THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 42, Issue of October 20, pp. 32940–32949, 2000
Printed in U.S.A.

Oxygen Requirement for the Biosynthesis of the *S*-2-Hydroxymyristate Moiety in *Salmonella typhimurium* Lipid A

FUNCTION OF LpxO, A NEW Fe^{2+}/α-KETOGLUTARATE-DEPENDENT DIOXYGENASE HOMOLOGUE*

Received for publication, June 30, 2000, and in revised form, July 13, 2000
Published, JBC Papers in Press, July 19, 2000, DOI 10.1074/jbc.M005779200

Henry S. Gibbons‡§, Shanhua Lin¶, Robert J. Cotter¶, and Christian R. H. Raetz‡‖

From the ‡Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and the ¶Middle Atlantic Mass Spectrometry Laboratory, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Lipid A molecules of certain Gram-negative bacteria, including *Salmonella typhimurium* and *Pseudomonas aeruginosa*, may contain secondary *S*-2-hydroxyacyl chains. *S. typhimurium* has recently been shown to synthesize its *S*-2-hydroxymyristate-modified lipid A in a PhoP/PhoQ-dependent manner, suggesting a possible role for the 2-OH group in pathogenesis. We postulated that 2-hydroxylation might be catalyzed by a novel dioxygenase. Lipid A was extracted from a PhoP-constitutive mutant of *S. typhimurium* grown in the presence or absence of O_2. Under anaerobic conditions, no 2-hydroxymyristate-containing lipid A was formed. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of lipid A from cells grown in the presence of $^{18}O_2$ confirmed the direct incorporation of molecular oxygen into 2-hydroxyacyl-modified lipid A. Using several well characterized dioxygenase protein sequences as probes, tBLASTn searches revealed unassigned open reading frame(s) with similarity to mammalian aspartyl/asparaginyl β-hydroxylases in bacteria known to make 2-hydroxyacylated lipid A molecules. The *S. typhimurium* aspartyl/asparaginyl β-hydroxylase homologue (designated *lpxO*) was cloned into pBluescriptSK and expressed in *Escherichia coli* K-12, which does not contain *lpxO*. Analysis of the resulting construct revealed that *lpxO* expression is sufficient to induce O_2-dependent formation of 2-hydroxymyristate-modified lipid A in *E. coli*. LpxO very likely is a novel Fe^{2+}/α-ketoglutarate-dependent dioxygenase that catalyzes the hydroxylation of lipid A (or of a key precursor). The *S. typhimurium lpxO* gene encodes a polypeptide of 302 amino acids with predicted membrane-anchoring sequences at both ends. We hypothesize that 2-hydroxymyristate chains released from lipopolysaccharide inside infected macrophages might be converted to 2-hydroxymyristoyl coenzyme A, a well characterized, potent inhibitor of protein *N*-myristoyl transferase.

Salmonella typhimurium and related organisms are enteric Gram-negative pathogens. In human hosts, *S. typhimurium* infection causes gastroenteritis, but in mice, the outcome is a fatal, typhoid-like sepsis, characterized by dissemination of bacteria into spleen, liver, and blood (1). *S. typhimurium* initially invade intestinal epithelial cells and M cells of Peyer's patches and then pass into the lymphatic system by colonizing phagocytic cells (1). The bacteria survive and multiply within modified vacuoles of macrophages (2) and gradually induce macrophage apoptosis (3–5).

The ability of *S. typhimurium* to adapt to the acidic pH and the low divalent cation concentrations found inside macrophage vacuoles is critical to the infection process (6). The low Mg^{2+} concentration within phagolysosomes activates the PhoP/PhoQ two-component signal transduction system of *S. typhimurium*, triggering numerous responses needed for survival and persistence within macrophages (7). Phosphorylation of the transcriptional regulator PhoP (8) by the sensory kinase PhoQ under such conditions results in the activation or repression of as many as 40 *S. typhimurium* genes (9). The low pH of the phagolysosome, together with PhoP/PhoQ, also activates the PmrA/PmrB two-component system (10). The latter confers resistance to polymyxin and to many cationic antibacterial peptides (11).

Lipopolysaccharide (LPS)[1] is the principal constituent of the outer leaflet of the outer membranes of Gram-negative bacteria (12–14). In addition to its function as a protective permeability barrier (15), recognition of LPS by mammalian cells activates innate immune responses, including synthesis of cell adhesion proteins in endothelial cells (16) and of proinflammatory cytokines, like tumor necrosis factor-α and interleukin-1β, in monocytes (17, 18). Lipid A, the hydrophobic membrane anchor of LPS (Fig. 1), triggers most of these responses (13, 14). The acylated glucosamine disaccharide backbone of lipid A (Fig. 1*A*) is highly conserved in diverse Gram-negative bacteria (12, 19) and is detected by the pattern recognition receptor TLR4 of animal cells (20, 21).

Although previously thought to be a relatively static structure, recent studies of *S. typhimurium* and *Pseudomonas aeruginosa* have demonstrated that lipid A may be modified in a PhoP/PhoQ-dependent manner under conditions that mimic the phagolysosomal environment (22, 23). A palmitate group can be added in acyloxyacyl linkage to the *R*-3-hydroxymyristate residue at position 2 on *S. typhimurium* lipid A, and the amount of *S*-2-hydroxymyristate at position 3′ can be greatly increased (Fig. 1*B*) (22). Furthermore, 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) groups may

* This work was supported by National Institutes of Health Grants GM-51310 (to C. R. H. R.) and GM-54882 (to R. J. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
§ Supported by National Institutes of Health Grant 5 T32 GM07184–23 (to Duke University).
‖ To whom correspondence should be addressed: P.O. Box 3711, Dept. of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710. Tel.: 919-684-5326; Fax: 919-684-8885; raetz@biochem.duke.edu.

[1] The abbreviations used are: LPS, lipopolysaccharide; pEtN, phosphoethanolamine; L-Ara4N, 4-deoxy-4-amino-L-arabinose; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 4-morpholineethanesulfonic acid.

TABLE I
Strains and plasmids utilized in this study

Strain/Plasmids	Genotype	Source or reference
S. typhimurium		
14028	Virulent wild type	*Salmonella* Genetic Stock Center, University of Calgary, Canada
CS022	*pho-24* (PhoP-constitutive)	Ref. 38
E. coli		
XL1-BlueMR	Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*^{c}	Stratagene
Plasmids		
pBluescriptSK	Amp^R	Stratagene
pHSG1	pBluescript:*lpxO*; Amp^R	This work

be attached to the 4′- and/or 1-phosphates (Fig. 1*B*) once the PmrA/PmrB system is activated (22). While not required for bacterial growth in culture, these modifications may facilitate host-pathogen interactions. For instance, *S. typhimurium* mutants that are defective in the PhoP/Q-activated gene *pagP* do not incorporate the palmitate moiety into their lipid A and are more susceptible to the NP-1 defensin (24). Strains that cannot make L-Ara4N are unable to acquire resistance to polymyxin (25). In *Escherichia coli* K-12, modification of lipid A with L-Ara4N, pEtN, and/or palmitate is seen in polymyxin-resistant mutants (26) or in wild type cells treated with metavanadate (27), but 2-hydroxymyristate is not made (28).

The stimulation of *S*-2-hydroxymyristate biosynthesis at low Mg^{2+} concentrations and its absence in PhoP null mutants suggest a function for 2-hydroxylation in pathogenesis (22, 29). Although 2-hydroxy fatty acids have been used as taxonomic markers (28, 30), the enzymatic pathway for the biosynthesis of the *S*-2-hydroxymyristate moiety in lipid A of *S. typhimurium* and other organisms is unknown. Early studies of hydroxyacyl composition, conducted prior to the elucidation of the covalent structure and biosynthesis of lipid A, suggested that the 2-OH (but not the 3-OH) groups of *Pseudomonas* might be derived from O_2 (31, 32).

We now show that the presence of the 2-hydroxymyristate residue in *S. typhimurium* lipid A is O_2-dependent and that $^{18}O_2$ is directly incorporated into 2-hydroxymyristate-containing lipid A. We also report the discovery, cloning, and heterologous expression of a novel gene from *S. typhimurium*, designated *lpxO*, encoding a 302-amino acid polypeptide with significant sequence similarity to mammalian aspartyl/asparaginyl β-hydroxylase, an Fe^{2+}/α-ketoglutarate-dependent dioxygenase (33, 34). LpxO shares more subtle structural features with other Fe^{2+}/α-ketoglutarate-dependent dioxygenases, including deacetoxycephalosporin C synthase from *Streptomyces clavuligerus* (35). Heterologous expression of *S. typhimurium lpxO* in *E. coli* K-12, which does not contain the gene, results in the aerobic biosynthesis of 2-hydroxymyristate-modified lipid A. The *lpxO* gene is highly homologous to a family of bacterial genes that may be responsible for the biosynthesis of 2-hydroxy fatty acids found in lipid A molecules of certain other Gram-negative pathogens, including *Klebsiella pneumonia*, *P. aeruginosa*, *Bordetella pertussis*, *Legionella pneumophila*, and all types of *Salmonella*. The release of 2-hydroxyacyl chains from lipid A within phagolysosomes, known to be catalyzed by mammalian acyloxyacyl hydrolase (36), might allow animal cells to synthesize 2-hydroxyacyl-coenzyme A species, some of which are very potent inhibitors of protein *N*-myristoylation (37).

