coli are due to the structural characteristics of their respective lipid A molecules. Identification of the L. interrogans genes and enzymes responsible for some of these structural variations should provide helpful tools for investigating this hypothesis. Here, it is reported that a novel L. interrogans membrane enzyme, designated LmtA, catalyzes the selective transfer of a methyl group to the 1-phosphate residue of Kdo₂-lipid A. When LmtA is expressed in E. coli, a modified lipid A species is synthesized in vivo, which is shown to be the 1-phosphomethyl derivative of E. coli lipid A. Lipid A methylation probably occurs on the cytoplasmic face of the inner membrane, since it is independent of MsbA function. The addition of the methyl group to E. coli lipid A does not alter its potent, TLR4-specific bioactivity.

**EXPERIMENTAL PROCEDURES**

Materials—[γ-32P]ATP, [32P]γ-[glycerol-U-14C]phosphatidate acid, and [glycerol-U-14C]glycerol-3-phosphate were purchased from PerkinElmer Life Sciences, and Silica Gel 60 (0.25-mm) TLC plates were obtained from Merck. Trypsin and yeast extract were from Difco, whereas chloroform, ammonium acetate, and sodium acetate were from EM Science. Triton X-100 and the BCA protein determination kit were purchased from Pierce. LPS derived from E. coli strain O111:B4 was purchased from Sigma and reextracted by phenol chloroform as described (24). Pam2CysK4 was from EMC Microcollection GmbH (Tübingen, Germany), and human IL-1β was purchased from Peprotech (Rocky Hill, NJ). All other reagent grade chemicals were obtained from Sigma or Mallinkrodt.

**Bacterial Strains and Growth Conditions**—All bacterial strains used in this study are described in Table I. L. interrogans serovar interohaemorrhagiae (strain Verdun) cell pellets were kindly provided by Cath-
FIG. 2. Proposed biosynthetic pathway for L. interrogans Kdo₂-lipid A. Most of these reactions are catalyzed by orthologues of the E. coli Lpx enzymes. However, GnnA and GnnB act at the beginning of the pathway to make the unique sugar nucleotide, UDP-GlcNAc3N, whereas the methyltransferase and the 4'-phosphatase function in the later stages. GlcN3N, 2,3-diamino-2,3-dideoxy-ß-galactopyranose.

### TABLE I

Relevant bacterial strains and plasmids

| Strains | Description | Source or reference |
|---------|-------------|---------------------|
| **Strains** | | |
| L. interrogans | Avirulent variant of serovar icterohaemorrhagiae | Ref. 23 |
| Strain Verdun | | |
| E. coli | | |
| XL1 Blue-MR | mcrABC recA1 endA1 gyrA96 relA1 supE44 thi-1 lac | Stratagene |
| W3110 | Wild-type, F−, λ, araBAD: Tn10 | E. coli Genetic Stock Center (Yale) |
| W3110A | Wild-type, F−, λ, araBAD: Tn10 mshA (A270T) | Ref. 37 |
| WD2 | | Ref. 36 |
| **Plasmids** | | |
| pET23a | Expression vector, T7lac promoter, ampr | Novagen |
| pMBH8 | pET23a expressing lmtA | This work |
| pWSK29 | Low copy expression vector, lac promoter, ampr | Ref. 27 |
| pLmtA | pWSK29 expressing lmtA | This work |

**Plasmids were isolated using the QIAquick Gel Extraction Kit.** DNA fragments were recovered from agarose gels using the QIAquick Gel Extraction Kit. Pfu DNA polymerase (Stratagene), T4 DNA ligase (Invitrogen), restriction endonucleases (New England Biolabs), and shrimp alkaline phosphatase (U. S. Biochemical Corp.) were used according to the manufacturers’ instructions. The Duke University DNA Analysis Facility sequenced double-stranded DNA with an ABI Prism 377 instrument. All primers were obtained from MWG-Biotech. Chemically competent cells for transformations were prepared by the method of Inoue et al. (26).

