

An Inner Membrane Dioxygenase that Generates the 2-Hydroxymyristate Moiety of *Salmonella* Lipid A[†]

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ABSTRACT: The lipid A residues of certain Gram-negative bacteria, including most strains of *Salmonella* and *Pseudomonas*, are esterified with one or two secondary *S*-2-hydroxyacyl chains. The *S*-2 hydroxylation process is O₂-dependent in vivo, but the relevant enzymatic pathways have not been fully characterized because in vitro assays have not been developed. We previously reported that expression of the *Salmonella* *lpxO* gene confers upon *Escherichia coli* K-12 the ability to synthesize 2-hydroxymyristate modified lipid A (*J. Biol. Chem.* (2000) 275, 32940–32949). We now demonstrate that inactivation of *lpxO*, which encodes a putative Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenase, abolishes *S*-2-hydroxymyristate formation in *S. typhimurium*. Membranes of *E. coli* strains expressing *lpxO* are able to hydroxylate Kdo₂-[4'-³²P]-lipid A in vitro in the presence of Fe²⁺, O₂, α-ketoglutarate, ascorbate, and Triton X-100. The Fe²⁺ chelator 2,2'-bipyridyl inhibits the reaction. The product generated in vitro is a monohydroxylated Kdo₂-lipid A derivative. The [4'-³²P]-lipid A released by mild acid hydrolysis from the in vitro product migrates with authentic *S*-2-hydroxylated lipid A isolated from ³²P-labeled *S. typhimurium* cells. Electrospray ionization mass spectrometry and gas chromatography/mass spectrometry of the in vitro product are consistent with the 2-hydroxylation of the 3'-secondary myristoyl chain of Kdo₂-lipid A. LpxO contains two predicted trans-membrane helices (one at each end of the protein), and its active site likely faces the cytoplasm. LpxO is an unusual example of an integral membrane protein that is a member of the Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenase family.

The *Salmonella typhimurium* genome encodes several enzymes (Figure 1) that catalyze the covalent modification of the Kdo₂-lipid A region of lipopolysaccharide (LPS)¹ (1, 2). Activation of the PmrA/PmrB two-component system by growth at low pH or as the result of point mutations in PmrA (3–8) induces the transcription of the enzymes EptA and ArnT, which attach phosphoethanolamine (pEtN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) units to lipid A, respectively (Figure 1) (9–11). The active sites of EptA and ArnT are located on the outer surface of the inner membrane (12). Activation of the PhoP/PhoQ two-component system by growth of cells at low divalent cation concentrations (13–15), or in the presence of cationic antimicrobial peptides (16), induces the genes encoding the outer membrane enzymes

PagP (17–19) and PagL (20, 21), which remodel the acyl chains of lipid A (Figure 1). The addition of a pEtN unit to the outer Kdo residue by EptB (Figure 1) is independent of PmrA/PmrB and PhoP/PhoQ, but instead is induced by 5 to 50 mM Ca²⁺ ions (22, 23).

The PmrA/PmrB and PhoP/PhoQ two-component systems are both activated following endocytosis of live *S. typhimurium* cells by RAW 264.7 macrophage tumor cells, resulting in multiple partial covalent modifications of lipid A (5). Addition of the L-Ara4N and palmitate moieties to lipid A confers increased resistance to polymyxins and β-defensins, respectively (4, 15, 24). The remodeling of the acyl chains (Figure 1) also reduces the potency of lipid A as an agonist against TLR-4 (25).

The enzymes that add the L-Ara4N, pEtN and palmitate groups to lipid A are present both in *Escherichia coli* K-12 and *S. typhimurium* (1). However, PagL (20), LpxR (26), and LpxO (27) (Figure 1) are restricted to *Salmonella*. LpxR cleaves the 3'-acyloxyacyl moiety of lipid A in the presence of Ca²⁺ ions (26). LpxO is involved in generating the *S*-2-hydroxy group present on the 3'-secondary myristoyl chain of *Salmonella* lipid A (27), provided that the cells are grown in the presence of O₂ (Figure 1). Although the existence of *S*-2-hydroxymyristate in *S. typhimurium* lipid A has been known for many years (28), its enzymatic synthesis has not been fully elucidated (27). The *S*-2-hydroxymyristate moiety may increase hydrogen bonding between adjacent lipid A units, enhancing the outer membrane's ability to resist

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¹ Abbreviations: L-Ara4N, 4-amino-4-deoxy-L-arabinose; BSA, bovine serum albumin; EI/MS, electron impact/mass spectrometry; ESI/MS, electrospray ionization/mass spectrometry; GC/MS, gas chromatography/mass spectrometry; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; IPTG, isopropyl thio-β-D-galactoside; Kdo, 3-deoxy-D-manno-octulosonic acid; LPS, lipopolysaccharide; pEtN, phosphoethanolamine; TIC, total ion chromatogram; TLC, thin layer chromatography; TMS, trimethylsilyl.

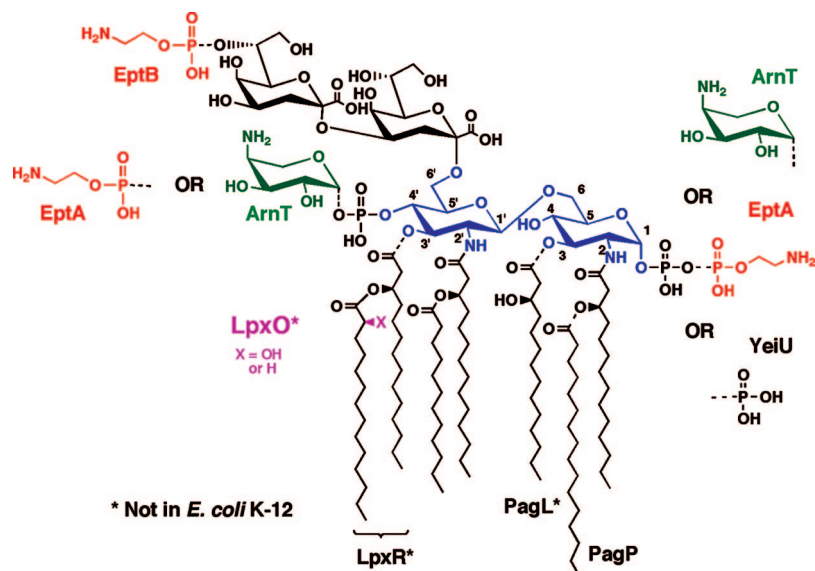


FIGURE 1: Regulated covalent modifications of *S. typhimurium* and *E. coli* Kdo₂-lipid A. Covalent modifications of the lipid A phosphate groups are regulated by the PmrA/PmrB two-component signaling system (1). ArnT attaches L-Ara4N mainly to the 4'-position, whereas EptA predominantly adds pEtN mainly to the 1-position. However, the selectivity of these enzymes is reversed in the absence of Kdo (73, 74), and minor species are formed in which both positions are modified either with pEtN or with L-Ara4N (1, 50). The PagP-dependent palmitoylation (1, 17–19) and the PagL catalyzed deacylation (1, 20) of the lipid A acyl chains are under the control of the PhoP/PhoQ two-component system (1). Other lipid A modifications, such as those catalyzed by another pEtN transferase EptB (26) or the 3'-O-deacylase LpxR (1, 23), are induced by high concentrations of Ca²⁺. The formation of 2-hydroxymyristate on lipid A by LpxO (1, 27), observed when cells are grown in the presence of O₂, is not dramatically regulated by PhoP/PhoQ under our conditions (5). The properties of the indicated enzymes responsible for these modifications are reviewed elsewhere (1). The YeiU gene, which encodes a novel phosphotransferase, was recently renamed LpxT to reflect its enzymatic function (77).

penetration by organic ions, such as ethidium, under some growth conditions (29, 30).

In previous work, we identified the *Salmonella lpxO* gene, the occurrence of which correlates with the presence of S-2-hydroxyacylated lipid A in diverse Gram-negative bacteria (27). Expression of *Salmonella lpxO* in *E. coli* K-12 resulted in robust O₂-dependent formation of *E. coli* lipid A containing 2-hydroxymyristate (27), implicating LpxO as the enzyme responsible for lipid A 2-hydroxylation. The LpxO protein shares similarity with the superfamily of Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenases (27), which participate in important processes, such as collagen cross-linking, transcription factor inactivation, herbicide degradation, and taurine biosynthesis (31, 32). Many Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenases catalyze hydroxylation reactions, but some catalyze ring expansions, dehydrations, and halogenations (33–35). The active sites of these enzymes contain a single ferrous ion, usually coordinated by a His-X-Asp/Glu-X_n-His facial triad motif in which *n* denotes at least 40 consecutive amino acid residues (36). The existence of a similar sequence motif in LpxO (27) suggested a possible enzymatic mechanism for lipid A 2-hydroxylation (37). However, an in vitro assay for LpxO was not developed (27), and therefore, the substrates for the 2-hydroxylation process remained obscure.