EXPERIMENTAL PROCEDURES

Materials—$^{32}P_i$ was purchased from NEN Life Science Products. $^{18}O_2$ (97% isotopic enrichment) was purchased from Isotec. Pyridine, methanol, 88% formic acid, and KH_2PO_4 were from Mallinckrodt, while chloroform, KCl, and $(NH_4)_2SO_4$ were purchased from EM Science. MES buffer and sodium fumarate were from Sigma. A dissolved oxygen test kit was purchased from Lamotte. Glass-backed Silica Gel thin layer chromatography plates (0.25 mm) were obtained from Merck. Stainless steel tubing and brass fittings used in the $^{18}O_2$ delivery system were from Supelco.

Bacterial Strains—All bacterial strains used in this study are described in Table I. *E. coli* XL1-BlueMR was from Stratagene. *S. typhimurium* (CS14028) was obtained from ATCC. The *S. typhimurium* phoP^c (CS022) strain was kindly provided by Dr. Samuel I. Miller (University of Washington) (38). Except where stated, bacterial shaking cultures were grown at 37 °C in LB medium containing 10 g of Tryptone, 5 g of yeast extract, and 10 g of NaCl per liter (39). When needed, the concentration of ampicillin was 100 μg/ml.

Isolation of Genomic DNA from S. typhimurium—Genomic DNA was isolated according to the method of Meade *et al.* (40). Briefly, 4 ml of an overnight culture of *S. typhimurium* (CS14028) was centrifuged and resuspended in 2 ml of TE buffer (41). A 100-μl portion of 2 mg/ml lysozyme stock solution was added, and the mixture was incubated at 37 °C for 15 min. Next, 180 μl of 10% SDS, 45 μl of 20 mg/ml proteinase K solution, and 3 μl of 500 μg/ml RNase were added. The final mixture was incubated an additional 1 h at 37 °C. The solution was then transferred to a glass vial, and 2.5 ml of chloroform/phenol/isoamyl alcohol (25:24:1, v/v/v) was added. The tube was inverted gently 15–20 times and centrifuged briefly at room temperature to separate the phases. The lower phase was removed, and the upper phase was reextracted three times with fresh lower phase. After the third extraction, the upper phase was transferred into a fresh tube and extracted six times (until the interface was clear) with chloroform/isoamyl alcohol (24:1, v/v). Finally, 150 μl of 3 M sodium acetate at pH 5.0 and 4 ml of 100% ethanol were added to the final upper phase. The DNA was allowed to precipitate at −20 °C overnight. The precipitate was collected by centrifugation at 4 °C for 5 min at 4000 rpm in a Beckman JS4.3 rotor. The pellet was air-dried, redissolved in 300 μl of TE buffer, checked for purity based on the A_{280}/A_{260} ratio, and stored at −20 °C.

Cloning of lpxO from S. typhimurium Genomic DNA—Primers corresponding to the 5′- and 3′-ends of the *lpxO* open reading frame coding for the putative lipid A 2-hydroxylase were designed as follows: for the 5′-end, BHyd5 (5′-CCGCCGGAATTCCATATGTTCGCAGCAATCATTATCGG-3′); for the 3′-end, BHyd3 (5′-CCGCTCGAGTCAGAGGAGGCTGAAAAGGAT-3′). The *lpxO* open reading frame was amplified by polymerase chain reaction from genomic DNA under the following conditions: 50-μl total reaction volume, 200 nM each primer, 200 μM dNTPs, 1.5 μg of genomic DNA, 4 mM $MgCl_2$, 2.5 units of *Pfu* DNA polymerase (Stratagene) with buffers supplied by the manufacturer. The temperature program was as follows: 94 °C for 7 min, a cycle of 45 s each at 94, 50, and 72 °C repeated 25 times, followed by 7 min at 72 °C. The polymerase chain reaction product and the pBluescriptSK vector DNA were then digested with *Eco*RI and *Xho*I, gel-purified with the QIAEX-II kit (Qiagen), and ligated together with T4 DNA ligase (Life Technologies, Inc.) to form pHSG1, which was transformed into $CaCl_2$-competent cells of *E. coli* strain XL1-BlueMR. Plasmid DNA from the resulting colonies (selected on ampicillin) was prepared from overnight cultures in LB with 100 μg/ml ampicillin (QIAQUICK Spin Miniprep kit, Qiagen) and screened by restriction enzyme analysis, using *Eco*RI and *Xho*I. The nucleotide sequence of the final construct was confirmed from four candidate clones at the Duke University Nucleotide Sequencing Core Facility using the T7 promoter and T3 promoter primers to the pBluescriptSK plasmid (Stratagene).

$^{32}P_i$ Labeling of E. coli and S. typhimurium Cultures—Radioactive labeling of lipid A species was performed as described previously (27) at 5 μCi of $^{32}P_i$/ml of culture medium. *E. coli* was grown on LB broth (39), and *S. typhimurium* was grown on minimal G56F (see below) with 10 mM $MgCl_2$. After extraction and 100 °C hydrolysis at pH 4.5 (27), 2000 cpm of the released lipid A species were spotted onto a 10 × 20-cm silica gel TLC plate, which was developed in chloroform/pyridine/88% formic

acid/water (50:50:16:5, v/v/v/v). The plates were dried, and separated lipid A species were visualized by overnight exposure to a PhosphorImager screen (Molecular Dynamics, Inc., Sunnyvale, CA).

Anaerobic Growth of S. typhimurium—*S. typhimurium* cells were grown in low phosphate G56 minimal medium (42) supplemented with fumarate (designated G56F). This medium contains 45 mM MES, pH 7.3, 0.3 mM KH_2PO_4, 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 10 mM glucose, 40 mM sodium fumarate, 10 mM $MgCl_2$, and 0.4% casamino acids. To exclude oxygen, sterile 50-ml polypropylene centrifuge tubes were filled to the top with culture medium and inoculated using a 1:500 dilution of an overnight culture of *S. typhimurium* phoP^c CS022 grown on LB broth. The tubes were tightly capped and incubated at 37 °C without shaking. The concentration of dissolved oxygen was determined at 1-h intervals using the azide-modified Winkler titration (Lamotte) (43). After 1 h of growth under these conditions, no dissolved oxygen was detected. For lipid A preparations from anaerobic cells, four 50-ml culture tubes were used. Aerobic cultures were grown in 200 ml of the same medium in a 1-liter culture flask at 37 °C with shaking at 200 rpm.

Isolation of the Lipid A 1,4′-Bisphosphate Components of CS022 by Chromatography on DEAE-cellulose—In our protocol, 200 ml of bacteria grown either aerobically or anaerobically to $A_{600} \sim 0.6$ were harvested by centrifugation at 4 °C. Each cell pellet was resuspended in 80 ml of phosphate-buffered saline to which was added 100 ml of chloroform and 200 ml of methanol to make a single phase Bligh/Dyer mixture (44). After extraction of the glycerophospholipids and centrifugation, crude lipid A components were released from the Bligh/Dyer insoluble pellet by hydrolysis at pH 4.5 in the presence of SDS (27, 45). To purify the major lipid A 1,4′-bisphosphate species (lacking the L-Ara4N or pEtN substituents), the crude lipid A samples from CS022 were fractionated based on charge using DEAE-cellulose column chromatography (27, 45). Typically, the crude lipid A from a 200-ml culture was redissolved in 5 ml of chloroform/methanol/water (2:3:1, v/v/v) with the aid of a bath sonicator. A 1-ml DEAE-cellulose column in the acetate form (Whatman DE52) (46) was prepared and washed with 20 ml of chloroform/methanol/water (2:3:1, v/v/v) prior to loading the sample. After application of the sample at the natural flow rate, the column was eluted with increasing concentrations of ammonium acetate (45). The major, unmodified lipid A 1,4′-bisphosphate components emerged with chloroform/methanol/240 mM aqueous ammonium acetate (2:3:1, v/v/v) (45). The desired components were detected by thin layer chromatography in the solvent chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v) and charring either with 10% sulfuric acid in ethanol or with ethanol/*p*-anisaldehyde/H_2SO_4/acetic acid (89:2.5:4:1, v/v/v/v) (45, 47). Fractions containing the lipid A 1,4′-bisphosphate components were pooled and converted to two-phase Bligh/Dyer systems by the addition of the appropriate amounts of chloroform and water. The lower phases were collected, dried under nitrogen, and stored at −80 °C.