**Cloning of lmtA from L. interrogans Genomic DNA**—The L. interrogans lmtA gene was cloned into pET23a (Novagen) behind the T7lac promoter to generate pMBH8. First, the gene was amplified by PCR from L. interrogans serovar ichterohemorrhagiae (strain Verdun) genomic DNA, kindly provided by Catherine Werts (Institut Pasteur). The primers were designed based on the DNA sequence of L. interrogans serovar I (strain 56601), which is 99.7% identical to serovar ichterohemorrhagiae (strain Verdun) at the DNA level and 100% at the protein level for LmtA. The forward primer consisted of a clamp region and an NdeI site (underlined) that overlaps with the first 28 base pairs of the lmtA gene (start codon in boldface type). The reverse primer contained a clamp region, a BamHI site (underlined), and the last 24 base pairs of lmtA (stop codon in boldface type). Sequences of the forward and reverse primers were 5'-GGCCATATGGCTTGTAGCTGAAA-GATTGAAACTC-3' and 5'-GGCAGACTCTAAACGACCACATC-ATTAAAG-3', respectively. The PCR consisted of 100 ng of genomic DNA template, 250 ng of each primer, 200 μM each of dNTPs, 1× Pfu buffer (20 mM Tris-HCl, pH 8.8, 2 mM MgSO₄, 10 mM KCl, 10 mM (NH₄)₂SO₄, 0.1 mg/ml bovine serum albumin (BSA), 0.1% Triton X-100), and 5 units of Pfu DNA polymerase in a total reaction volume of 100 μl. The reaction conditions were as follows: 94°C denaturation for 1 min followed by 25 cycles of 94°C (denaturation) for 1 min, 50°C (annealing) for 1 min, and 72°C (extension) for 1 min. This was followed by a 10-min run-off at 72°C. The gel-purified PCR product was digested with NdeI and BamHI and ligated into NdeI-BamHI-digested and shrimp alkaline phosphatase-treated pET23a vector. The resulting pMBH8 was transformed into XL-1 Blue cells (Stratagene). The accession number for the lmtA DNA coding sequence is DQ097086.

The lmtA gene was also cloned into the lac-inducible, low copy expression vector, pWSK29 (27). Using XbaI and BamHI, the fragment containing lmtA and the upstream ribosome-binding site was excised. This fragment was ligated into XbaI/BamHI-digested and shrimp alkaline phosphatase-treated pWSK29. This plasmid, pLmtA, was then transformed into E. coli W3110.

**Preparation of Cell-free Extracts and Washed Membranes**—L. interrogans was provided as a frozen cell pellet. E. coli W3110 cells, harboring either pLmtA or pWSK29, were grown in 100-ml cultures that were harvested by centrifugation (3500 × g, 20 min, 4°C). Cells were resuspended in 4 ml of ice-cold 50 mM HEPES, pH 7.5, and lysed by two passages through a French pressure cell at 10,000 p.s.i. The lysate was cleared by centrifugation at 10,000 × g for 20 min at 4°C. A small portion of the resulting supernatant (cell-free extract) was saved at −80°C. Membranes were prepared from the remaining supernatant by
ultracentrifugation at 100,000 × g for 60 min at 4 °C, and the resulting high speed supernatant (cytosol) was saved at −80 °C. The membranes were washed in 8 ml of 50 mM HEPES, pH 7.5, and subjected to an additional ultracentrifugation step. The final pellet was resuspended in 750 μl of 50 mM HEPES, pH 7.5, and stored at −80 °C. The BCA assay was used to determine protein concentration.

Preparation of Lipid Substrates—The radiolabeled substrates, [4-32P]Kdo₂-lipid A, [4-32P]lipid IV₃, and [4-32P]-Kdo₂-lipid IV₃, were prepared in vitro following a published procedure (28, 29). Phosphatidyl-

In vitro glycerophosphate was prepared by enzymatic synthesis using CDP-diacylglycerol and [γ-32P]-glycerol-3-phosphate (30). Unlabeled Kdo₂-lipid A was isolated from the heptose-deficient E. coli strain WBB06, as described (31). Unlabeled lipid IV₃ and Kdo₂-lipid IV₃ were obtained following a published procedure (32).