We now demonstrate that membranes of *E. coli* cells expressing *Salmonella lpxO* catalyze the hydroxylation of Kdo₂-lipid A (38) in accordance with the proposed scheme shown in Figure 2A, whereas membranes from the vector control strain do not. LpxO appears to be localized to the inner cytoplasmic membrane. LpxO activity is stimulated in vitro by the addition of Fe²⁺ ions and is absolutely dependent upon the presence of α-ketoglutarate and a nonionic detergent. LpxO possesses two putative trans-

membrane domains (one at its N-terminus and the other at its C-terminus) (39). Its central catalytic domain (Figure 2B) is predicted to face the cytoplasm, consistent with the fact that LpxO activity in living cells is independent of the lipid A transporter MsbA (12, 40).

MATERIALS AND METHODS

Reagents and Materials. ³²P_i and [γ-³²P]-ATP were purchased from Perkin-Elmer Life Sciences, Waltham, MA. Silica gel 60 glass-backed TLC plates (0.25 mm) were obtained from E. Merck (Darmstadt, Germany). Pyridine, methanol, 88% formic acid, and KH₂PO₄ were from Mallinckrodt, Hazelwood, MO. Chloroform, KCl, and NaCl were from EM Science, Gibbstown, NJ. Dithiothreitol (DTT), ascorbic acid, α-ketoglutarate, Fe(NH₄)₂(SO₄)₂, catalase, and bovine serum albumin were from Sigma (St. Louis, MO). Triton X-100 was purchased as the SurfactAmp 10% solution in water from Pierce/Endogen (Rockford, IL). *E. coli* phospholipids were obtained from Avanti Polar Lipids, Alabaster, AL. DL-3-hydroxymyristic acid and DL-2-hydroxymyristic acid were from Sigma (St. Louis, MO), and Tri-Sil reagent was from Pierce (Rockford, IL).

Bacterial Strains and Cultures. Unless otherwise stated, bacteria were grown in Luria-Bertani (LB) broth (41). Antibiotics were added at concentrations of 100 μg/mL (ampicillin) and 30 μg/mL (chloramphenicol and kanamycin).

Construction of an LpxO Overexpression Vector and Isolation of Membranes from Overexpressing Strains. The *lpxO* gene was excised from pHSG1 (27) using NdeI and XhoI and cloned into pET21a⁺ (Novagen) using T4 DNA ligase (Boehringer Mannheim). The resulting plasmid (pHSG2) was transformed into *E. coli* XL1-Blue (Stratagene). The insert size was

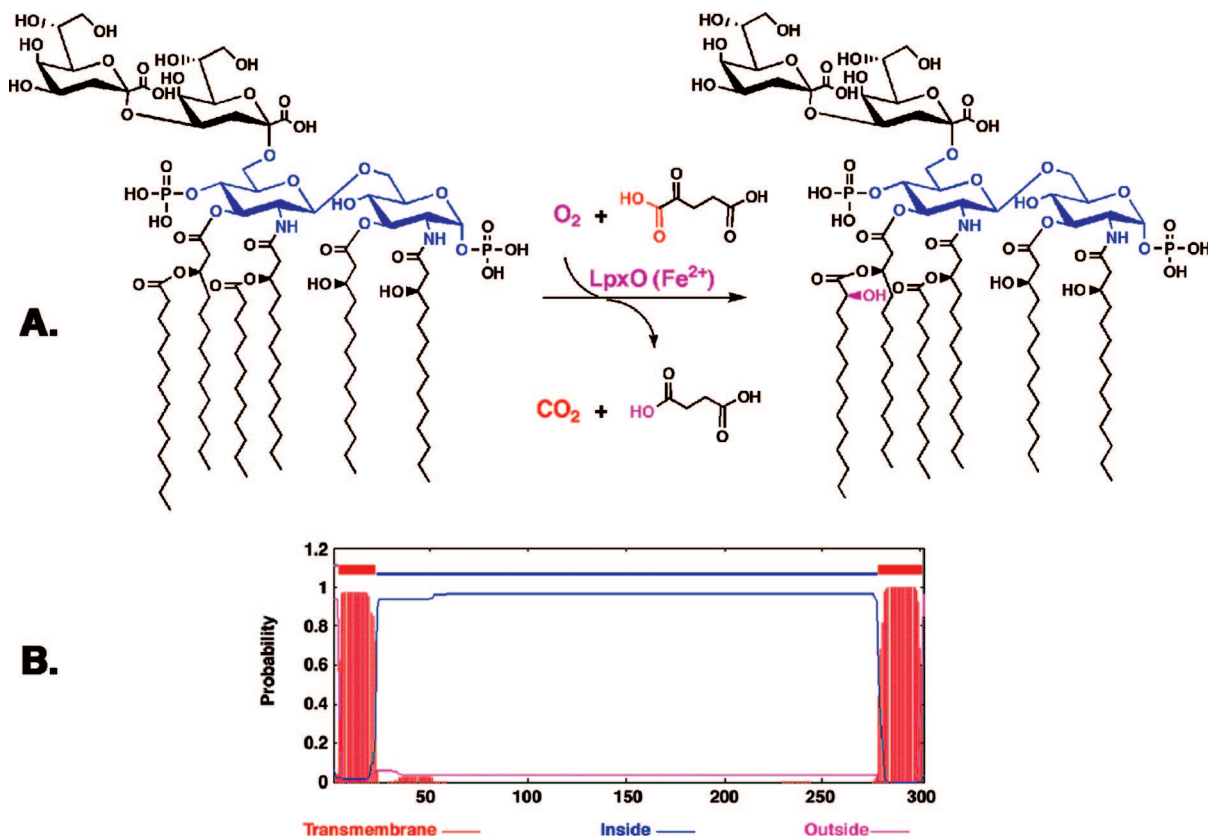


FIGURE 2: Proposed reaction catalyzed by LpxO. (A) The LpxO reaction is proposed to proceed by a mechanism similar to that used by other Fe²⁺/O₂/α-ketoglutarate dependent dioxygenases, most likely catalyzing the direct 2-hydroxylation of the 3'-myristate chain of hexa-acylated Kdo₂-lipid A (38), as demonstrated in this study. (B) Predicted topology and trans-membrane segments of LpxO based on the TMHMM algorithm (39).

confirmed by restriction enzyme digest analysis. pHSG2 was subsequently transformed into *E. coli* BLR(DE3)/pLysS (Novagen).

To express LpxO, stationary phase cultures in LB broth, containing ampicillin and chloramphenicol, were diluted 1:100 into 1 L of fresh LB broth containing ampicillin and chloramphenicol, and grown at 37 °C until the A₆₀₀ value reached 0.5. Isopropyl thio-β-D-galactoside (IPTG) (1 mM) was then added, and growth was continued for 3 h. The cells were harvested by centrifugation (5000g for 20 min). The cell pellets were washed, resuspended in 50 mM HEPES at pH 7.5, and broken in a French pressure cell. Cell debris was removed by centrifugation at 4000g for 20 min. Membranes were isolated by centrifuging the clarified lysates at 100,000g for 1 h. The membrane pellet was resuspended in 50 mM HEPES at pH 7.5 and centrifuged at 100,000g for 1 h. The washed membranes were resuspended in 50 mM HEPES at pH 7.5, to a concentration of 1–5 mg/mL of membrane protein, as judged by the bicinchoninic acid assay with bovine serum albumin as the standard (42).