Purification of Unmodified and 2-Hydroxymyristate-modified Lipid A from E. coli Cells Expressing S. typhimurium lpxO—Crude lipid A species from 1 liter of *E. coli* XL1-BlueMR(pHSG1) grown on LB medium to $A_{600} \sim 2.0$ were extracted following pH 4.5 hydrolysis as described above. Total lipid A 1,4′-bisphosphates were first prepared by chromatography on a 2-ml DEAE-cellulose column, equilibrated and eluted proportionally as described above. To separate the lipid A species with the *S*-2-hydroxymyristate residue at the 3′-acyloxyacyl position from the unmodified lipid A, half of the DEAE-purified sample was redissolved in ~100 μl of chloroform/methanol (4:1, v/v) and spotted in 2-μl portions along the origins of two 20 × 20-cm silica gel TLC plates. The plates were then developed in chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v). As the plates were drying at room temperature, two closely migrating lipid A species were resolved and could be seen transiently as distinct white zones on the plates. The more slowly migrating component contains the 2-hydroxymyristate substituent. Both of the zones were marked with a pencil. The plates were dried for another ~20 min to remove solvents. After moistening the surface with a water spray, the silica within the zones was scraped off with a scalpel, and the chips were collected in separate glass tubes. The chips in each tube were extracted four times with 6-ml portions of chloroform/methanol/50 mM aqueous ammonium acetate adjusted to pH 1.5 with HCl (1:2:0.8, v/v/v). Following each extraction, large chips were removed by low speed centrifugation, and the supernatants were passed through a column of glass wool in a Pasteur pipette. The filtered samples were converted into two-phase Bligh/Dyer systems, consisting of chloroform/methanol/water (2:2:1.8, v/v/v), by the addition of appropriate amounts of water and chloroform. The pooled lower phases were neutralized with 24 drops of high pressure liquid chromatography grade pyridine, cleared by adding 30 drops of methanol, and evaporated under nitrogen. To remove any remaining fine silica particles, each of the lipid A components was redissolved in 5 ml of chloroform/methanol/water (2:3:1, v/v/v) and purified over another 2-ml DEAE-cellulose column, as described above. The intact lipids were analyzed by TLC and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Fatty acid compositions were determined by preparation of methyl esters, which were resolved by gas chromatography and detected with flame ionization.

$^{18}O_2$ Labeling of Lipid A—The procedure for growing bacteria in an $^{18}O_2$ atmosphere was adapted from that described for *Pseudomonas ovalis* by Kawahara and co-workers (32). A sealed 1-liter culture flask, containing 200 ml of LB medium inoculated by 1:100 dilution from an overnight culture of the PhoP-constitutive *S. typhimurium* mutant CS022, was evacuated to 27 inches of mercury, as determined on a Marsh Instrument Co. model 1305-12 in line vacuum gauge. The flask was flushed with nitrogen and reevacuated twice to eliminate residual oxygen in the culture medium. Following a third application of the vacuum to 27 inches of mercury, the headspace was brought to 20 inches Hg with $^{18}O_2$ and then to ambient pressure with nitrogen (resulting in an atmosphere of approximately 26% $^{18}O_2$ at 97% isotopic enrichment). The sealed flask was removed from the apparatus and shaken at 37 °C and 220 rpm for 4 h until the culture reached an A_{600} of ~0.8, after which the lipid A 1,4′-bisphosphate species were isolated as described above. A control culture was prepared by sealing a flask that was not evacuated immediately after inoculation, so that the bacteria could grow under otherwise identical conditions with ambient air.

Fatty Acid Compositions of Purified Lipid A Components—Purified lipid A samples were sent to Microbial ID, Inc. (Newark, DE) for fatty acid analysis according to standard procedures. In brief, samples were esterified in 2 M methanolic HCl, extracted into petroleum ether, and concentrated. Next, 2-μl samples were injected with a 100:1 split onto a gas chromatography column. Fatty acid methyl esters were detected by flame ionization, and their elution times were compared with a mixture of known fatty acid methyl ester standards run prior to analysis. Retention times were analyzed by the Sherlock software package. Peaks appearing outside of a defined retention time window were labeled as unknowns.

MALDI-TOF Mass Spectrometry—Spectra were acquired in the negative ion linear mode by using a Kratos Analytical (Manchester, United Kingdom) MALDI-TOF mass spectrometer equipped with a 337-nm nitrogen laser, a 20-kV extraction voltage, and time-delayed extraction. Each spectrum was the average of 50 shots. The matrix was a mixture of saturated 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v). The lipid A samples were dissolved in a mixture of chloroform/methanol (4:1 v/v) and mixed with the matrix on a slide. The sample mixtures were allowed to dry at room temperature prior to mass analysis. Hexa-acylated lipid A 1,4′-bisphosphate from wild-type *E. coli* (purchased from Sigma) was used as an external standard for calibration.

RESULTS

Effects of O_2 on Growth and Biosynthesis of Lipid A Species in Salmonella CS022—*S. typhimurium* CS022 is PhoP-constitutive (38) and efficiently incorporates L-Ara4N, pEtN, palmitate, and 2-hydroxymyristate moieties into its lipid A (Fig. 1*B*), even in the presence of 10 mM $MgCl_2$ in the medium. To test the effects of O_2 on the formation of 2-hydroxymyristate-containing lipid A, CS022 cells were grown either aerobically or anaerobically in 200 ml of G56F medium. The cells reached an A_{600} of ~0.8 after 6 h of anaerobic growth. Under these conditions, approximately 90% of the cell mass was generated after all measurable O_2 had been depleted from the medium (data not shown).

Lipid A was isolated from solvent-extracted cells by hydrolysis at 100 °C in SDS at pH 4.5. To simplify the interpretation of the mass spectra, the crude lipid A released from the cells was first fractionated on DEAE-cellulose columns (data not shown). Only those lipid A species containing unsubstituted 1- and 4′-bisphosphate moieties, which elute with chloroform/methanol/240 mM aqueous ammonium acetate (2:3:1, v/v/v) (27), were analyzed further. Two major and one minor lipid A 1,4′-bisphosphate species were resolved from cells grown in the presence of O_2 by TLC (Fig. 2, *lane 1*). The slowly migrating

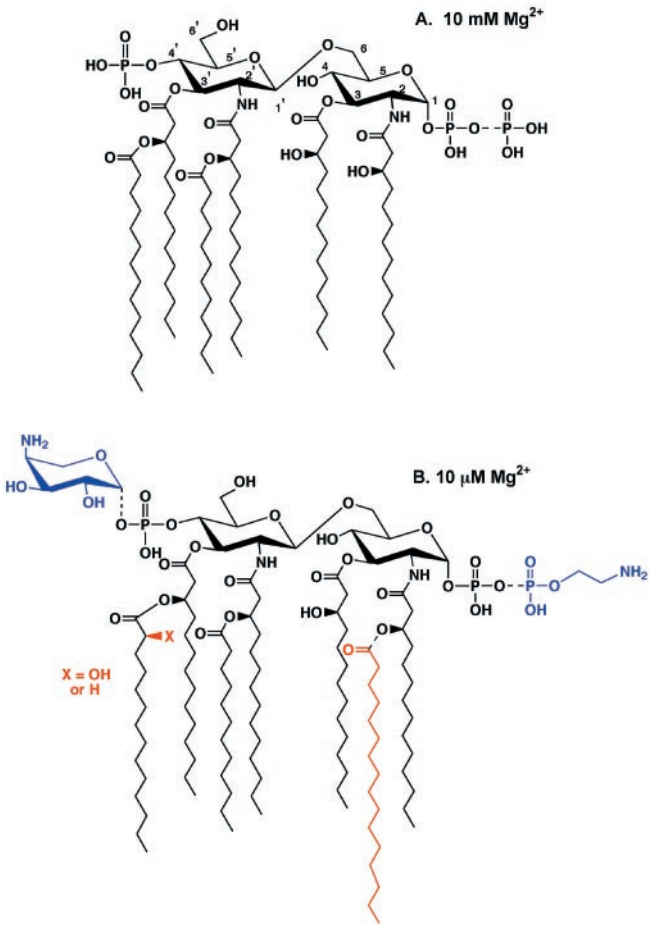

FIG. 1. **Regulated covalent modifications of lipid A in *S. typhimurium*.** *A*, *E. coli* lipid A isolated from cells grown in LB medium or in the presence of 1–10 mM Mg^{2+} consists mostly of a 1,4′-bisphosphate species bearing six acyl chains. About one-third of the *E. coli* lipid A is recovered as the 4′-phosphate/1-pyrophosphate, as indicated by the *dashed line* (27, 76). Lipid A species are released from cells (or purified LPS) by hydrolysis at pH 4.5 in the presence of SDS at 100 °C (27, 76). The same 1,4′-bisphosphate is also a major component of *S. typhimurium* lipid A, but it may be modified with additional substituents (see below), the biosynthesis and attachment of which are regulated by the PhoP/PhoQ (*red*) and PmrA/PmrB (*blue*) two-component systems (22, 26). The pyrophosphate variant is seen only in $PhoP^-$ *S. typhimurium* grown at high Mg^{2+} concentrations (Z. Zhou and C. R. H. Raetz, unpublished observations). *B*, *S. typhimurium* lipid A becomes heavily modified with covalently attached pEtN and/or L-Ara4N moieties, as indicated, when grown at low Mg^{2+} concentrations (~10 μM), a condition that resembles the intraphagosomal environment and activates PhoP/PhoQ (22). In addition, the lipid A is modified by incorporation of an extra palmitoyl chain and/or a 2-hydroxymyristoyl group (in place of myristate) at the 2- and 3′-acyloxyacyl positions, respectively (22, 24). Different combinations of these substituents account for the 16 or more lipid A molecular species seen in PhoP-constitutive *S. typhimurium* mutants. Minor forms exist in which the pEtN and L-Ara4N groups are attached in the opposite manner (not shown), or in which two pEtN or two L-Ara4N groups are present (Z. Zhou and C. R. H. Raetz, manuscript in preparation).

major component (*A*) was not seen when lipid A was prepared from CS022 cells grown without O_2 (Fig. 2, *lane 2*).