Methyltransferase Assay—The activity of LmtA in either cell-free extracts, cytosol, or membranes was assayed under optimized conditions in 10–35 mM reaction volume with the substrates Kdo₂-lipid A and SAM. Assays included 50 mM HEPES, pH 7.5, 0.1% Triton X-100, 5 mM EDTA, 1 mg/ml BSA, 10 mM SAM, 500 cpm/μl [4-32P]Kdo₂-lipid A, and 1 μM unlabeled Kdo₂-lipid A. Assays conducted with E. coli membranes did not include 5 mM EDTA in order to minimize the formation of the PagP product, palmitoyl-Kdo₂-lipid A (33). Reactions were incubated at 30 °C for varying times and terminated by spotting 4 μl onto a TLC plate. Gels 60 TL2 (2:3, v/v) were separated in chloroform/methanol/water/acetic acid (25:15:4:4, v/v/v/v). The entire sample was spotted onto a TLC plate, and the lipid A mixture was converted into a two-phase Bligh/Dyer system by adding 200 μl of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS. The mixture was then boiled at 100 °C for 30 min to hydrolyze the Kdo residues. The resulting solution was converted into a two-phase Bligh/Dyer system by adding 200 μl each of chloroform and methanol, and it was centrifuged for 5 min. The lower phase (containing the free lipid A species) was removed, dried under vacuum, and resuspended in 10 μl of chloroform/methanol (2:1, v/v). The entire sample was spotted onto a TLC plate, and the lipid A molecules containing containing 1% SDS were resolved in chloroform/methanol/water (2:3:1, v/v/v) and DNA was washed two times with 3 ml of a single-phase Bligh/Dyer solution (chloroform/methanol/water, 1:2:0.8, v/v/v) and incubated at room temperature for 60 min in order to extract the phospholipids. The sample was centrifugated, and the pellet (containing the LPS, proteins, and DNA) was washed two times with 3 ml of a single-phase Bligh/Dyer mixture. Then the pellet was boiled at 100 °C in 3 ml of 12.5 mM sodium acetate, pH 4.5, containing 1% SDS, for 30 min to release the lipid A from the LPS core residues. The lipid A molecules were extracted by conversion into a two-phase Bligh/Dyer system (chloroform/methanol/water, 2:2:1.8, v/v/v). The lower phase, containing the lipid A, was removed after centrifugation, and the remaining upper phase was washed twice with chloroform/methanol. The losalbumin equivalents were transferred to a 2-ml DEAE-cellulose column (Whatman DE-52), which fractions contained the desired lipid. These fractions were pooled, and the lipid was dissolved in 0.35 ml of CDCl₃/CD₃OD/D₂O (2:3:1, v/v/v) and it was centrifuged at 15,000 rpm at 4 °C. The resulting solution was deposited immediately on top of the sample spot, and the two solutions were allowed to dry together at room temperature. The matrix utilized for all analyses was a saturated solution of 6-aza-2-thiouridine in 50% acetonitrile and 10% tribasic ammonium citrate (9.1, v/v). Spectra for both positive and negative ions were acquired in linear mode. Each spectrum represents the average of 100 laser shots.

NMR Spectroscopy Analysis—A 0.3-mg sample of 1-phosphomethyl-lipid A was dissolved in 0.35 ml of CDCl₃/CD₃OD/D₂O (2:3:1, v/v/v) in a 5-mm D Rwanda NMR tube. Proton and 31P NMR spectra were run utilizing a 20-kV extraction voltage and time-delayed excitation. Dried lipid A samples were dissolves in chloroform/methanol (4:1, v/v) and prepared for MALDI-TOF analysis by depositing 0.3 μl of the sample solution on the sample plate. An equal volume of matrix solution was deposited immediately on top of the sample spot, and the two solutions were allowed to dry together at room temperature. The matrix utilized for all analyses was a saturated solution of 6-aza-2-thiouridine in 50% acetonitrile and 10% tribasic ammonium citrate (9.1, v/v). Spectra for both positive and negative ions were acquired in linear mode. Each spectrum represents the average of 100 laser shots.