Isolation of Lipid A from *S. typhimurium* Cultures. Cultures of *S. typhimurium* 14028s (wild type) and HG002 (5) were grown with shaking at 250 rpm in 200 mL of LB medium at 37 °C to A₆₀₀ = 1.0. Cells were harvested by centrifugation at 5000g for 30 min. Pellets were washed once with 30 mL of phosphate-buffered saline (pH 7.4) (43), and the cells were centrifuged again at 5000g for 30 min. The final cell pellet was resuspended in 20 mL of phosphate-buffered saline; chloroform (25 mL) and methanol (50 mL) were then added to generate a single-phase Bligh/Dyer mixture (44). After

1 h at room temperature, the LPS-containing precipitate was collected by centrifugation (2500g for 20 min). The pellet was then washed twice with 50 mL of a single-phase Bligh/Dyer mixture (44) and collected by centrifugation. The washed pellet was dispersed in 25 mL of 50 mM sodium acetate (pH 4.5) using a Branson probe sonicator. The suspension was heated to 100 °C for 30 min in a boiling water bath. After cooling to room temperature, chloroform (28 mL) and methanol (28 mL) were added to make a two-phase Bligh/Dyer mixture (44). The lower organic phase was collected after separation of the phases by centrifugation (2500g for 20 min). The aqueous phase was re-extracted with fresh pre-equilibrated lower-phase, and again, the organic layer was collected after centrifugation. The organic phases were pooled and dried using a rotary evaporator. The final lipid A preparations were stored at –80 °C. Labeling of *S. typhimurium* cells with ³²P_i and isolation of the lipid A fraction were carried out as described previously (27).

Enzymatic Synthesis and Purification of LpxO Substrates. Preparation of radiolabeled Kdo₂-[4'-³²P]-lipid A was performed according to published procedures (45). Unlabeled Kdo₂-lipid A was purified from WBB06 according to the method of Raetz et al. (38) or purchased from Avanti Polar Lipids.

In Vitro Assay of LpxO Activity. An in vitro system for LpxO, using Kdo₂-lipid A as the putative acceptor substrate, was developed on the basis of assays previously reported for other Fe²⁺/O₂/α-ketoglutarate-dependent hydroxylases (46, 47). The reaction conditions, unless otherwise indicated, included 50 mM HEPES at pH 7.5, 1 mM α-ketoglutarate,

2 mM ascorbate, 10 μM Fe(NH₄)₂(SO₄)₂, 0.2% Triton X-100, 4 mM DTT, 0.5 mg/mL *E. coli* phospholipid, and 4 μM Kdo₂-[4'-³²P]-lipid A (20,000 cpm/reaction or 100,000 cpm/nmol). Assays were carried out at 30 °C in a final volume of 50 μL. Reactions were initiated by adding *E. coli* membranes (0.01–0.1 mg/mL final concentration). In some cases, catalase (0.1 mg/mL) was also included to scavenge H₂O₂.

To monitor the hydroxylation of lipid A, the reaction was quenched by removing a 10 μL portion of the assay mixture and adding it to 170 μL of a hydrolysis buffer, consisting of 12.5 mM sodium acetate (pH 4.5) and 1% SDS. This mixture was heated at 100 °C for 30 min to cleave off the Kdo residues (48). Following hydrolysis, 400 μL of CHCl₃/MeOH (1:1, v/v) was added to make a two-phase Bligh/Dyer mixture. After mixing, the phases were separated by brief centrifugation (13,000 rpm) in a tabletop microcentrifuge. The upper phase was discarded, and the lower phase containing the released lipid A was vacuum-dried, redissolved in 10 μL of CHCl₃/MeOH (4:1, v/v), and spotted at the origin of a 20 cm × 20 cm Silica Gel 60 TLC plate. Hydroxylated lipid A was resolved from unmodified lipid A by chromatography in the solvent CHCl₃/pyridine/88% formic acid/water (50:50:16:5, v/v). The hydroxylated lipid A product migrates more slowly than unmodified lipid A, being resolved just enough to permit quantification (27). Lipid A species separated by TLC were quantified using a PhosphorImager, equipped with ImageQuant software (Molecular Dynamics).

Purification of Hydroxylated Kdo₂-lipid A Generated in Vitro. The LpxO hydroxylation reaction was scaled up to a 4.5 mL reaction volume under conditions otherwise identical to the assay system described above, except that the radioactive tracer was omitted and the concentration of Kdo₂-lipid A was raised up to 100 μM. Reactions were carried out in the presence of 0.1 mg/mL membrane protein, using membranes from IPTG-induced BLR(DE3)/pLysS cells containing either pET21a⁺ or pHSG2. After 2 h at 30 °C, the reaction mixture was converted into a single-phase Bligh/Dyer system by the addition of 5.6 mL of CHCl₃ and 11.3 mL of methanol. The Kdo₂-lipid A acceptor substrate is soluble in this single-phase solvent mixture (38), whereas the endogenous LPS present in the membranes of the host strain is not. The Kdo₂-lipid A was retrieved by adding appropriate volumes of CHCl₃ and aqueous HCl to generate a two-phase Bligh/Dyer system, which consists of CHCl₃/MeOH/0.1 M HCl (2:2:1.8, v/v) (44). After thorough mixing, the phases were separated by low-speed centrifugation for 10 min. The lower phase, which contains Kdo₂-lipid A and its hydroxylated product, was removed, washed once with fresh pre-equilibrated acidic upper phase, and then dried under N₂. The Kdo₂-lipid A species were then separated from glycerophospholipids by anion exchange chromatography on DEAE cellulose (22) and analyzed by electrospray ionization-mass spectrometry (ESI/MS) in the negative mode.

ESI/MS Analysis of Kdo₂-lipid A and Lipid A Sample. Mass spectra were acquired on a QSTAR-XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciex, Foster City, CA), equipped with an ESI source. Spectra were acquired in the negative-ion mode and typically were the accumulation of 60 scans over the range of 200–2500 atomic mass units (38). For MS analysis, the extracted lipids were

dissolved in 500 μL of chloroform/methanol/water (2:3:1, v/v), containing piperidine (1%, v/v), and infused into the ion source at 5–10 μL/min. The negative-ion ESI was carried out at –4200 V. Data acquisition and analysis were performed using the AnalystQS software.

GC/MS Analysis of Hydroxylated Fatty Acids. The DEAE-purified Kdo₂-lipid A species derived from the large scale *in vitro* reactions described above were hydrolyzed in acidic methanol, *N*-acetylated, and then converted to trimethylsilyl ethers. DL-3-Hydroxymyristic acid and DL-2-hydroxymyristic acid standards were processed and analyzed in parallel with the samples. Typically, 0.5–1.0 mg of sample was thoroughly dried in a Reacti-vial equipped with a Teflon-lined screw cap. Samples were hydrolyzed by adding 300 μL of 1 M HCl in methanol and heated at 80 °C for 15 h. The reaction mixtures were then cooled, and the solvents were removed under a stream of nitrogen. Next, 200 μL of anhydrous methanol, 40 μL of pyridine, and 40 μL of acetic anhydride were added to the vial. The reaction mixtures were mixed and incubated overnight at room temperature. The solvents were evaporated under a stream of nitrogen. Finally, silylation of free OH groups to generate the trimethylsilyl (TMS) derivatives was achieved by adding 200 μL of Tri-Sil reagent to the dried samples, mixing, and incubating at room temperature for an hour. The samples were dried under a gentle stream of nitrogen, redissolved in 100 μL of hexane, and transferred to new vials for gas chromatography/mass spectrometry (GC/MS) analysis.

GC/MS was performed using a Finnigan Trace MS instrument coupled with a Trace GC 2000 gas chromatography system. The column was a 30 m RTX-5MS (0.25 μm internal diameter and 0.25 μm phase thickness) from Restek (Bellefonte, PA). The temperature program of the GC was as follows: the column oven temperature was initially held at 100 °C for 3 min, increased to 150 °C at a rate of 20 °C/min, then increased to 200 °C at a rate of 1 °C/min, further increased to 335 °C at a rate of 30 °C/min, and finally held at 335 °C for 2 min. The total run time was 62 min. The injector was operated in the split mode (1:20 split), and the temperature of the injection port was kept at 200 °C. Helium was the carrier gas with a constant flow rate of 1 mL/min. The instrument was operated in the electron impact (EI) mode with the electron energy set at 70 eV.