Mass Spectrometry of the Lipid A 1,4′-Bisphosphates Isolated from S. typhimurium CS022 Grown with or without O_2—Negative mode MALDI-TOF mass spectrometry of the lipid A 1,4′-bisphosphates, isolated by DEAE chromatography from aerobically grown cells, showed major peaks at *m/z* 1796.9 and 1813.0 (Fig. 3*A*). The peak at *m/z* 1796.9 is seen in lipid A of both *E. coli* and *S. typhimurium*. It corresponds to $[M - H]^-$ of a hexa-acylated lipid A 1,4′-bisphosphate bearing laurate and myristate as secondary acyl chains at positions 2′ and 3′, respectively (Fig. 1*A*). The peak at *m/z* 1813.0 is 16.1 atomic mass units higher, and it presumably corresponds to $[M - H]^-$ of the species bearing a secondary 2-hydroxymyristoyl chain in place of myristate at position 3′, as is seen in PhoP-constitutive *S. typhimurium* (Fig. 1*B*). In addition, minor species are observed

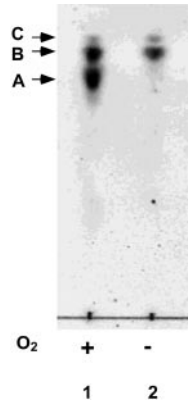

FIG. 2. **Effect of anaerobic growth on composition of lipid A 1,4′-bisphosphate species in a PhoP-constitutive strain of *S. typhimurium*.** The unmodified lipid A 1,4′-bisphosphate fraction was isolated by chromatography on DEAE-cellulose from PhoP-constitutive *S. typhimurium* CS022 cells, grown in the presence (*lane 1*) or the absence (*lane 2*) of oxygen. DEAE-cellulose-purified lipid A (2–5 μg) was spotted onto a TLC plate, which was developed in chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v). Lipids were visualized by spraying the plate with 10% sulfuric acid in ethanol and charring on a hot plate. Based on previous studies and mass spectrometry (27), lipid A species could be identified tentatively as hepta-acylated lipid A 1,4′-bisphosphate (*C*), hexa-acylated lipid A 1,4′-bisphosphate (*B*), and hexa-acylated lipid A 1,4′-bisphosphate(s) bearing the 2-hydroxymyristate substituent (*A*). Further evidence for this is presented below.

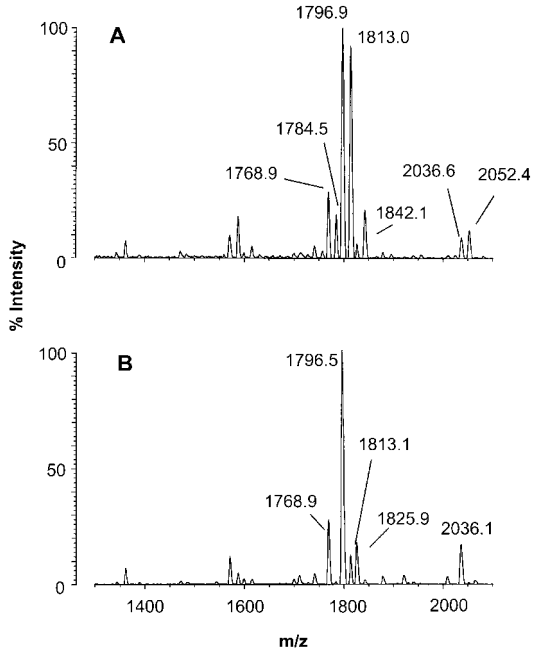

FIG. 3. **Mass spectrometry of lipid A 1,4′-bisphosphates from a PhoP-constitutive mutant of *S. typhimurium* grown with or without O_2.** *A*, lipid A species isolated from aerobically grown bacteria. The peaks at *m/z* 1796.9 and 1813.0 are interpreted as $[M - H]^-$ of the hexa-acylated lipid A 1,4′-bisphosphates. The latter contains an extra oxygen atom, presumably as part of the 2-hydroxymyristate residue. The peaks at *m/z* 2036.6 and 2052.4 represent $[M - H]^-$ of the corresponding hepta-acylated species, bearing an additional palmitate chain. *B*, lipid A species isolated from an anaerobic culture. The peaks at *m/z* 2052.4 and 1813.0 are significantly diminished in size, compared with *A*, suggesting a greatly reduced rate of biosynthesis of 2-hydroxymyristate in the absence of oxygen.

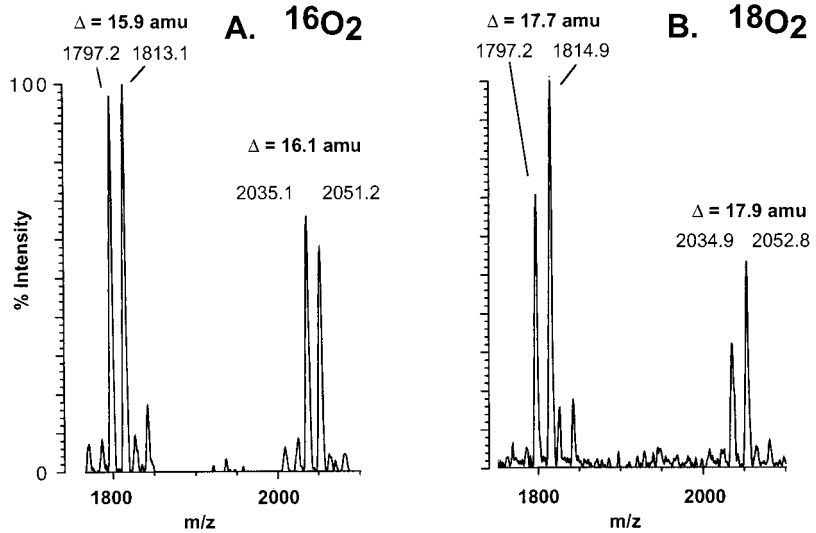

FIG. 4. **$^{18}O_2$ labeling of the lipid A 1,4′-bisphosphate species in a PhoP-constitutive mutant of *S. typhimurium*.** MALDI-TOF mass spectrometry was used to analyze the DEAE-cellulose-purified lipid A 1,4′-bisphosphates isolated from 200-ml cultures of CS022, grown in a sealed 1 liter flask containing either air (*A*) or a mixture of 26% $^{18}O_2$ (97% isotope-enriched) and 74% N_2 (*B*). An increase of ~2 atomic mass units in the difference in the masses (Δ) between the myristate and the 2-hydroxymyristate-containing lipid A species is seen when the cells are grown on $^{18}O_2$. This increase in Δ under the labeling conditions with $^{18}O_2$ demonstrates unequivocally the direct incorporation of molecular oxygen into the 2-hydroxymyristate-containing lipid A species. There is no effect of $^{18}O_2$ on the masses of the lipid A species lacking 2-hydroxymyristate, indicating that all other oxygen atoms in lipid A of *S. typhimurium* are derived from water.

at *m/z* 2036.6 and 2052.4 (Fig. 3*A*). These are interpreted as corresponding to $[M - H]^-$ of hepta-acylated lipid A species that are identical to those at *m/z* 1796.9 and 1813.0, respectively, except that they are further acylated with a palmitoyl substituent at position 2 (Fig. 1*B*). Lipid A 1,4′-bisphosphates isolated from anaerobic cultures revealed strong peaks $[M - H]^-$ at *m/z* 1796.5 and at 2036.1, corresponding to the hexa- and hepta-acylated lipid A components without the 16-atomic mass unit substituent. The fact that peaks at *m/z* 1813.0 and 2052.4 are greatly reduced in lipid A prepared from anaerobically grown cells suggests that the 2-OH group of the 2-hydroxymyristate moiety is indeed O_2-derived.

Positive mode MALDI-TOF mass spectrometry is consistent with the results shown in Fig. 3 and confirms that the extra oxygen (when present) is located on the distal glucosamine unit (data not shown). Gas chromatography of the fatty acid methyl esters prepared from the DEAE-cellulose-purified lipid A 1,4′-bisphosphates confirmed the presence of 2-hydroxymyristate in the aerobically grown cells and its absence in anaerobic cells (data not shown).

$^{18}O_2$ Labeling of the S. typhimurium Lipid A 1,4′-Bisphosphates—To confirm the hypothesis that molecular oxygen is directly incorporated into the 2-hydroxymyristoyl chain of *S. typhimurium* lipid A, CS022 cells were grown in the presence of $^{18}O_2$. In a procedure adapted from an earlier study with *P. ovalis* (32), 200-ml LB broth cultures of CS022 were grown at 37 °C in sealed 1-liter Erlenmeyer flasks with either ambient air or a mixture of 26% $^{18}O_2$ and 74% N_2 in the headspace. The cells were harvested at A_{600} ~ 0.8. The lipid A 1,4′-bisphosphates were purified by DEAE chromatography and analyzed by MALDI-TOF mass spectrometry. In the negative mode, the mass difference (Δ) between the hydroxylated and nonhydroxylated lipid A species of the culture grown with ambient air (Fig. 4*A*) was 15.9 atomic mass units for the hexa-acylated and 16.1 atomic mass units for the hepta-acylated subtypes, respectively. When grown under $^{18}O_2$, the difference (Δ) increased to 17.7 atomic mass units and 17.9 atomic mass units, respectively (Fig. 4*B*), as expected if molecular O_2 is incorporated into the 2-OH group.