MALDI-TOF Mass Spectrometry Analysis—Lipids were analyzed using an AXIMA-CFR (Kratos Analytical, Manchester, UK) matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer utilizing 20-kV extraction voltage and time-delayed excitation. Dried lipid A samples were dissolved in chloroform/methanol (4:1, v/v) and prepared for MALDI-TOF analysis by depositing 0.3 μl of the sample solution on the sample plate. An equal volume of matrix solution was deposited immediately on top of the sample spot, and the two solutions were allowed to dry together at room temperature. The matrix utilized for all analyses was a saturated solution of 6-aza-2-thiouridine in 50% acetonitrile and 10% tribasic ammonium citrate (9.1, v/v). Spectra for both positive and negative ions were acquired in linear mode. Each spectrum represents the average of 100 laser shots.

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RESULTS

Methyltransferase Activity Associated with L. interrogans Membranes—Both cell-free extracts and membranes isolated from L. interrogans appear to modify 1 µM Kdo2-lipid A in vitro in the presence of the methyl donor SAM (10 mM). When the reaction is monitored by TLC, a new SAM-dependent product band appears, which migrates faster than Kdo2-[4-32P]lipid A (Fig. 3A). This substance is not formed when high speed supernatant (cytosol) is used as the enzyme source, suggesting that the putative methyltransferase is membrane-bound. The more rapid migration of the SAM-dependent product on the TLC plate suggests the addition of a hydrophobic moiety, such as the methyl group found in leptospiral lipid A (Fig. 1B). Product formation by L. interrogans membranes was linear for 30 min at 0.25 mg/ml protein (specific activity of 0.064 nmol/min/mg).

The Addition of the Presumed Methyl Group to the Lipid A Portion of the Molecule—To rule out the possibility that the methyl group is added to the Kdo residues of Kdo2-lipid A, further analysis was required. L. interrogans membranes were assayed for various time intervals, and then the Kdo residues were removed by hydrolysis at 100 °C in 12.5 mM sodium acetate, pH 4.5, containing 1% SDS. The released lipid A molecules were extracted and analyzed by TLC. As illustrated in Fig. 3B, the more rapidly migrating component is present in the lipid A portion of the modified Kdo2-[4-32P]lipid A, verifying that the putative methyltransferase is occurring on the lipid A moiety. No lipid A modification occurs in the absence of SAM.

Identification and Cloning of the Candidate Methyltransferase Gene, lmtA—A possible gene encoding the lipid A methyltransferase was identified using bioinformatics. There are no available sequences for enzymes that methylate phosphate residues with which to search the L. interrogans genome. However, it was reasoned that the chemical environment required for the methylation of a phosphate group at a membrane surface might share some common features with the methylation of a carboxylate moiety. The latter reaction is catalyzed by the Saccharomyces cerevisiae isoprenylcysteine carboxyl methyltransferase, Ste14p (44, 45), a member of the same enzyme family that methylates Ras. A BLAST search revealed a distant orthologue of Ste14p in L. interrogans serovar lai (E value of 10−5), which had been annotated as a “putative protein-S-isoprenylcysteine methyltransferase” (17). Several properties of this protein suggested that it might actually be the leptomembrane lipid A methyltransferase, now designated LmtA. First, orthologues were not found in E. coli, which lacks the 1-phosphomethyl group. Likewise, no orthologues were detected in the other spirochetes, which do not synthesize lipid A. Like Ste14p, leptomembrane LmtA was predicted to have six transmembrane segments.

L. interrogans lmtA was expressed behind the lac promoter on the plasmid pWSK29 in E. coli W3110. The SDS-PAGE gel in Fig. 4A clearly demonstrates successful heterologous expression. An additional protein with the expected molecular mass (~27 kDa) is seen in both cell-free extracts and membranes of E. coli W3110 harboring pLmtA, but not in the vector controls (W3110/pWSK29). Membranes of W3110/pLmtA catalyzed time- and SAM-dependent synthesis of the presumed methyltransferase product with a specific activity of 2.95 nmol/min/mg when Kdo2-lipid A was used as the substrate (Fig. 4B). Methyltransferase activity was linear with respect to protein (data not shown). The identical migration during TLC of the product band generated by W3110/pLmtA membranes compared with leptomembrane membranes strongly suggests that LmtA is indeed the relevant lipid A methyltransferase. W3110/pLmtA membranes were also assayed under identical conditions using alternative lipid substrates (lipid IVα, Kdo2-lipid IVα, phosphatidyglycerophosphate, and phosphatidic acid). Specific activities were less than 1% of that reported for Kdo2-lipid A (data not shown).