RESULTS

A Mutant of *S. typhimurium* Lacking 2-Hydroxymyristate-Modified Lipid A. *S. typhimurium* mutant HG002 (*lpxO::kan*) was previously shown to lack 2-hydroxymyristate modified lipid A species, as judged by TLC analysis of ³²P_i-labeled cells (5). This conclusion was confirmed by high-resolution ESI/MS of crude lipid A species (Figure 3) obtained from wild-type or HG002 cells grown on LB broth. As shown in Figure 3A, the predominant [M – 2H]^{2–} ions in the spectrum of wild type are consistent with enteric hexa-acylated lipid A species, bearing either myristate (*m/z* 897.623) or 2-hydroxymyristate (*m/z* 905.622) as the secondary acyl chain at position 3'. Minor ions can be attributed to penta- or hepta-acylated lipid A species, to variations in acyl chain lengths, or to sodium adducts (Figure 3A). The prominent peak at *m/z* 905.622 (Figure 3A) is absent in the spectrum of the mutant lipid A (Figure 3B), consistent with a defect in the

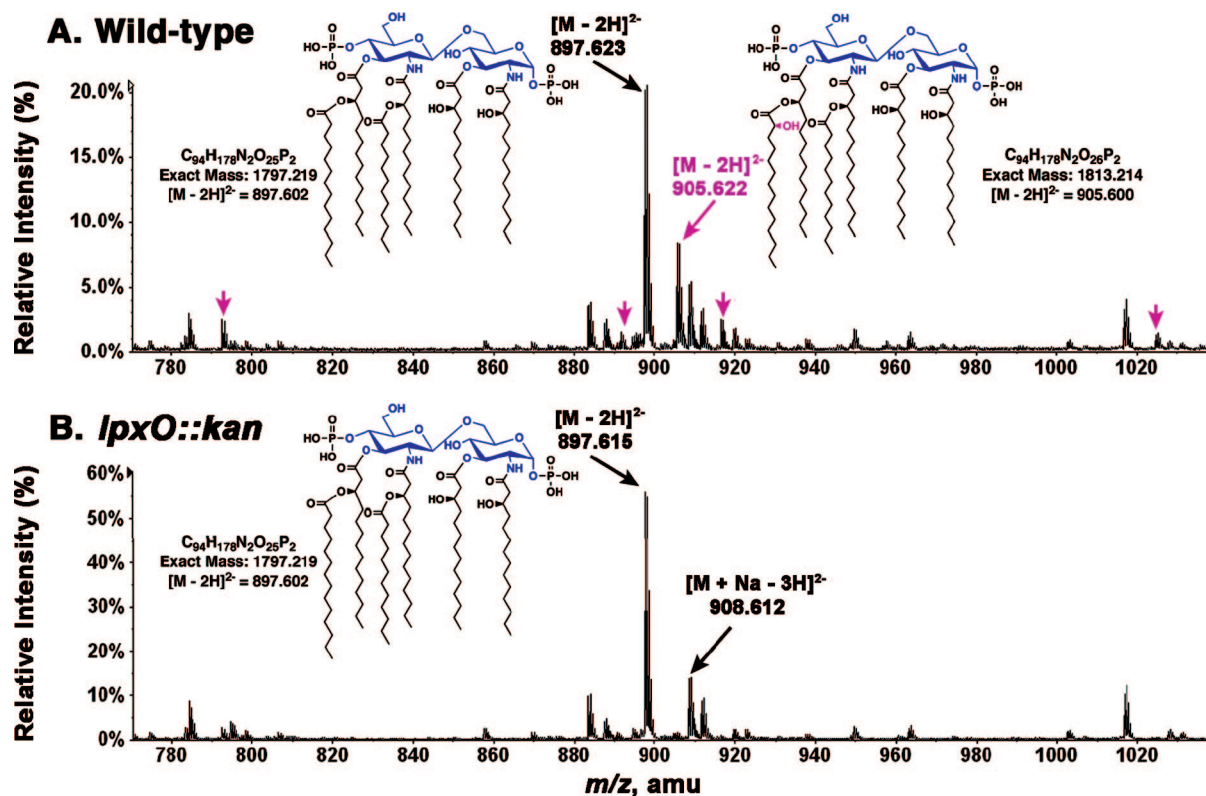


FIGURE 3: The *lpxO* mutant HG002 lacks lipid A species containing 2-hydroxymyristate. ESI/MS spectra of crude lipid A species isolated from the wild-type *S. typhimurium* 14028s (A) or from the *lpxO::kan* mutant HG002 (B). The minor peaks shown in magenta in panel A are proposed to arise from the following covalent modifications or adducts of the parent compound with $[M - 2H]^{2-}$ at m/z 905.6; $[M + C16:0-2H]^{2-}$ at m/z 1024.7; $[M + Na - 3H]^{2-}$ at m/z 916.6; $[M - 2CH_2 - 2H]^{2-}$ at m/z 891.6; and $[M - 3OHC14 - 2H]^{2-}$ at m/z 792.5.

2-hydroxylation of lipid A. Several minor molecular species, predicted to contain 2-hydroxymyristate based on their exact masses, are likewise missing in mutant HG002 (Figure 3A versus 3B, magenta arrows).

Deletion of *lpxO* did not appreciably compromise outer membrane integrity or growth of cells either on LB broth or on low- Mg^{2+} N-minimal medium (data not shown). Sensitivity to erythromycin, rifampicin, streptomycin, or bacitracin was not increased, as judged by disk diffusion assays (data not shown). The ability of HG002 to penetrate and multiply inside of mouse RAW 264.7 macrophage tumor cells was unaffected (5). The virulence of HG002 was comparable to that of wild-type *S. typhimurium*, when tested in a mouse infection model by either intravenous or oral administration (Romilanus, E., and Maskell, D. J., personal communication).

Membrane Localization of Overexpressed LpxO in *E. coli*. The *lpxO* gene was overexpressed behind the T7-*lac* promoter on the hybrid plasmid pHSG2 (Table 1) in *E. coli* BLR(DE3)/pLysS. Crude extracts of *lpxO*-induced cultures displayed a faint protein band on SDS gels near 35 kDa, consistent with the predicted molecular weight of LpxO (Figure 4A). This band was absent in the cytosol but was enriched in the membrane fraction (Figure 4A), confirming the predicted subcellular localization of LpxO (Figure 2B) (39), which is unusual among the Fe^{2+} / α -ketoglutarate dependent hydroxylases.

An In Vitro Assay for LpxO. Given its sequence similarity to the Fe^{2+} / α -ketoglutarate-dependent dioxygenases and the O_2 requirement for the formation of 2-hydroxymyristate and other 2-hydroxyacyl chains in vivo (27, 49), it seemed

Table 1: Bacterial Strains and Plasmids Used in This Study

strain/plasmid	description	source
<i>Escherichia coli</i>		
XL1-BlueMR	$\Delta(mcrA)183\Delta(mcrCB-hsdSMR-mrr)173\ endA1\ supE44\ thi-1\ recA1\ gyrA96\ relA1\ lac$	Stratagene
BLR(DE3)pLysS WBB06	T7- <i>lac</i> expression strain, <i>recA</i> $\Delta rfaC\ rfaF::tet$	Novagen ref 76
<i>Salmonella typhimurium</i>		
14028s	virulent wild-type	S. I. Miller ref 5
HG002	<i>lpxO::kan</i> derivative of 14028s	
plasmids		
pHSG1	pBluescript II SK containing the <i>lpxO</i> gene	ref 27
pET21a ⁺	T7- <i>lac</i> expression vector	Novagen
pHSG2	pET21a ⁺ containing <i>lpxO</i> gene in NdeI and XhoI sites	this work

plausible that LpxO might catalyze the in vitro hydroxylation of hexa-acylated *E. coli* Kdo₂-lipid A (38) in the presence of appropriate cofactors and O_2 (Figure 2A). We therefore prepared an aerobic assay system to probe for LpxO activity, consisting of 4 μ M Kdo₂-[4'-³²P]-lipid A, 1 mM α -ketoglutarate, 2 mM ascorbate, 10 μ M $Fe(NH_4)_2(SO_4)_2$, 0.2% Triton X-100, 4 mM DTT, and 0.5 mg/mL *E. coli* phospholipids in 50 mM Hepes at pH 7.5. The reaction was initiated by adding either soluble protein or membranes (typically 0.01 to 0.1 mg/mL, as indicated) from the vector control or the LpxO overexpressing strain (with or without IPTG induction). Incubation of the complete reaction mixture at 30 °C resulted in LpxO-dependent formation of a new, more slowly migrating ³²P-labeled lipid A species (Figure 4B), as judged

