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

FUNCTION OF LpxO, A NEW Fe²⁺/α-KETOGLUTARATE-DEPENDENT DIOXYGENASE HOMOLOGUE*

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-hydroxyristate-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 O₂. Under anaerobic conditions, no 2-hydroxyristate-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 ¹⁸O₂ 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 O₂-dependent formation of 2-hydroxyristate-modified lipid A in E. coli. LpxO very likely is a novel Fe³⁺/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-hydroxyristate chains released from lipopolysaccharide inside infected macrophages might be converted to 2-hydroxyristoyl 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 Mg²⁺ 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) 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. 1A) 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-hydroxyristate residue at position 2 on S. typhimurium lipid A, and the amount of S-2-hydroxyristate at position 3’ can be greatly increased (Fig. 1B) (22). Furthermore, 4-amino-4-deoxy-t-arabinose (t-Ara4N) and phosphoethanolamine (pEtN) groups may

* This work was supported by National Institutes of Health Grants GM-51910 (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; t-Ara4N, 4-deoxy-4-amino-t-arabinose; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; MES, 4-morpholineethanesulfonic acid.
be attached to the 4'- and/or 1-phosphates (Fig. 1B) 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 Mg2+ 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 hydroxycarboxylic 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 O2 (31, 32).

We now show that the presence of the 2-hydroxymyristate residue in S. typhimurium lipid A is O2-dependent and that 18O2 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 asparyl/asparaginyl 

### Identification and Role of lpxO in Lipid A Hydroxylation

#### Table I

| Strain/Plasmids | Genotype | Source or reference |
|-----------------|----------|---------------------|
| S. typhimurium 14028 | Virulent wild type | Salmonella Genetic Stock Center, University of Calgary, Canada Ref. 38 |
| CS022 E. coli | pho-24 (PhoP-constitutive) | Stratagene |
| XL1-BlueMR | Δ(mcrA183 Δ(mcrBC-hsdSMR-mrr) 173 endA1 supE44 thi-1 recA1 | Stratagene |
| Plasmids | | |
| pBluescriptSK | AmpR | Stratagene |
| pHSIG1 | pBluescript/lpxO; AmpR | This work |

Theoretical and experimental studies suggest a function for 2-hydroxylation in pathogenesis (22, 29). Bacterial Strains—All bacterial strains used in this study are described in Table I. E. coli XL1-BlueMR was from Stratagene. S. typhi-

The lpxO strain was kindly provided by Dr. Samuel J. 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 ml sodium acetate at pH 5.0 and 4 ml of 10% ethanol were added. 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 A260/A280 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 following the 5′-end, BHyd5 (5′-CCGCCGGAATTCCATATGTTCGCAGCAATCATT-3′); for the 3′-end, BHyd3 (5′-CCGCTCGAGTCAGAGGAGGC-TTCCGTA-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 MgCl2, 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 °C for 72 min. The polymerase chain reaction product and the pBluescriptSK vector DNA were then digested with EcoRI and XhoI, gel-purified with the QIAEX-II kit (Qiagen), and ligated together with T4 DNA ligase (Life Technologies, Inc.) to form pHSIG1, which was transformed into CaCl2-

### EXPERIMENTAL PROCEDURES

**Materials—**32P, was purchased from NEN Life Science Products. 18O2 (97% isotopic enrichment) was purchased from Isotec. Pyridine, methanol, 88% formic acid, and KH2PO4 were from Mallinckrodt, while chloroform, KCl, and (NH4)2SO4 were purchased from EM Science. MES buffer and sodium formate 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 18O2 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. typhi-

**Plasmids**

- pBluescriptSK
- pHSIG1

**Strains and plasmids utilized in this study**

- Strains: S. typhimurium
- Plasmids: pBluescriptSK, pHSIG1

**Identification and Role of lpxO in Lipid A Hydroxylation**

**Materials—**32P, was purchased from NEN Life Science Products. 18O2 (97% isotopic enrichment) was purchased from Isotec. Pyridine, methanol, 88% formic acid, and KH2PO4 were from Mallinckrodt, while chloroform, KCl, and (NH4)2SO4 were purchased from EM Science. MES buffer and sodium formate 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 18O2 delivery system were from Supelco.
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 glucose (40 mM), 0.3 mM KH$_2$PO$_4$, 10 mM KCl, 15 mM (NH$_4$)$_2$SO$_4$, 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 1:50 dilution of an overnight culture of S. typhimurium phoPCS022 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, 40 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 Component of CS022 by Chromatography on DEAE-cellulose—In our protocol, 200 mL of bacteria grown either aerobically or anaerobically to A$_{600}$ ~ 1.0 were harvested by centrifugation at 4 °C. Each cell pellet was re-suspended in 50 mL of phosphate-buffered saline to which was added 100 mL of chloroform/methanol/water (2:3:1, v/v/v) 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 1-Ara4N or P6EtN 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 component 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-Annisaldehyde/H$_2$SO$_4$/acetic acid (89:2.5:4.1, v/v/v) (45, 47). After charring, the lipids were dissolved in chloroform/methanol (4:1, v/v) and spotted on a glass 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) ionizing the lipid A samples 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 1-Ara4N, P6EtN, palmitate, and 2-hydroxyacylrate moieties into its lipid A (Fig. 1B), even in the presence of 10 mM MgCl$_2$ in the medium. To test the effects of O$_2$ on the formation of 2-hydroxyacyl- and lipid A-containing 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 further 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 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 minor forms in which the pEtN and/or 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).