LmtA Expressed in E. coli Is Located in the Inner Membrane—The outer and inner membranes of E. coli W3110 ex-
pressing LmtA were separated by isopycnic sucrose gradient centrifugation. Protein concentration, outer membrane phospholipase A, and inner membrane NADH oxidase assays revealed that the membranes were well resolved (Fig. 5). The profile of LmtA activity mirrors that of the NADH oxidase, revealing that LmtA is an inner membrane protein when expressed in E. coli. Given its amino acid sequence, it also is likely to be an inner membrane enzyme in L. interrogans. A similar membrane separation performed with the vector control W3110/pWSK29 did not yield fractions with measurable methyltransferase activity (data not shown).

Modification of Lipid A in E. coli Cells Expressing LmtA—A 32P labeling study was conducted to determine whether expression of LmtA modifies E. coli lipid A in living cells. Both W3110/pLmtA and W3110/pWSK29 were grown in the presence of 32P, and crude LPS was recovered in the residue of a single-phase Bligh/Dyer extraction of the cell pellet. Lipid A was released from the Kdo residues by hydrolysis at pH 4.5 in the presence of SDS, extracted, and analyzed by TLC. As shown in Fig. 6A, an additional modified lipid A species is observed in E. coli cells harboring pLmtA, but not in the empty vector control. The higher migration of the extra band is consistent with a methylated lipid A species, which accounts for ~30% of the total. Unmodified hexa-acylated lipid A and its 1-diphosphate variant are also present.

LmtA Activity Is MsbA-independent—MsbA is an essential ABC transporter that acts as the LPS flippase within the inner membrane (36, 37). The temperature-sensitive strain WD2, harboring a point mutation in MsbA, accumulates lipid A on the inner face of its inner membrane at the nonpermissive temperature (36, 37). The temperature-sensitive strain WD2, and its lipid A species were labeled at both the permissive (30 °C) and nonpermissive (44 °C) temperatures. Control labeling studies were performed at 44 °C with W3110/pLmtA and W3110/pWSK29, which possess wild-type MsbA. The vector control, WD2/pWSK29, was also labeled in parallel. Fig. 6B shows the TLC analysis of the lipid A species extracted from each culture. Comparable amounts of the putative methylated lipid A species are present in WD2/pLmtA at both temperatures. Therefore, even when MsbA is not functioning to flip newly synthesized lipid A to the periplasmic face of the inner membrane at 44 °C, LmtA is still active. Consequently, it is very likely that LmtA methylates lipid A on the cytoplasmic face of the inner membrane. The W3110A/pLmtA control likewise synthesizes modified lipid A at the elevated temperature, whereas neither strain harboring the vector control produces modified lipid A.

Demonstration of a Methyl Group at the 1-Position of Lipid A by Mass Spectrometry—Based on the published structure of L. interrogans lipid A (18), it is presumed that LmtA is catalyzing the addition of a methyl group solely to the 1-position of Kdo_2-lipid A. However, detailed structural analysis was needed.
to verify this hypothesis. The modified lipid A from W3110/pLmtA was isolated from 1 liter of cells grown to late log phase. A DEAE-cellulose column was used to separate the putative methylated lipid A from the unmodified lipid A species. Negative ion MALDI-TOF mass spectroscopy revealed a prominent \([M-\text{H}]^-\) ion at \(m/z\) 1812.3 (Fig. 7A), consistent with the presence of one extra methyl group in the modified lipid A (Fig. 1C). Additional structural information is revealed in the positive ion mode spectrum (Fig. 7B). The \(B_1^-\) oxonium ion, derived from the distal sugar unit, is formed by cleavage of the glycosidic linkage, and the \(B_2^-\) ion is formed by the loss of the substituent attached to the 1-position in the proximal sugar (Fig. 1C). The \(B_1^-\) ion at \(m/z\) 1086.6 and the \(B_2^-\) ion at \(m/z\) 1701.0 do not differ from those observed with unmodified wild-type \(E. coli\) lipid A (data not shown). Therefore, the methylphosphate moiety must be located at the 1-position. These results confirm the tentative identification of LmtA as the lipid A methyltransferase.