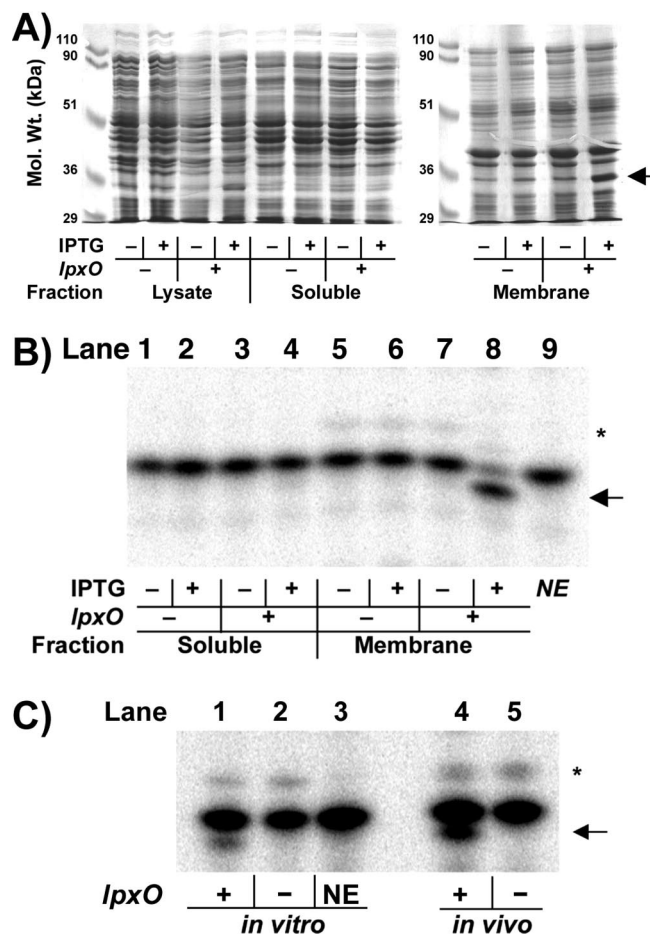


FIGURE 4: Overexpression and catalytic activity of membrane-associated LpxO. (A) Coomassie-blue stained SDS gel of subcellular fractions from LpxO-overexpressing *E. coli*. The LpxO protein (arrow) migrates as a 35-kDa protein and is localized in the membrane fraction. (B) Incubation of *E. coli* Kdo₂-[4'-³²P]-lipid A in the LpxO assay system, described in Materials and Methods, with 0.1 mg/mL cytosol (lanes 1–4) or 0.1 mg/mL membranes (lanes 5–8) of *E. coli* BLR(DE3)/pLysS harboring pET21a+ (lanes 1, 2, 5, and 6) or pHSG2 (lanes 3, 4, 7, and 8) for 60 min. Lipid A species were separated by TLC in CHCl₃/pyridine/formic acid/H₂O (50:50:16:5, v/v) after mild acid hydrolysis of the reaction product at pH 4.5 to remove the Kdo disaccharide (75). The hydroxylated lipid A product is highlighted by the arrow. NE indicates (lane 9) the no-enzyme control. (C) Chromatographic comparison of lipid A 1,4'-bis-phosphate species generated in vitro by LpxO-overexpressing membranes (lane 1) at 0.1 mg/mL for 5 min to matched vector or no-enzyme controls (lanes 2 and 3), and to the lipid A species obtained from ³²P_i-labeled wild-type (lane 4) or *lpxO::kan* mutant *S. typhimurium* (lane 5). The asterisk indicates the hepta-acylated product formed by the outer membrane enzyme PagP (17).

by TLC, after the removal of the Kdo disaccharide by hydrolysis at pH 4.5. The *R_f* of the modified lipid A species is consistent with the behavior of 2-hydroxymyristate-containing lipid A isolated by hydrolysis at pH 4.5 from *S. typhimurium* LPS (Figure 4C) (27, 50).

The formation of the slowly migrating [4'-³²P]-lipid A product was time- and protein concentration-dependent (Figure 5), although linearity of product formation with time was gradually lost after 5 min (Figures 5 and 6A). The specific activity of membranes from LpxO-overexpressing cells, assayed at 0.01 mg/mL for 5 min, was 6.9 nmol/min/mg with Kdo₂-[4'-³²P]-lipid A as the substrate. No products were generated when LpxO was incubated with Kdo₂-lipid

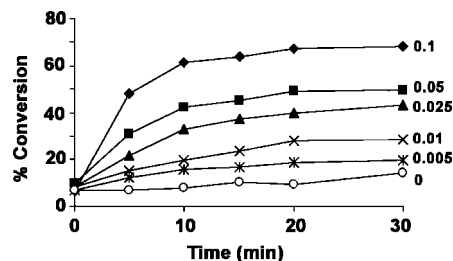


FIGURE 5: Dependence of LpxO product formation on time and protein concentration. Increasing amounts of LpxO membrane protein were added to the assay cocktail, and the formation of hydroxylated lipid A was monitored with time. Membrane protein concentrations (mg/mL) in the final assay mixture are indicated. ♦, 0.1 mg/mL; ■, 0.05 mg/mL; ▲, 0.025 mg/mL; ×, 0.01 mg/mL; *, 0.005 mg/mL; ○, no enzyme control. The small amount of apparent product formation at time 0 and in the no-enzyme control is caused by slight streaking of the substrate band during TLC, which migrates just above the hydroxylated product band (Figure 4B).

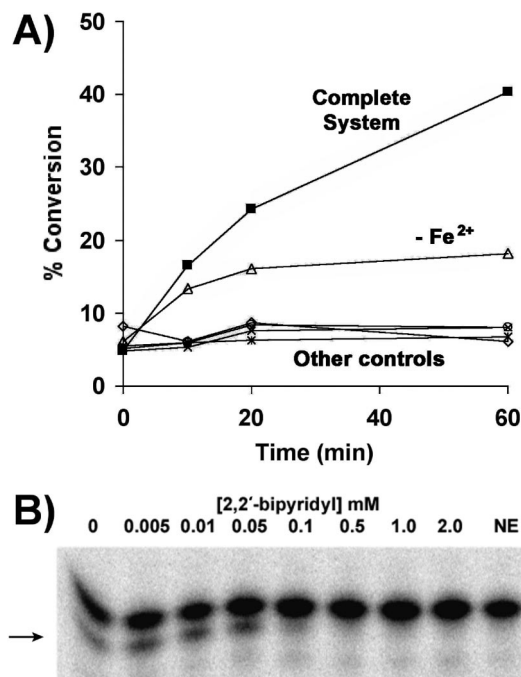


FIGURE 6: Dependence of LpxO activity on soluble cofactors. (A) A standard LpxO assay mixture containing 0.01 mg/mL membrane protein was prepared in which either α-ketoglutarate, ascorbate, or iron was omitted. Full activity was evident only when all the cofactors were present (■). Omission of α-ketoglutarate (◇) or ascorbate (○) eliminated lipid A hydroxylation completely, as in the vector and no enzyme controls (+ and *). Omission of added Fe²⁺ resulted in lower activity (△). (B) Inhibition of LpxO activity by increasing concentrations of 2,2'-bipyridyl, an Fe²⁺ chelator. The standard reaction mixture containing 0.01 mg/mL protein (5 min of incubation) was supplemented with the indicated concentrations of 2,2'-bipyridyl. The arrow indicates the position of the LpxO product.

IV_A (I), lipid IV_A (I) or hexa-acylated lipid A (lacking the Kdo disaccharide) under otherwise identical assay conditions (data not shown).

Dependence of the LpxO Reaction on Cofactors. When either α-ketoglutarate or ascorbate was omitted from the reaction mixture (Figure 6A, ◇ and ○, respectively), no LpxO product was formed, as in the no-enzyme and vector controls (Figure 6A, + and *). Omission of iron from the assay cocktail reduced the extent of lipid A 2-hydroxylation relative to the complete reaction mixture, but it did not entirely

abolish enzymatic activity (Figure 6A, Δ). The membranes used to initiate the reaction probably contained some iron. When the reaction mixture was supplemented with catalase to scavenge H_2O_2 , partial lipid A hydroxylation was observed in the absence of ascorbate, indicating that ascorbate is not absolutely necessary for LpxO activity (data not shown).

If LpxO requires Fe^{2+} for activity, then 2,2'-bipyridyl, an Fe^{2+} chelator, should inhibit lipid A hydroxylation. When 2,2'-bipyridyl was included in the reaction mixture, dose-dependent inhibition of LpxO product formation was indeed observed (Figure 6B). Taken together, the results demonstrate a strict requirement for α -ketoglutarate and Fe^{2+} in the LpxO catalyzed hydroxylation of lipid A.