A Homologue of Bovine Aspartyl/Asparaginyl β-Hydroxylase in S. typhimurium and Some Other Gram-negative Bacteria—Several cloned, well characterized dioxygenases, representing different mechanistic families including cytochrome P450-, dinuclear non-heme iron-, and Fe^{2+}/α-ketoglutarate-dependent enzymes, were used as probes in tBLASTn searches (48) against the available microbial genome data bases. The rationale was to identify a unique class of dioxygenase homologues in those bacteria that synthesize 2-hydroxyacyl modified lipid A (28, 31, 49–52). A tBLASTn search with the protein sequence of bovine aspartyl/asparaginyl β-hydroxylase (33, 34), a type of Fe^{2+}/α-ketoglutarate-dependent dioxygenase, as the probe fit this criterion. When compared by tBLASTn with the *S. typhimurium* genome, the catalytic domain of bovine aspartyl/asparaginyl β-hydroxylase revealed significant similarity (*E* value of $\sim 10^{-10}$) to a previously unidentified open reading frame on the *S. typhimurium* chromosome, mapping next to *fdhF* at 92.8 min (53). This novel gene (designated *lpxO*) codes for a putative 302-amino acid polypeptide (Fig. 5) with hydrophobic N- and C-terminal sequences (Fig. 5, *shading*). The predicted LpxO amino acid sequence shares several important features with the aspartyl/asparaginyl β-hydroxylase catalytic domain, including four conserved histidine side chains (*red*) and several aspartate and glutamate residues (*blue*). Histidine 675 of bovine aspartyl/asparaginyl β-hydroxylase (Fig. 5, *arrow*) has in fact been shown to be an iron ligand (34) and is conserved. LpxO also contains a His-*X*-Asp-$X_{\sim 50}$-His motif (Fig. 5, *yellow*) (54), resembling the Fe^{2+} binding site deacetoxycephalosporin C synthase (35). The latter is the only Fe^{2+}/α-ketoglutarate-dependent dioxygenase for which a high resolution crystal structure is available (35). The His-*X*-Asp-$X_{\sim 50}$-His motif is also present in TfdA (55) and TauD (56), two additional bacterial Fe^{2+}/α-ketoglutarate-dependent dioxygenases.

The GC content of *lpxO* and its flanking DNA does not deviate from the *S. typhimurium* average of 52%. It is therefore unlikely that *S. typhimurium lpxO* resides within a pathogenicity island. As noted above, however, homologues of aspartyl/asparaginyl β-hydroxylase are seen in other bacteria that synthesize 2-hydroxyacyl chains (Table II), including *Salmonella typhi, Salmonella paratyphi*, *K. pneumonia, B. pertussis*, *Bordetella bronchiseptica*, *P. aeruginosa*, *Pseudomonas putida*,

FIG. 5. **Bioinformatic identification of LpxO, a putative hydroxylase involved in lipid A biosynthesis, in *S. typhimurium*.** To find LpxO, tBLASTn searches (48, 58) against the available microbial genomes were conducted using well characterized protein sequences of various cloned dioxygenases as probes. Only one enzyme, the aspartyl/asparaginyl β-hydroxylase of animal cells, a type of Fe^{2+}/α-ketoglutarate-dependent dioxygenase, yielded a distinct family of homologues restricted to those bacteria known to make 2-hydroxyacylated lipid A species. LpxO of *S. typhimurium* shown above displayed an *E* value of $\sim 10^{-10}$ against the catalytic domain of bovine aspartyl/asparaginyl β-hydroxylase. The proposed histidine 675 iron ligand of the bovine aspartyl/asparaginyl β-hydroxylase is indicated by the *arrow* and is present at position 155 in *S. typhimurium* LpxO and in the other bacterial homologues (not shown). *Red letters* show other conserved histidine residues, while *blue letters* indicate conserved aspartate and glutamate residues, some of which might function as iron ligands. Hydrophobic segments are *shaded* in *gray*, whereas the putative His-*X*-Asp-$X_{\sim 50}$-His motif (54, 57), also seen in the other bacterial LpxO homologues, is *shaded* in *yellow*.

TABLE II
A distinct family of S. typhimurium LpxO homologues in the NCBI microbial data base

Homologues in the NCBI Microbial Genomes Blast Databases (available on the World Wide Web) were identified with the tBLASTn algorithm (48, 58), using the predicted S. typhimurium LpxO protein sequence as the probe. *S. paratyphi* and *Salmonella enteritidis* contain single homologues (not shown) that are essentially the same as the one in *S. typhi*.

Organism (gene no. if >1)	Homology[a]	*E* values
S. typhi	300/302/302	10^{-178}
K. pneumonia	180/220/290	10^{-104}
P. aeruginosa (1)	169/216/295	6×10^{-99}
P. aeruginosa (2)	146/200/299	5×10^{-82}
P. putida (1)	166/217/294	5×10^{-99}
P. putida (2)	143/201/303	1×10^{-77}
B. pertussis	160/212/290	4×10^{-95}
B. bronchiseptica	160/212/290	4×10^{-95}
L. pneumophila	63/106/211	3×10^{-23}

[a] Homology is given as number identities/number positives/total number of amino acid residues compared with *S. typhimurium* LpxO, a hypothetical protein of 302 amino acid residues.

and *L. pneumophila*, all of which make 2-hydroxyacyl-containing lipid A species (28, 31, 49–52). *P. aeruginosa* contains two LpxO homologues, consistent with the fact that its lipid A contains two distinct 2-hydroxylaurate chains (50). The bacterial LpxO homologues are all closely related to each other (*E* values from $\sim 10^{-178}$ to 10^{-23} when probed with *S. typhimurium* LpxO), and are all about the same length. LpxO homologues are not present in Gram-positive bacteria or in Gram-negatives that do not make 2-hydroxyacylated lipid A. Despite the presence of the His-*X*-Asp-$X_{\sim 50}$-His motif (54, 57) in LpxO, which was noted by visual inspection (Fig. 5), PSI-BLAST searches (58) comparing *S. typhimurium* LpxO with the complete nonredundant data base failed to reveal significant similarity to other Fe^{2+}/α-ketoglutarate-dependent dioxygenase besides the mammalian aspartyl/asparaginyl β-hydroxylases.

Cloning of S. typhimurium lpxO and Expression in E. coli K12—The *lpxO* gene was amplified by polymerase chain reaction from genomic DNA prepared from wild type *S. typhimurium*. The polymerase chain reaction product, prepared with primers containing appropriate restriction sites, was digested with *Eco*RI and *Xho*I and ligated into pBluescriptSK so that expression of *lpxO* would be driven by the *lac* promoter. The resulting hybrid plasmid, pHSG1, was transformed into competent *E. coli* XL1-BlueMR. The correct coding sequence of the plasmid was confirmed by nucleotide sequencing in both directions.

Lipid A Hydroxylation in E. coli Cells Expressing S. typhimurium lpxO—Although not optimized for maximal expression of LpxO activity, the hybrid plasmid pHSG1 confers upon *E. coli* the ability to synthesize lipid A molecules containing 2-hydroxymyristate. ^{32}P-Labeled lipid A species purified from XL1-

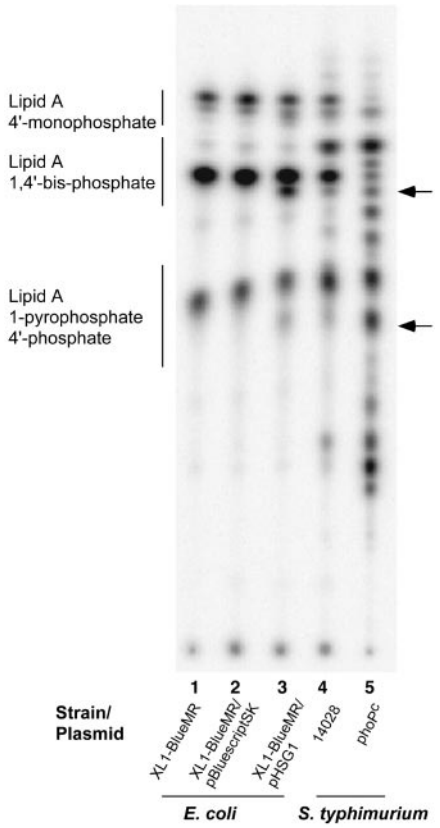

FIG. 6. **Expression of *S. typhimurium lpxO* in *E. coli* results in formation of a subset of new lipid A species also seen of *S. typhimurium*.** The *S. typhimurium lpxO* gene was cloned between the *Eco*RI and *Xho*I sites of pBluescriptSK and expressed behind the *lac* promoter in *E. coli*. Lipid A species were isolated from cells labeled with $^{32}P_i$ and separated by thin layer chromatography in chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v). After drying the plate, labeled lipid A species were detected with a PhosphorImager. *Lanes 1* and *2*, *E. coli* wild-type and vector controls, respectively. *Lane 3*, *E. coli* XL1-BlueMR expressing *S. typhimurium lpxO*. LpxO-dependent lipid A species are indicated with *arrows*. *E. coli* were grown in LB medium. *Lanes 4* and *5*, *S. typhimurium* 14028 and CS022, respectively, grown in G-56F minimal medium with 10 mM $MgCl_2$.