**NMR Spectroscopy of the Methylated Lipid A Species**—The 800-MHz \(^1\text{H}\) NMR spectrum of the putative 1-phosphomethyl-lipid A from W3110/pLmtA, dissolved in CDCl\(_3\)/CD\(_3\)OD/D\(_2\)O (2:3:1, v/v/v), reveals sharp and well-resolved resonances in the sugar (3.5–5.5 ppm) region (Fig. 8A), similar to previous 500-MHz NMR spectra of unmodified \(E. coli\) lipid A in the same solvent system (40, 41). However, the spectrum of the modified lipid A shows an unusual doublet at 3.57 ppm, which integrates to three protons and shows a splitting of 11.0 Hz. A similar doublet signal, which arises from the proximal methyl group, is observed for L. inter撞击s (18). The 3.57-ppm doublet signal in the \(^1\text{H}\) NMR spectrum of the modified \(E. coli\) lipid A, like that of \(L.\) inter撞击s lipid A, did not manifest any cross-peaks from homonuclear coupling in COSY, ZQCOSY, or TOCSY spectra (data not shown). \(^{31}\text{P}\) NMR spectroscopy at 202 MHz of the modified \(E. coli\) lipid A revealed two \(^{31}\text{P}\) resonances, one at 0.149 and the other at 1.325 ppm (data not shown). To investigate the possibility of heteronuclear coupling of the doublet arising from the presumed methyl group to one of the phosphorus atoms, selective inverse decoupling difference spectroscopy was implemented (40, 41). In this analysis, the difference spectra generated by subtracting on- and off-resonance \(^{31}\text{P}\)-decoupled \(^1\text{H}\) NMR lipid A spectra reveal the protons that are spin-coupled to the irradiated phosphate group. As noted above, the expanded sugar region of the modified lipid A in the absence of \(^{31}\text{P}\) decoupling (control) clearly reveals a doublet suggestive of a methyl group at 3.57 ppm (Fig. 8A). The difference spectrum of two selective, \(^{31}\text{P}\)-decoupled, \(^1\text{H}\) spectra (on- and off-resonance) for the 1.325-ppm \(^{31}\text{P}\) signal shows the simplification of the H-4' signal (4.14 ppm) into a triplet (Fig. 8B). Therefore, the \(^1\text{H}\) observed, \(^{31}\text{P}\)-decoupled
Neither lipid A preparation activated TLR2-YFP-expressing cells the 31P NMR signal at 0.149 ppm. The difference spectrum reveals the simultaneous collapse of the 3.57-ppm methyl doublet to a singlet and the subtraction of 1H spectra with selective on- and off-resonance decoupling of the 31P NMR signal at 1.325 ppm. The difference spectrum reveals the HEK293 cells stably transfected with TLR2-YFP or TLR4-YFP/MD2 were used in a luciferase reporter assay, as reported previously (43). Both the 1-phosphomethyl-lipid A and the unmodified E. coli lipid A showed dose-dependent activation of TLR4-YFP/MD2-expressing cells (Fig. 9, A and B, respectively). Neither lipid A preparation activated TLR4-YFP-expressing cells or cells harboring the empty vector. Furthermore, the cell lines responded to their respective positive control ligands as expected (Fig. 9C).