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 hepta-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-hydroxymyristoyl 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.

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. 3A). 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. 1A). 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. 1B). In addition, minor species are observed...
Identification and Role of lpxO in Lipid A Hydroxylation

Fig. 4. \textsuperscript{18}O\textsubscript{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% \textsuperscript{18}O\textsubscript{2} (97% isotope-enriched) and 74% N\textsubscript{2} (B). An increase of ~2 atomic mass units in the difference in the masses (\(\Delta\)) between the myristate and the 2-hydroxymyristate-containing lipid A species is seen when the cells are grown on \textsuperscript{18}O\textsubscript{2}. This increase in \(\Delta\) under the labeling conditions with \textsuperscript{18}O\textsubscript{2} demonstrates unequivocally the direct incorporation of molecular oxygen into the 2-hydroxymyristate-containing lipid A species. There is no effect of \textsuperscript{18}O\textsubscript{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.

A Homologue of Bovine Aspartyl/Asparaginyl \(\beta\)-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\(^{2+}\)/\(\alpha\)-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 \(\beta\)-hydroxylase (33, 34), a type of Fe\(^{2+}\)/\(\alpha\)-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 \(\beta\)-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 \textit{fdhF} at 92.8 min (53). This novel gene (designated \textit{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 \(\beta\)-hydroxylase catalytic domain, including four conserved histidine side chains (red) and several aspartate and glutamate residues (blue). Histidine 675 of bovine aspartyl/asparaginyl \(\beta\)-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\(_{\text{50}}\)-His motif (Fig. 5, yellow) (54), resembling the Fe\(^{2+}\)/\(\alpha\)-ketoglutarate-dependent dioxygenase for which a high resolution crystal structure is available (35). The latter is the only Fe\(^{2+}\)/\(\alpha\)-ketoglutarate-dependent dioxygenase for which a high resolution crystal structure is available (35). The His-X-Asp-X\(_{\text{50}}\)-His motif is also present in TdA (55) and Tau (56), two additional bacterial Fe\(^{2+}\)/\(\alpha\)-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 pathogenic island. As noted above, however, homologues of aspartyl/asparaginyl \(\beta\)-hydroxylase are seen in other bacteria that synthesize 2-hydroxyacyl chains (Table II), including \textit{Salmonella typhi}, \textit{Salmonella paratyphi}, \textit{K. pneumonia}, \textit{B. pertussis}, \textit{Borrelia bronchiseptica}, \textit{P. aeruginosa}, \textit{Pseudomonas putida},...
and L. pneumophila, all of which make 2-hydroxacyl-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 $10^{-178}$ 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$_{50}$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 database failed to reveal significant similarity to other Fe$^{2+}$/o-ketoglutarate-dependent dioxygenases besides the mammalian aspartyl/asparaginyl $\beta$-hydroxylase of animal cells, a type of Fe$^{2+}$/o-ketoglutarate-dependent dioxygenase.

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 EcoRI and XhoI 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-
Identification and Role of lpxO in Lipid A Hydroxylation

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. 7A), 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. 7B).

**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).

**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 i-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.
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,

**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/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,
Identification and Role of lpxO in Lipid A Hydroxylolation

we cannot yet exclude the alternative possibility that 2-hydroxyxymristate 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-hydroxyxymristoyl 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$^2+/lo$-ketoglutarate-dependent hydroxylases. Those dioxygenases with homologues in bacteria known to make 2-hydroxyxylated 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$^2+/lo$-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$^{275}$ 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$_{50}$-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-hydroxyxymristoyl-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 (Fig. 7). 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-hydroxyxymristoyl biosynthesis on the PhoPQ system (22), no genes mapping to lpxO have been found in searches for PhoPQ-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-hydroxyxymristoyl-modified lipid A has not been investigated. We propose a “Troyan horse” hypothesis for 2-hydroxyxymristoyl function during S. typhimurium infections (Fig. 9). Neutrophils and monocytes are known to deacetylate purified LPS (72, 73) and can even remove the secondary acyl chains from LPS in whole bacterial cells (74). The relevant acylxoyacyl hydroxylase has been well characterized and is thought to detoxify LPS from diverse Gram-negative bacteria (36, 72, 73). During S. typhimurium infections, acylxoyacyl hydroxylase would be expected to release 2-hydroxyxymristoyl from LPS, which might allow mammalian cells to synthesize 2-hydroxyxymristoyl coenzyme A, a potent inhibitor (K$\sim$ 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-hydroxyxymristoyl 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-hydroxyxymristoyl-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-hydroxyxymristoyl-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, 9633–9637
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. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5189–5193
7. Fields, P. I., Groisman, E. A., and Heffron, F. (1998) Science 243, 1059–1062
8. Groisman, E. A., Chiao, E., Lipps, C. J., and Heffron, F. (1998) Proc. Natl. Acad. Sci. U. S. A. 96, 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. Rietzel, E. T., Kirikae, T., Schade, F. U., Mumat, U., Schmidt, G., Lopponn, H., Ulmer, A. J., Zahringer, U., Di Padova, P., 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., 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. Zachariae, U., Lindner, B., and Rietzel, 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., Kim, R. B., Gunn, J. S., Bainbridge, B., Darveau, R. P., Hackett, M.,

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