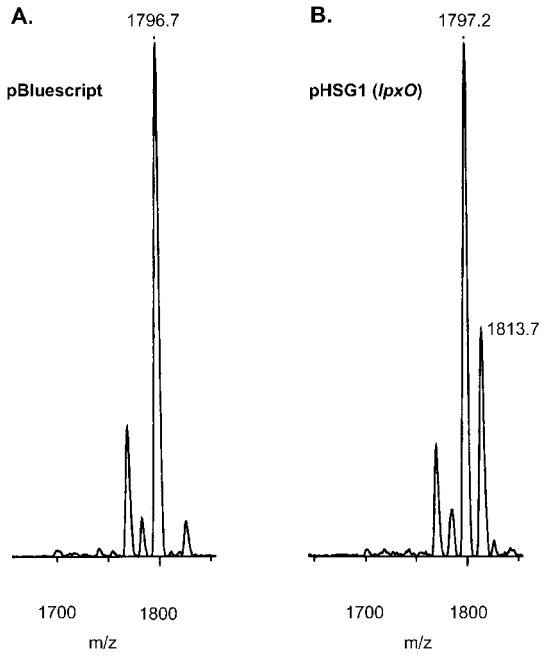

FIG. 7. **Heterologous expression of *S. typhimurium lpxO* induces the biosynthesis of a hydroxylated lipid A variant in *E. coli*.** MALDI-TOF mass spectrometry was used to analyze the lipid A 1,4′-bisphosphates extracted from *E. coli* XL1-BlueMR carrying either pBluescriptSK (*A*) or pHSG1, a pBluescript-derived hybrid plasmid bearing the *S. typhimurium lpxO* gene (*B*).

BlueMR containing either no vector, pBluescriptSK, or pHSG1 were analyzed by TLC and were compared with similarly radiolabeled lipid A species from *S. typhimurium* wild type or CS022 (Fig. 6). Lipid A from wild-type or vector control *E. coli* (*lanes 1* and *2*) exhibited only those spots characteristic of wild type *E. coli* K-12 grown under normal conditions, *i.e.* mostly the hexa-acylated lipid A 1,4′-bisphosphate with some 1-pyrophosphate and small amounts of 4′ monophosphate (a byproduct of hydrolysis). *E. coli* containing pHSG1 (*lane 3*) showed at least two additional species, indicated by the *arrows* on the *right*, that migrated more slowly than the lipid A species in the vector control. Interestingly, these pHSG1-dependent lipid A variants migrated at the same R-factors as certain bands in crude *S. typhimurium* lipid A prepared by pH 4.5 hydrolysis (Fig. 6, *lanes 4* and *5*).

The MALDI-TOF mass spectrometry of the DEAE-cellulose-purified lipid A 1,4′-bisphosphates from *E. coli* XL1-Blue containing pBluescriptSK shows a single major peak at *m/z* 1796.7 (Fig. 7*A*), typical of wild-type cells. Expression of the *Salmonella lpxO* on pHSG1, however, confers upon *E. coli* the ability to add an additional OH group to lipid A, as judged by the appearance of the peak at *m/z* 1813.7 (Fig. 7*B*).

Purification and Fatty Acid Composition of Nonhydroxylated and Hydroxylated Lipid A from E. coli Expressing S. typhimurium lpxO—For conclusive confirmation that *lpxO* encodes a protein that makes the 2-hydroxymyristate moiety of *S. typhimurium* lipid A, hydroxylated and nonhydroxylated lipid A species from *E. coli* cells expressing *lpxO* were separated from each other by preparative TLC and anion exchange chromatography, as described under "Experimental Procedures." The TLC plate of the purified lipid A species is shown in Fig. 8*A*. MALDI-TOF mass spectra of these species (Fig. 8, *B* and *C*) confirm their purity and identity. Gas chromatography of the corresponding fatty acid methyl esters (Fig. *8*, *insets*) unambiguously demonstrates the presence of 2-hydroxymyristate only in the slowly migrating hydroxylated lipid A sample with $[M - H]^-$ at *m/z* 1813.5, derived from *E. coli* expressing pHSG1. The appearance of the 2-hydroxymyristate group is accompanied by a corresponding decline in myristate content. The analysis excludes the formal possibility that LpxO incorporates a hydroxyl group at some site other than carbon 2 of the myristate chain of *E. coli* lipid A.

DISCUSSION

Although 2-hydroxy fatty acids have long been recognized as components of lipid A molecules in a select subset of Gram-negative bacteria (28, 31, 49–52), little is known about their biosynthesis and function (32). The recent discovery that formation of the 2-hydroxymyristate moiety in *S. typhimurium* lipid A is under the control of the PhoP/PhoQ two-component regulatory system raises the intriguing possibility that this substituent might play a role in pathogenesis (22). Although other lipid A modifying groups, such as L-Ara4N, pEtN, and C16:0 moieties, are similarly regulated (22) and are common to both *E. coli* and *S. typhimurium* (27), the 2-hydroxymyristate substituent is seen only in *S. typhimurium*. Mutants defective in PhoP/PhoQ do not make any of these substituents (22). Such strains are much less virulent than wild type and are susceptible to cationic anti-microbial peptides (24, 25). The biological functions of each of the lipid A modifying groups are difficult to assess, however, because the enzymes that catalyze their formation have not yet been identified.

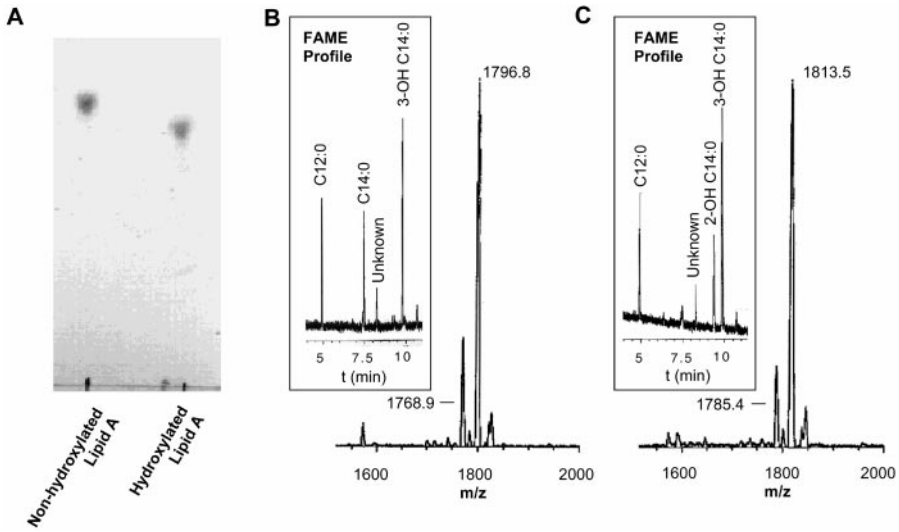

FIG. 8. **Mass spectrometry and fatty acid composition of nonhydroxylated and hydroxylated lipid A species from *E. coli* expressing *lpxO*.** The nonhydroxylated and the hydroxylated lipid A 1,4′-bisphosphate species derived from *E. coli* XL1-BlueMR expressing *S. typhimurium lpxO* were resolved by preparative thin layer chromatography, as described under "Experimental Procedures." *A* shows the thin layer analysis of ~5-μg samples of purified lipid A species developed with chloroform/pyridine/88% formic acid/water (50:50:16:5, v/v/v/v) and charred with 10% sulfuric acid in ethanol. *B* shows the MALDI-TOF mass spectrum of the nonhydroxylated lipid A 1,4′-bisphosphate, and *C* shows the MALDI-TOF mass spectrum of the hydroxylated lipid A 1,4′-bisphosphate. *Insets* show the profiles of the fatty acid methyl esters (*FAME*) derived from the purified lipid samples by gas chromatography with flame ionization detection. The analyses clearly indicate the presence of 2-hydroxymyristate only in the hydroxylated lipid A species at the expense of myristate. Fatty acid methyl esters were identified by their retention times in comparison with standards. An unknown component is labeled with an *asterisk*.