Detergent-Requirement and Stabilization of LpxO by Phospholipids. Because LpxO is an integral membrane enzyme with a hexa-acylated lipid substrate, the reaction should display an absolute requirement for detergent. Indeed, no hydroxylation was observed when the detergent was omitted, whereas 0.1–0.2% Triton X-100 provided optimal stimulation of enzyme activity (Figure 7A). Product formation was reduced at higher detergent concentrations (Figure 7A), because of either surface dilution of the substrate (51) or inactivation of the enzyme.

Preincubation of LpxO-containing membranes with Triton X-100 and assay buffer, prior to initiation of the reaction by addition of the remaining reagents, resulted in reduced LpxO activity (Figure 7B). Enzymes such as LpxO sometimes inactivate themselves by hydroxylating tyrosine residues near their active sites (52). Alternatively, solubilization of LpxO at low protein concentrations and removal of tightly bound lipids might also inactivate the enzyme. Supplementation of the reaction mixture with additional *E. coli* phospholipids partially stabilized LpxO activity and improved linearity of product formation with time, but higher concentrations of phospholipids were inhibitory (Figure 7C).

ESI/MS of the Product Generated by LpxO-Catalyzed Hydroxylation of Kdo₂-lipid A. To confirm that the 3'-secondary acyl chain of Kdo₂-lipid A is selectively hydroxylated in our *in vitro* LpxO assay system, a 4.5 mL reaction mixture, containing 0.1 mg/mL membrane protein and 100 μ M Kdo₂-lipid A (38), was incubated for 2 h at 30 °C. The Kdo₂-lipid A species (unresolved substrate and hydroxylated product) were repurified from the crude reaction mixture by Bligh/Dyer extraction followed by anion-exchange chromatography on DEAE cellulose (22). The Kdo₂-lipid A species were then analyzed directly without pH 4.5 hydrolysis by negative ion ESI/MS (Figure 8). The vector control sample (Figure 8A) yielded peaks at m/z 1117.681 and m/z 744.790, which are interpreted as $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ of unmodified Kdo₂-lipid A, respectively (38). The sample incubated with the LpxO-overexpressing membranes as the enzyme source (Figure 8B) yielded peaks at m/z 1125.682 and m/z 750.124, which are interpreted as the $[M - 2H]^{2-}$ and $[M - 3H]^{3-}$ ions of monohydroxylated Kdo₂-lipid A, respectively. The prominent triply charged ions near m/z 668.72, present in both spectra, arise from neutral loss of the 3'-secondary acyl chains during ESI/MS, as previously observed for Kdo₂-lipid A (38). The extent of neutral loss from the triply charged ions is much higher than that from the doubly charged ions, which is likely attributed to the fact that the triply charged ions are subjected to more energetic collisional activation than the doubly charged ions

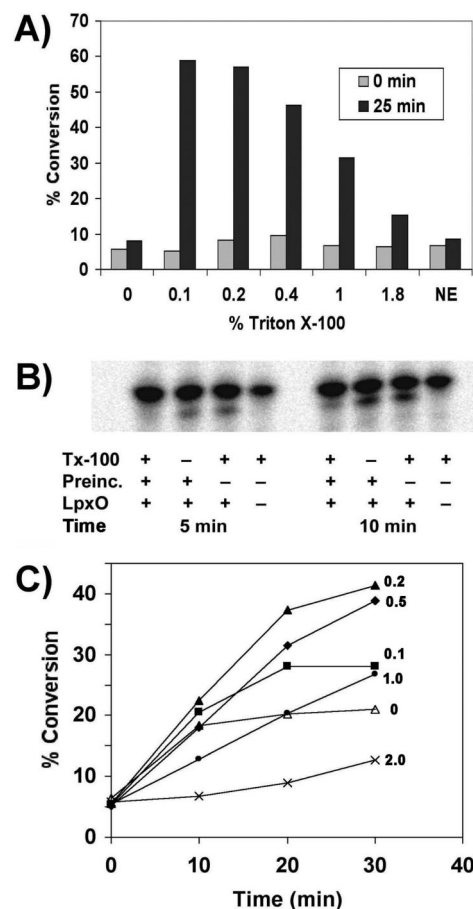


FIGURE 7: Detergent and phospholipid dependence of LpxO activity. (A) LpxO-catalyzed conversion of Kdo₂-lipid A to its hydroxylated derivative was monitored after 25 min in the presence of increasing amounts of Triton X-100 (% v/v) at 0.1 mg/mL membrane protein. NE, no-enzyme control. (B) Inactivation of LpxO by preincubation for 30 min at 0.02 mg/mL membrane protein in assay buffer containing Triton X-100 prior to initiation of the reaction by addition of the full set of cofactors and substrates to give a final membrane protein concentration of 0.01 mg/mL. (C) Addition of *E. coli* phospholipids to the LpxO assay cocktail partially stabilizes LpxO activity, when assayed at 0.01 mg/mL membrane protein. Time courses of product formation (without preincubation) were followed for assay mixtures supplemented with 0 (Δ), 0.1 (\blacksquare), 0.2 (\blacktriangle), 0.5 (\blacklozenge), 1.0 (\bullet), or 2 mg/mL (\times) of exogenous *E. coli* phospholipids (as indicated by the numbers on the graph). The rate and extent of product formation with time were deemed optimal with 0.2 or 0.5 mg/mL phospholipids.

in the ion source region. The identical mass of this species in the spectra of both the substrate and the product provides unequivocal evidence that the LpxO-catalyzed hydroxylation of Kdo₂-lipid A is restricted to the 3'-secondary myristate chain, as previously observed in living cells (27).

GC/MS Analysis of the Hydroxylated Fatty Acid Generated *in Vitro*. To prove that 2-hydroxymyristate is indeed generated in our *in vitro* system, the hydroxylated Kdo₂-lipid A product formed by the LpxO-expressing membranes was subjected to GC/MS analysis, as described in Materials and Methods, and compared to unmodified Kdo₂-lipid A reisolated from the vector control reaction mixture, as well as to the standards (3-hydroxymyristic acid and 2-hydroxymyristic acid). The vector control material (Figure 9A) yielded only the TMS derivative of 3-hydroxymyristoylmethyl ester, as judged by its retention time, whereas the material from the LpxO-expressing membranes contained an additional shoul-