**DISCUSSION**

Four significant differences are apparent when the structure of L. interrogans lipid A (18) is compared with that of E. coli lipid A (Fig. 1). Due to the presence of GnnA, GnnB, and a highly selective LpxA acyltransferase, leptospiral lipid A contains four N-linked hydroxyacyl chains (Fig. 2), whereas that of E. coli has only two. Furthermore, L. interrogans lipid A has two unsaturated secondary acyl chains and lacks the 4'-phosphate moiety. Last, the lipid A of L. interrogans contains a methylphosphate group at the 1-position, leading to the proposal that L. interrogans lipid A biosynthesis must include a novel methylation reaction (Fig. 2). This structural variation has not been observed in the lipid A of any other Gram-negative bacteria (5). In fact, there are few well characterized examples of methylated phosphate groups in all of biology. The N-linked oligosaccharides on the lysosomal enzymes of Dictyostelium discoideum contain mannose 6-phosphomethyl residues (20), and a γ-monomethyl-phosphate cap at the 5’ end of 7SK, B2, and U6 small RNAs in eukaryotes has also been reported (21). The only well documented example in lipid biochemistry is the methylated analogue of phosphatidylglycerophosphate seen in H. salinarium (22). The relevant methyltransferases and their structural genes have not been identified in any of these systems. The L. interrogans enzyme transfers the methyl group of SAM to the 1-phosphate group of E. coli Kdo2-lipid A in vitro. Presumably, the Kdo2-lipid A analogue with the structure shown in Fig. 2, synthesized by the leptospiral orthologues of the E. coli lpx gene products (17), functions as the natural substrate for LmtA in L. interrogans.

Based on the assumption that phosphate group methylation is chemically analogous to carboxylate methylation, bioinformatics was used to identify the L. interrogans lipid A methyl-
transferase gene. Database searching revealed a distant orthologue of S. cerevisiae Ste14p in L. interrogans. This yeast membrane metalloenzyme (45) is a member of the isoprenyl-cysteine carboxyl methyltransferase (ICMT) family and performs the last step of CAAX box protein processing, which involves C-terminal carboxylmethylation of farnesylated or geranylgeranylated substrates (44, 45). A detailed topological and genetic analysis predicted that Ste14p is composed of six transmembrane helices, with both the N and C termini facing the cytosol (44). The fifth and sixth transmembrane segments are proposed to form a helix-turn-helix (helical hairpin) within the membrane. Orthologues of Ste14p are found in the genomes of Schizosaccharomyces pombe, Xenopus laevis, Caenorhabditis elegans, mice, rats, and humans. Sequence alignments revealed a consensus sequence at the C terminus (44). Interestingly, this consensus motif also appears in yeast phosphatidylethanolamine N-methyltransferases, certain ergosterol biosynthetic enzymes (presumed to be methyltransferases), and open reading frames of unknown function from various bacteria. The sequence of LmtA had not yet been deposited in the data base when this consensus motif was first reported (44).

Fig. 10 shows the primary sequence alignment of LmtA with several members of the ICMT family obtained using ClustalW software (available on the World Wide Web at www.ebi.ac.uk/clustalw). Red boxes indicate amino acid identity, blue boxes indicate conserved amino acid substitutions, and green boxes indicate semiconserved amino acid substitutions. The bars represent the predicted transmembrane domains of Ste14p (44). The dashed arrow indicates the start of the C-terminal consensus sequence.
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throughout the sequence, including in the C-terminal consensus sequence (dashed arrow in Fig. 10). Like Ste14p, LmA is predicted to have six transmembrane segments, which approximately coincide in the sequence alignment (Fig. 10, black lines). The two C-terminal transmembrane segments of LmA are predicted to be more distinct from each other than those of Ste14p, suggesting that the helical hairpin motif has a larger connecting loop in LmA. Although the mechanistic significance of the C-terminal region in the ICMT family members has not been established, it might include one or more substrate binding sites. Members of this family lack the usual tripartite consensus sequences believed to comprise the SAM binding sites found in the large majority of SAM-dependent enzymes (46). An alternative SAM binding site might exist in the cytoplasmic regions of the ICMT consensus sequence (residues 136–175 and 207–239 of Ste14p in Fig. 10). Mutagenesis studies of both the ICMTs and LmA are needed to determine the significance of the conserved C-terminal region. Within the ICMT family, however, there is significantly more similarity between lines

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