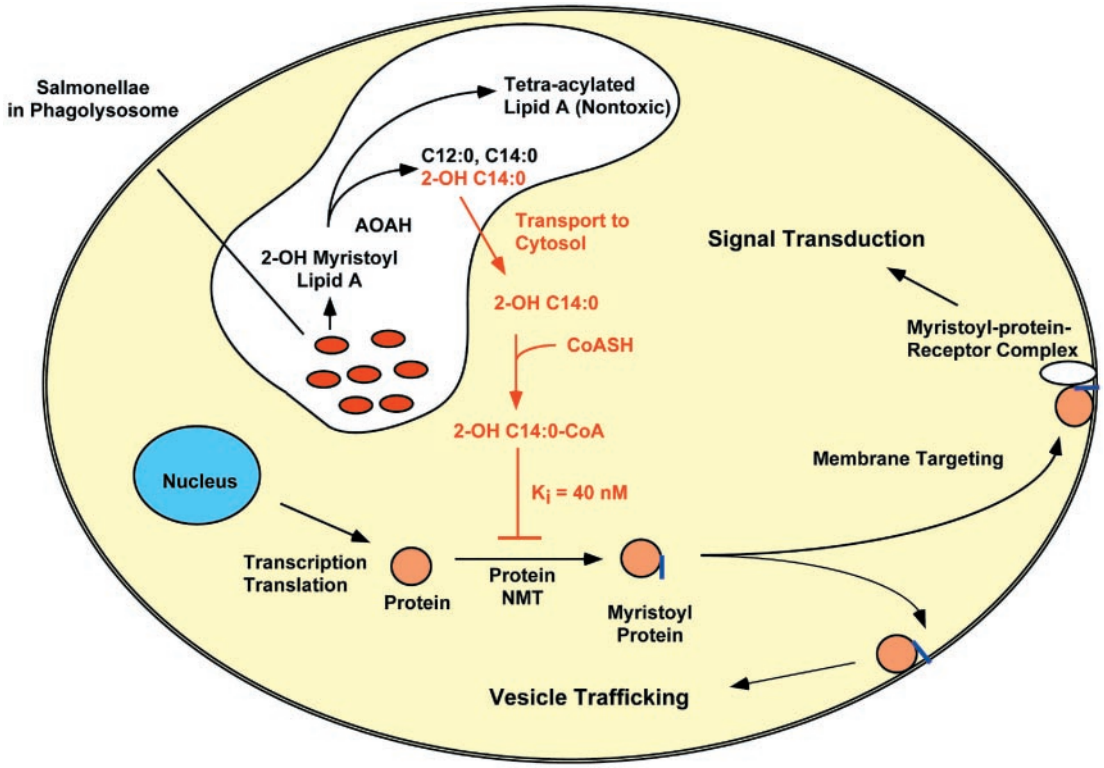

FIG. 9. **A "Trojan horse" model for release of the 2-hydroxymyristate moiety and inhibition of host cell signaling.** The *diagram* represents a macrophage or epithelial cell harboring *S. typhimurium* in its phagolysosome. Bacteria are represented as *red ovals*. *AOAH*, acyloxyacyl hydrolase; *Protein NMT*, myristoyl-coenzyme A/protein *N*-myristoyltransferase. The normal pathway of protein myristoylation is shown with *black arrows*, while the effect of the *S. typhimurium* Trojan horse is shown with *red arrows*. Given that the 2-hydroxymyristate substituent can probably be released in animal cells from *S. typhimurium* lipid A by the action of acyloxyacyl hydrolase (36), it is plausible that it might be converted to 2-hydroxymyristoyl coenzyme A, a very potent inhibitor of protein *N*-myristoyl transferase (37). The consequence might be suppression of host cell signaling functions, permitting a more prolonged survival of the bacteria inside the host cell.

We have now established that the formation of the 2-hydroxymyristate moiety in *S. typhimurium* lipid A is oxygen-dependent, as is the case for 2-hydroxylaurate in *P. ovalis* (32). When *S. typhimurium* cells are grown under anaerobic conditions, myristate is incorporated into lipid A in place of 2-hydroxymyristate. The 2-hydroxymyristate residue is probably generated by hydroxylation of the myristate chain after its incorporation into nascent lipid A by MsbB (13, 59). However,

we cannot yet exclude the alternative possibility that 2-hydroxymyristate is formed while still attached to acyl carrier protein, *i.e.* by hydroxylation of myristoyl-acyl carrier protein. Development of an *in vitro* assay should resolve this question.

We used a bioinformatic approach to identify the putative hydroxylase responsible for the formation of the 2-hydroxymyristoyl moiety of *S. typhimurium* lipid A. We first probed all of the available microbial genomic data bases for uncharacterized open reading frames with sequence similarity to well characterized, cloned dioxygenases, such as selected P450-, dinuclear iron-, and Fe^{2+}/α-ketoglutarate-dependent hydroxylases. Those dioxygenases with homologues in bacteria known to make 2-hydroxyacylated lipid A species were considered further. Of the various dioxygenases used to search the microbial data bases, only the mammalian aspartyl/asparaginyl β-hydroxylase yielded the desired pattern. This enzyme hydroxylates certain aspartyl and asparaginyl residues in clotting factors and other proteins (60, 61). Aspartyl/asparaginyl β-hydroxylase (33, 34, 60, 61) belongs to the larger family of Fe^{2+}/α-ketoglutarate-dependent dioxygenases, which include prolyl and lysyl hydroxylases (62), deacetoxycephalosporin C synthase (35), taurine hydroxylase (56), and thymine hydroxylase (63, 64). In a single tBLASTn search (48, 58), however, only the aspartyl/asparaginyl β-hydroxylase (33, 34) produced significant matches with the relevant bacterial species (*K. pneumonia*, *P. aeruginosa*, *P. putida*, *B. bronchiseptica*, *L. pneumophila*, and all types of *Salmonella*) (Table II).

The bovine aspartyl/asparaginyl β-hydroxylase and *S. typhimurium* LpxO share several important conserved amino acid residues (Fig. 5). Of these, His^{155} of the *S. typhimurium* LpxO is of special interest, since it corresponds to His^{675} of bovine aspartyl/asparaginyl β-hydroxylase (Fig. 5), a residue identified by site-directed mutagenesis to be critical for iron binding and catalysis (34). The His-*X*-Asp-$X_{\sim 50}$-His motif (Fig. 5, *yellow shading*), a structural feature of many non-heme iron active sites (54, 57), appears to be present as well in *S. typhimurium* LpxO (Figs. 5) and all other bacterial LpxO homologues, with the possible exceptions of *Legionella* LpxO. Its presence in *S. typhimurium* LpxO was recognized by visual inspection. This motif is well characterized in the crystal structure of deacetoxycephalosporin C synthase, in which the two His residues and one Asp residue of the motif function as iron ligands (35). The crystal structure of the bovine aspartyl/asparaginyl β-hydroxylase itself has not yet been solved.

The ability of the *S. typhimurium lpxO* gene to enable the biosynthesis of 2-hydroxymyristate-modified lipid A in *E. coli* (Figs. 7 and 8) strongly suggests that *lpxO* is the structural gene for a novel membrane bound, α-ketoglutarate-dependent hydroxylase. The LpxO active site may face the cytoplasm. However, in view of the ability of *S. typhimurium* to secrete large amounts of α-ketoglutarate (>100 μM) into the medium under conditions of iron stress, the possibility of a periplasmic or outer membrane localization for LpxO cannot be excluded (65).

Considerable effort has been devoted in recent years to the discovery of genes expressed *in vivo* during infection. Studies with *Salmonella* have utilized both signature-tagged mutagenesis and *in vivo* expression technology (66–69). It is interesting that no *S. typhimurium* genes expressed *in vivo* have been mapped to minute 92.8. Furthermore, despite the dependence of 2-hydroxymyristate biosynthesis on the PhoP/Q system (22), no genes mapping to *lpxO* have been found in searches for PhoP/Q-regulated genes (70, 71). It may be that these genetic screens were not fully saturated.

Despite the important roles that lipid A plays in host cell signaling and the fact that modified lipid A structures can elicit different host responses (13, 14), the role of 2-hydroxylation of lipid A has not been investigated. We propose a "Trojan horse" hypothesis for 2-hydroxymyristate function during *S. typhimurium* infections (Fig. 9). Neutrophils and monocytes are known to deacylate purified LPS (72, 73) and can even remove the secondary acyl chains from LPS in whole bacterial cells (74). The relevant acyloxyacyl hydrolase has been well characterized and is thought to detoxify LPS from diverse Gram-negative bacteria (36, 72, 73). During *S. typhimurium* infections, acyloxyacyl hydrolase would be expected to release 2-hydroxymyristate from LPS, which might allow mammalian cells to synthesize 2-hydroxymyristoyl coenzyme A, a potent inhibitor ($K_i \sim 40$ nM) of myristoyl-coenzyme A/protein *N*-myristoyltransferase (37). Inhibition of the latter might result in mislocalization of numerous proteins that utilize myristoyl chains as membrane anchors (75), possibly interfering with signal transduction and/or vesicle trafficking. Our hypothesis is contingent upon the transport of the released 2-hydroxymyristate from the phagolysosome to the cytosol. Interference with protein myristoylation could provide *S. typhimurium* with a way to modify the intracellular environment and facilitate the infection process.

The cloning of the *lpxO* gene now provides the means to purify large quantities of 2-hydroxymyristate-modified lipid A and to construct mutants of *S. typhimurium* and other Gram-negative organisms lacking *lpxO*. Preliminary characterization of such mutants in our laboratory indicates that 2-hydroxymyristate-modified lipid A is absent in *S. typhimurium* mutants lacking *lpxO*.[2] The discovery and characterization of *lpxO* should advance our understanding of the enzymology of lipid A modification and its involvement in pathogenesis.

Acknowledgment—We thank Dr. Sam Miller for providing strain CS022.

REFERENCES

1. Jones, B. D., and Falkow, S. (1996) *Annu. Rev. Immunol.* **14,** 533–561
2. Garcia-Del Portillo, F. (1999) *Trends Microbiol.* **7,** 467–469
3. Lindgren, S. W., Stojiljkovic, I., and Heffron, F. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 4197–4201
4. Monack, D. M., Raupach, B., Hromockyj, A. E., and Falkow, S. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 9833–9838
5. Richter-Dahlfors, A., Buchan, A. M. J., and Finlay, B. B. (1997) *J. Exp. Med.* **186,** 569–580
6. Fields, P. I., Swanson, R. V., Haidaris, C. G., and Heffron, F. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5189–5193
7. Fields, P. I., Groisman, E. A., and Heffron, F. (1989) *Science* **243,** 1059–1062
8. Groisman, E. A., Chiao, E., Lipps, C. J., and Heffron, F. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 7077–7081
9. Garcia Vescovi, E., Soncini, F. C., and Groisman, E. A. (1996) *Cell* **84,** 165–174
10. Groisman, E. A. (1998) *Bioessays* **20,** 96–101
11. Groisman, E. A., Kayser, J., and Soncini, F. C. (1997) *J. Bacteriol.* **179,** 7040–7045
12. Raetz, C. R. H. (1990) *Annu. Rev. Biochem.* **59,** 129–170
13. Raetz, C. R. H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 1035–1063, American Society for Microbiology, Washington, D. C.
14. Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Ulmer, A. J., Zähringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) *FASEB J.* **8,** 217–225
15. Nikaido, H. (1996) in *Escherichia coli and Salmonella: Cellular and Molecular Biology* (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 29–47, American Society for Microbiology, Washington, D. C.
16. Montgomery, K. F., Osborn, L., Hession, C., Tizard, R., Goff, D., Vassallo, C., Tarr, P. I., Bomsztyk, K., Lobb, R., Harlan, J. M., and Pohlman, T. J. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 6523–6527
17. Beutler, B., and Cerami, A. (1988) *Annu. Rev. Biochem.* **57,** 505–518
18. Dinarello, C. A. (1991) *Blood* **77,** 1627–1652
19. Zähringer, U., Lindner, B., and Rietschel, E. T. (1999) in *Endotoxin in Health and Disease* (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., eds), pp. 93–114, Marcel Dekker, Inc., New York
20. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Huffel, C. V., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., Freudenberg, M., Ricciardi-Castagnoli, P., Layton, B., and Beutler, B. (1998) *Science* **282,** 2085–2088
21. Beutler, B. (2000) *Curr. Opin. Microbiol.* **3,** 23–28
22. Guo, L., Lim, K. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M.,