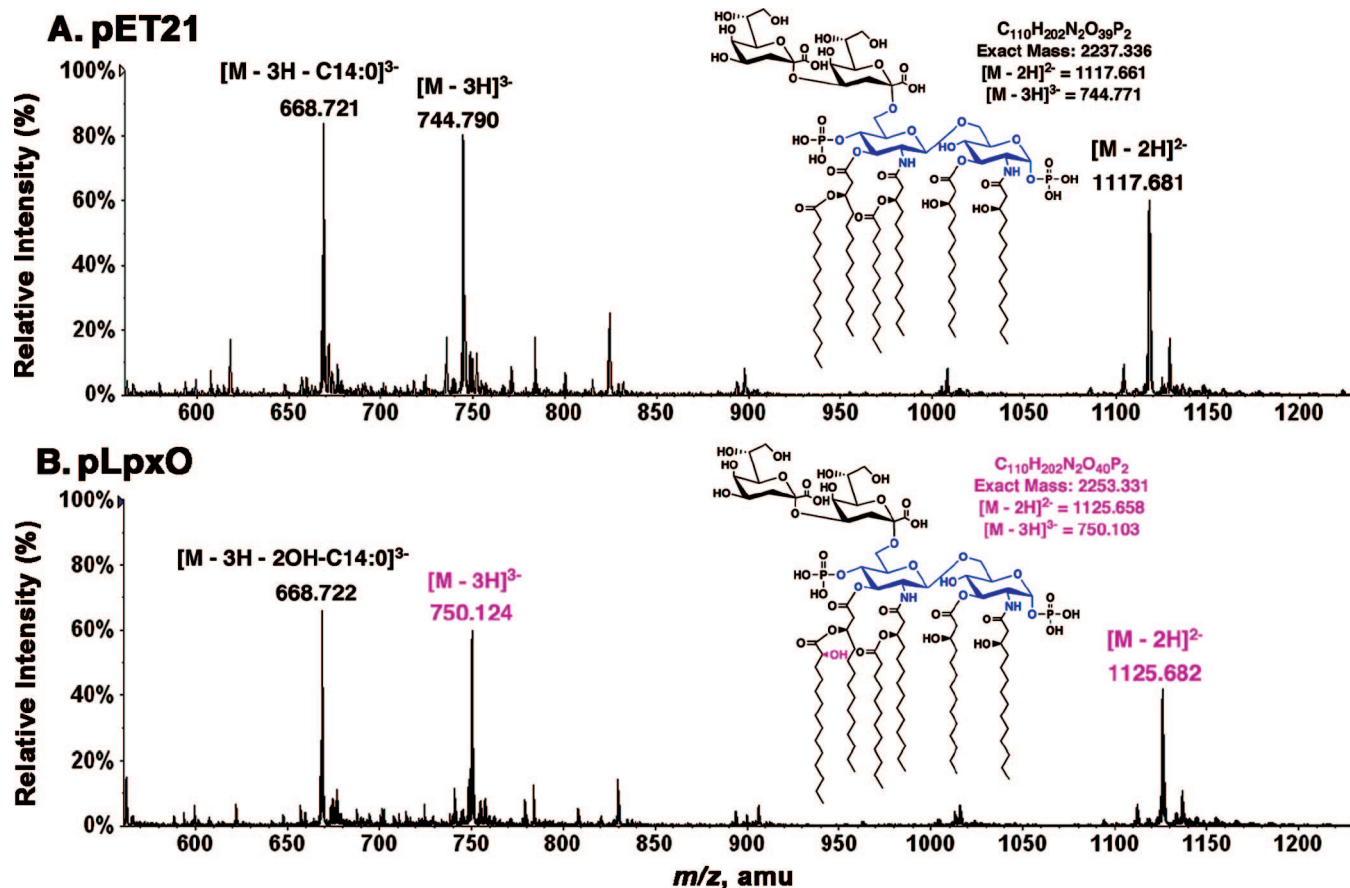


FIGURE 8: ESI/MS analysis of the LpxO reaction product. Negative-ion mode ESI/MS spectra were acquired for repurified Kdo₂-lipid A samples that had been incubated in vitro for 2 h under assay standard conditions with 0.1 mg/mL membranes from either the vector control strain harboring pET21a⁺ (A) or from the LpxO-overexpressing strain harboring pHSG2 (B). The $[M - 2H]^{2-}$ ions at *m/z* 1117.681 and at *m/z* 1125.682 correspond to the substrate (Kdo₂-lipid A) and its hydroxylated product, respectively. The $[M - 3H]^{3-}$ ions of these two species (at *m/z* 744.790 and *m/z* 750.124, respectively) are also very prominent, as noted previously (38). The triply charged ions near *m/z* 688.72, seen in both samples, arise by neutral loss of the 3'-secondary acyl chain from the triply charged Kdo₂-lipid A ions. The extensive neutral loss from the triply charged ions is likely due to the fact that the triply charged ions undergo more energetic collisional activation in the ion source region during ESI/MS than do the doubly charged ions (38). This unusual property of Kdo₂-lipid A (and its hydroxylated derivative) confirms that the LpxO-dependent hydroxylation of Kdo₂-lipid A occurs exclusively on the 3'-secondary acyl chain in vitro, as it does in vivo.

der with the retention time (Figure 9B) expected for the TMS derivative of 2-hydroxymyristoylmethylester. The EI/MS spectra of the major and minor peaks from Figure 9B are shown in Figure 9C and D, respectively. The spectra of these isomeric species are clearly different and correspond to the EI/MS spectra of the standards for a TMS derivative of 3-hydroxymyristoylmethylester and of a TMS derivative of 2-hydroxymyristoylmethylester, respectively (see Supporting Information, Figure S1). The key fragmentations are indicated in the inserted structures. The fact that a 2-hydroxymyristoyl group is generated by LpxO on Kdo₂-lipid A provides unequivocal evidence for the proposed location of the hydroxylation reaction (Figure 2), on the basis of the known structure of Kdo₂-lipid A. The only remaining ambiguity is whether the in vitro system generates the physiological S-hydroxymyristate moiety or a mixture of stereoisomers.

DISCUSSION

Although 2-hydroxyacyl chains have been recognized as constituents of some LPSs for over 30 years (28, 53–58), the biosynthesis and function of these moieties remain poorly characterized. A possible mechanism for its biosynthesis was

suggested by the discovery of the *Salmonella lpxO* gene (27), which encodes a novel member of the Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenase family. Expression of *lpxO* in *E. coli* K-12, which normally lacks this gene, confers upon *E. coli* the ability to synthesize 2-hydroxymyristate modified lipid A in the presence of O₂ (27). Conversely, *S. typhimurium* mutants lacking *lpxO* (5) lose the ability to generate the lipid A species containing 2-hydroxymyristate (Figure 3B).

In the present study, we have devised the first in vitro enzymatic assay system for detecting and quantifying LpxO activity, predicated on the assumption that LpxO is an Fe²⁺/O₂/α-ketoglutarate-dependent dioxygenase (Figure 2A). Hexaacylated Kdo₂-[4'-³²P]lipid A from *E. coli* (23, 38) was used as the acceptor substrate in the presence of appropriate cofactors (Figure 2A) and membranes from an *E. coli* strain that overexpresses *Salmonella* LpxO. As shown in Figures 5 and 6, LpxO activity is proportional to time and protein concentration, and it is dependent upon α-ketoglutarate and ascorbate (Figure 6A). It is stimulated by added Fe²⁺ (Figure 6A) and is inhibited by the chelator 2,2'-bis-pyridyl (Figure 6B). The inclusion of catalase in the assay system (59, 60) partially eliminates the ascorbate requirement (data not