[2] H. S. Gibbons and C. R. H. Raetz, manuscript in preparation.

and Miller, S. I. (1997) *Science* **276,** 250–253
23. Ernst, R. K., Yi, E. C., Guo, L., Lim, K. B., Burns, J. L., Hackett, M., and Miller, S. I. (1999) *Science* **286,** 1561–1565
24. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M., and Miller, S. I. (1998) *Cell* **95,** 189–198
25. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M., and Miller, S. I. (1998) *Mol. Microbiol.* **27,** 1171–1182
26. Nummila, K., Kilpelainen, I., Zähringer, U., Vaara, M., and Helander, I. M. (1995) *Mol. Microbiol.* **16,** 271–278
27. Zhou, Z., Lin, S., Cotter, R. J., and Raetz, C. R. H. (1999) *J. Biol. Chem.* **274,** 18503–18514
28. Bryn, K., and Rietschel, E. T. (1978) *Eur. J. Biochem.* **86,** 311–315
29. Ernst, R. K., Guina, T., and Miller, S. I. (1999) *J. Infect. Dis.* **179,** Suppl. 2, 326–330
30. Welch, D. F. (1991) *Clin. Microbiol. Rev.* **4,** 422–438
31. Humphreys, G. O., Hancock, I. C., and Meadow, P. M. (1972) *J. Gen. Microbiol.* **71,** 221–230
32. Kawahara, K., Uchida, K., and Aida, K. (1979) *Biochim. Biophys. Acta* **572,** 1–8
33. Jia, S., VanDusen, W. J., Diehl, R. E., Kohl, N. E., Dixon, R. A., Elliston, K. O., Stern, A. M., and Friedman, P. A. (1992) *J. Biol. Chem.* **267,** 14322–14327
34. Jia, S., McGinnis, K., VanDusen, W. J., Burke, C. J., Kuo, A., Griffin, P. R., Sardana, M. K., Elliston, K. O., Stern, A. M., and Friedman, P. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 7227–7231
35. Vålegard, K., van Scheltinga, A. C., Lloyd, M. D., Hara, T., Ramaswamy, S., Perrakis, A., Thompson, A., Lee, H. J., Baldwin, J. E., Schofield, C. J., Hajdu, J., and Andersson, I. (1998) *Nature* **394,** 805–809
36. Erwin, A. L., and Munford, R. S. (1990) *J. Biol. Chem.* **265,** 16444–16449
37. Paige, L. A., Zheng, G. Q., DeFrees, S. A., Cassady, J. M., and Geahlen, R. L. (1990) *Biochemistry* **29,** 10566–10573
38. Miller, S. I., and Mekalanos, J. J. (1990) *J. Bacteriol.* **172,** 2485–2490
39. Miller, J. R. (1972) *Experiments in Molecular Genetics*, p. 439, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
40. Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E., and Ausubel, F. M. (1982) *J. Bacteriol.* **149,** 114–122
41. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor, Cold Spring Harbor, NY
42. Galloway, S. M., and Raetz, C. R. H. (1990) *J. Biol. Chem.* **265,** 6394–6402
43. Cleresci, L. S., Greenberg, W. E., and Trussel, R. R. (eds) (1989) *Standard Methods for Examination of Water and Wastewater*, 17th Ed., American Public Health Association, Washington, D. C.
44. Bligh, E. G., and Dyer, J. J. (1959) *Can. J. Biochem. Physiol.* **37,** 911–918
45. Odegaard, T. J., Kaltashov, I. A., Cotter, R. J., Steeghs, L., van der Ley, P., Khan, S., Maskell, D. J., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 19688–19696
46. Raetz, C. R. H., and Kennedy, E. P. (1973) *J. Biol. Chem.* **248,** 1098–1105
47. Christ, W. J., McGuinness, P. D., Asano, O., Wang, Y., Mullarkey, M. A., Perez, M., Hawkins, L. D., Blythe, T. A., Dubuc, G. R., and Robidoux, A. L. (1994) *J. Am. Chem. Soc.* **116,** 3637–3638
48. Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990) *J. Mol. Biol.* **215,** 403–410
49. Hancock, J. C., Humphreys, G. O., and Meadow, P. M. (1970) *Biochim. Biophys. Acta* **202,** 389–391
50. Goldman, R. C., Doran, C. C., Kadam, S. K., and Capobianco, J. O. (1988) *J. Biol. Chem.* **263,** 5217–5223
51. Kawai, Y., and Moribayashi, A. (1982) *J. Bacteriol.* **151,** 996–1005
52. Zähringer, U., Knirel, Y. A., Lindner, B., Helbig, J. H., Sonesson, A., Marre, R., and Rietschel, E. T. (1995) *Prog. Clin. Biol. Res.* **392,** 113–139
53. Sanderson, K. E., Hessel, A., Liu, S., and Rudd, K. E. (1996) in *Escherichia coli and Salmonella* (Neidhardt, F. C., ed) Vol. 2, pp. 1903–1999, American Society for Microbiology Press, Washington, D. C.
54. Hegg, E. L., and Que, L. (1997) *Eur. J. Biochem.* **250,** 625–629
55. Hegg, E. L., Whiting, A. K., Saari, R. E., McCracken, J., Hausinger, R. P., and Que, L., Jr. (1999) *Biochemistry* **38,** 16714–16726
56. Eichhorn, E., van der Ploeg, J. R., Kertesz, M. A., and Leisinger, T. (1997) *J. Biol. Chem.* **272,** 23031–23036
57. Schofield, C. J., and Zhang, Z. (1999) *Curr. Opin. Struct. Biol.* **9,** 722–731
58. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) *Nucleic Acids Res.* **25,** 3389–3402
59. Clementz, T., Zhou, Z., and Raetz, C. R. H. (1997) *J. Biol. Chem.* **272,** 10353–10360
60. Gronke, R. S., VanDusen, W. J., Garsky, V. M., Jacobs, J. W., Sardana, M. K., Stern, A. M., and Friedman, P. A. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 3609–3613
61. Stenflo, J., Holme, E., Lindstedt, S., Chandramouli, N., Huang, L. H., Tam, J. P., and Merrifield, R. B. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 444–447
62. Kivirikko, K. I., and Pihlajaniemi, T. (1998) *Adv. Enzymol. Relat. Areas Mol. Biol.* **72,** 325–398
63. Holme, E. (1975) *Biochemistry* **14,** 4999–5003
64. Thornburg, L. D., and Stubbe, J. (1993) *Biochemistry* **32,** 14034–14042
65. Reissbrodt, R., Kingsley, R., Rabsch, W., Beer, W., Roberts, M., and Williams, P. H. (1997) *J. Bacteriol.* **179,** 4538–4544
66. Mahan, M. J., Slauch, J. M., and Mekalanos, J. J. (1993) *Science* **259,** 686–688
67. Mahan, M. J., Tobias, J. W., Slauch, J. M., Hanna, P. C., Collier, R. J., and Mekalanos, J. J. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 669–673
68. Heithoff, D. M., Conner, C. P., Hanna, P. C., Julio, S. M., Hentschel, U., and Mahan, M. J. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 934–939
69. Heithoff, D. M., Sinsheimer, R. L., Low, D. A., and Mahan, M. J. (1999) *Science* **284,** 967–970
70. Belden, W. J., and Miller, S. I. (1994) *Infect. Immun.* **62,** 5095–5101
71. Soncini, F. C., Garcia Vescovi, E., Solomon, F., and Groisman, E. A. (1996) *J. Bacteriol.* **178,** 5092–5099
72. Munford, R. S., and Hall, C. L. (1986) *Science* **234,** 203–205
73. Erwin, A. L., and Munford, R. S. (1992) in *Bacterial Endotoxic Lipopolysaccharides* (Morrison, D. C., and Ryan, J. L., eds) Vol. I, pp. 405–434, CRC Press, Inc., Boca Raton, FL
74. Katz, S. S., Weinrauch, Y., Munford, R. S., Elsbach, P., and Weiss, J. (1999) *J. Biol. Chem.* **274,** 36579–36584
75. Resh, M. D. (1999) *Biochim. Biophys. Acta* **1451,** 1–16
76. Zhou, Z., White, K. A., Polissi, A., Georgopoulos, C., and Raetz, C. R. H. (1998) *J. Biol. Chem.* **273,** 12466–124675