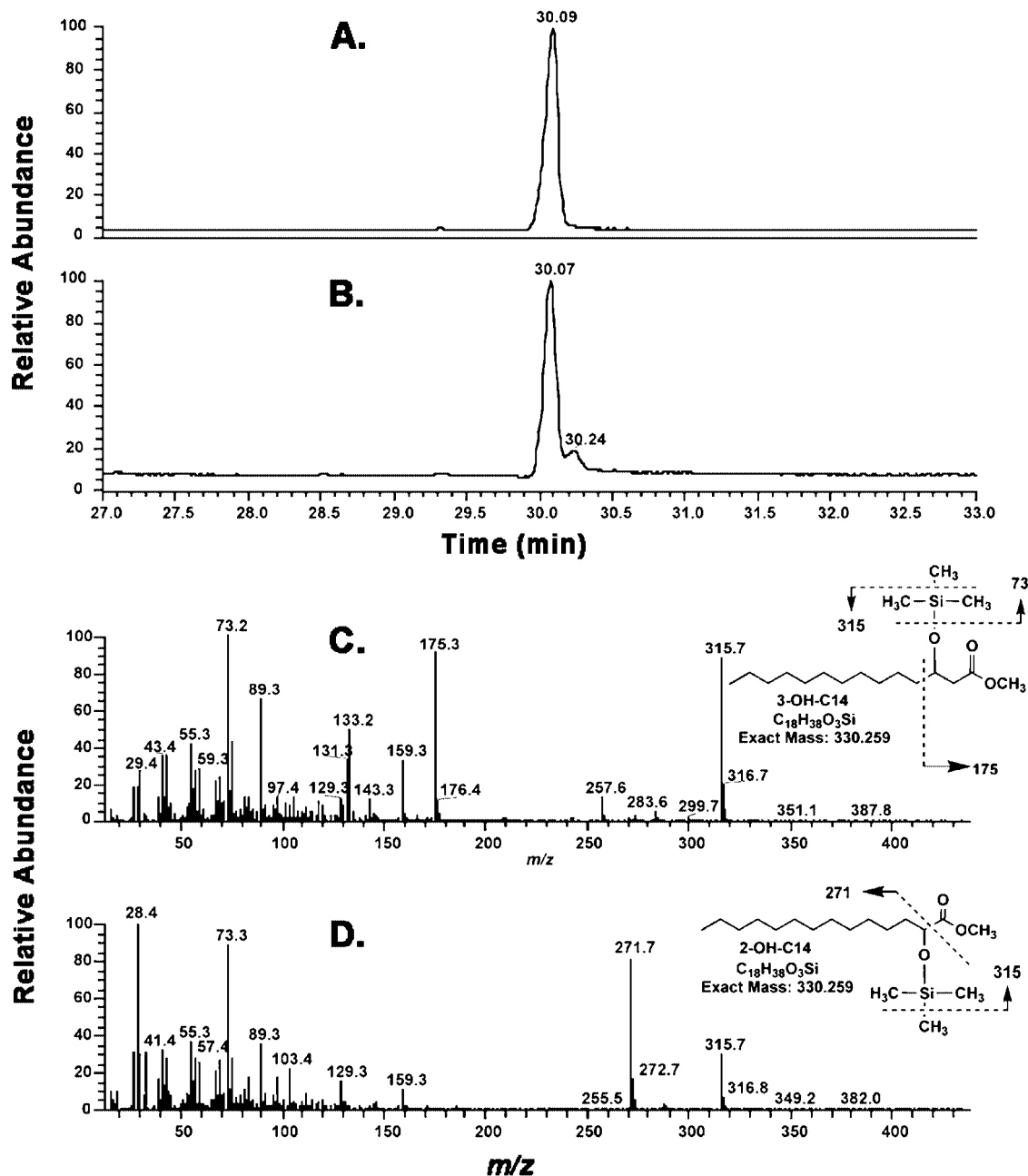


FIGURE 9: GC/MS analysis of the hydroxylated fatty acids in the *in vitro* product generated by LpxO. Kdo₂-lipid A was incubated with vector control or LpxO membranes, as described in Materials and Methods. The Kdo₂-lipid A or the hydroxylated product formed by LpxO was reisolated as a mixture from the reaction system, hydrolyzed, and converted to TMS derivatives to generate the fatty acid methylesters. (A) Total ion chromatogram (TIC) of the GC/MS analysis of the hydroxy fatty acids from the vector control reaction, showing the presence of a single peak consistent with a TMS-3-hydroxymyristoylmethylester. (B) TIC of the GC/MS analysis of the hydroxy fatty acids from the LpxO reaction system, showing the presence of a shoulder peak with the expected retention time for a TMS-2-hydroxymyristoylmethylester. The peak area ratio between the TMS-3-hydroxymyristoylmethylester and the TMS-2-hydroxymyristoylmethylester is about 5 to 1. (C) EI/MS spectrum of the major leading peak in panel B (retention time: 30.07 min), showing identity with a TMS-3-hydroxymyristoylmethylester standard (see Supporting Information, Figure S1). (D) EI/MS spectrum of the minor lagging peak in panel B (retention time, 30.24 min), showing identity with a TMS-2-hydroxymyristoylmethylester standard (see Supporting Information, Figure S1). The proposed origins of the major fragments are indicated.

shown), demonstrating that ascorbate is not absolutely necessary. Furthermore, *E. coli* cells do not synthesize ascorbate but nevertheless carry out lipid A 2-hydroxylation when *lpxO* is overexpressed from a hybrid plasmid (27). Some other endogenous reductant must be substituting for ascorbate *in vivo*.

LpxO activity is found exclusively in the particulate fraction (Figure 4A), consistent with the hydropathy analysis (Figure 2B). Its active site presumably is localized on the inner surface of the inner membrane. This topography would

give LpxO access to its water-soluble cosubstrates (Figure 2A), particularly to Fe^{2+} , which is readily converted to Fe^{3+} in an aqueous aerobic environment. Further evidence for the topography of LpxO comes from experiments in which LpxO is expressed in an *E. coli* strain harboring a temperature-sensitive point mutation in the ABC transporter MsbA (12). These mutants fail to transport newly synthesized core-lipid A across the inner membrane after 30 min at 44 °C (12, 40). However, under these conditions lipid A is still synthesized and normally 2-hydroxylated in this mutant (12). This finding

demonstrates that 2-hydroxylation occurs before MsbA-driven transport of core-lipid A to the periplasmic surface of the inner membrane (1, 40). The other covalent modifications of lipid A, shown in Figure 1, are catalyzed by extracellular enzymes, which are located either on the periplasmic surface of the inner membrane or in the outer membrane (1). Consequently, these enzymes are all MsbA-dependent in living cells (1).

How LpxO orthologues discriminate between the secondary acyl chains of lipid A, or for that matter between lipid A and glycerophospholipids, is uncertain. One important observation is that LpxO, like LpxL (61), is dependent upon the presence of the Kdo disaccharide, thereby targeting LpxO to LPS. In *S. typhimurium* and *S. minnesota* lipid A, the secondary 2-hydroxymyristate moiety appears to be attached exclusively to the 3'-R-3-hydroxymyristate chain (Figure 1) (14, 27, 62). *P. aeruginosa* contains two LpxO orthologues (63), and in fact, the two secondary acyl chains of its lipid A are both modified with 2-OH groups (64, 65). Each of these LpxO orthologues is probably responsible for hydroxylating only one of the two secondary acyl chains of *P. aeruginosa* lipid A, which are located at positions 2 and 2' (64). Purification of LpxO to homogeneity and structural studies will be needed to address the details of its substrate selectivity. The availability of pure enzyme would also facilitate the unequivocal demonstration of stoichiometric formation of succinate and CO₂ in conjunction with lipid A 2-hydroxylation (Figure 2A).

The phosphatidylethanolamine species of *Burkholderia cepacia* (66–68) and the ornithine amide lipids *Rhizobium tropici* (69) contain putative 2-hydroxyacyl chains. These organisms possess genes that are distantly related to *lpxO* (69). The single *lpxO* homologue present in *R. tropici* (known as *olsC*) is strictly required for the 2-hydroxylation of the ornithine lipids, as judged by the characterization of an *olsC* mutant (69). LpxO contains the canonical HXDX_{40–60}H motif, which is characteristic of most Fe²⁺/O₂/α-ketoglutarate-dependent hydroxylases. In primary sequence, OlsC (69) more closely resembles the mammalian aspartate β-hydroxylase (70–72), but in vitro assays for OlsC have not yet been reported.

The function of lipid A 2-hydroxylation remains uncertain. On the basis of its chromatographic properties, a large fraction of the lipid A species isolated from *S. typhimurium* grown inside of macrophage tumor cells is 2-hydroxylated (5). However, *Salmonella lpxO* mutants are fully virulent in RAW 264.7 macrophage tumor cells (5) and in a mouse infection model (Romilanus, E., and Maskell, D., personal communication). There are no gross defects in outer membrane permeability, as judged by disk diffusion assays with erythromycin or rifampicin on LB broth plates (data not shown). However, plate-based assays may not be sensitive enough to reveal subtle roles for *lpxO* in regulating outer membrane permeability. Recently, Nikaido and co-workers reported that an *lpxO* mutant bound more of a hydrophobic dye than did the isogenic wild-type strain (29, 30), implying a role for LpxO in the regulation of outer membrane permeability to some compounds. Testing the membrane permeability of strains containing the *lpxO::kan* mutation in combination with mutations of other genes involved in lipid A modification (Figure 1), as recently reported for ethidium uptake (30), might reveal additional phenotypes.

The relatively wide distribution of LpxO orthologues among diverse Gram-negative bacteria suggests that they must play an important biological role. A PSI-BLAST search of the current nonredundant protein database reveals close orthologues (>50% amino acid sequence identity) in strains of *Burkholderia*, *Pseudomonas*, *Serratia*, *Klebsiella*, *Xanthomonas*, *Ralstonia*, *Xylella*, *Acinetobacter*, *Chromobacterium*, *Bordetella*, *Azotobacter*, *Bradyrhizobium*, and many others. A mutant of *R. tropici* with a transposon insertion in the *olsC* gene lacks 2-hydroxylated ornithine amide lipids and is deficient in the colonization of root nodules (69). Like LpxO, with which it shares 35% sequence identity and 51% similarity over 168 amino acids, OlsC is predicted to be an inner membrane protein (69).

We are currently attempting to solubilize and purify *S. typhimurium* LpxO. We have generated a C-terminal hexahistidine fusion construct, the in vitro activity of which is indistinguishable from that of wild-type LpxO (unpublished results). Assuming that the enzyme can be stabilized in the presence of detergents, purification of LpxO and related hydroxylases to homogeneity should be possible, permitting direct assessment of its metal content and catalytic mechanism.

SUPPORTING INFORMATION AVAILABLE

GC/MS analysis of 3-hydroxymyristate and 2-hydroxymyristate standards as the TMS derivatives of their methyl esters. Sample preparation and GC/MS conditions are described in the manuscript. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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