An Outer Membrane Enzyme Encoded by Salmonella typhimurium lpxR That Removes the 3’-Acyloxyacyl Moiety of Lipid A*

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The Salmonella and related bacteria modify the structure of the lipid A portion of their lipopolysaccharide in response to environmental stimuli. Some lipid A modifications are required for virulence and resistance to cationic antimicrobial peptides. We now demonstrate that membranes of Salmonella typhimurium contain a novel hydrolase that removes the 3’-acyloxyacyl residue of lipid A in the presence of 5 mM Ca²⁺. We have identified the gene encoding the S. typhimurium lipid A 3’-O-deacylase, designated lpxR, by screening an ordered S. typhimurium genomic DNA library, harbored in Escherichia coli K-12, for expression of Ca²⁺-dependent 3’-O-deacylase activity in membranes. LpxR is synthesized with an N-terminal type I signal peptide and is localized to the outer membrane. Mass spectrometry was used to confirm the position of lipid A deacetylation in vitro and the release of the intact 3’-acyloxyacyl group. The heterologous expression of lpxR in the E. coli K-12 W3110, which lacks LpxR, resulted in production of significant amounts of 3’-O-deacetylated lipid A in growing cultures. Orthologues of LpxR are present in the genomes of E. coli 0157:H7, Yersinia enterocolitica, Helicobacter pylori, and Vibrio cholerae. The function of LpxR is unknown, but it could play a role in pathogenesis because it might modulate the cytokine response of an infected animal.

Salmonella typhimurium and related organisms are enteric Gram-negative bacteria. S. typhimurium cause gastroenteritis in human hosts but in mice can produce a fatal, typhoid-like syndrome, as well as in human hosts but in mice can produce a fatal, typhoid-like syndrome, as well as in human (1, 2). These bacteria invade the epithelial cells and M cells of Peyer patches and then pass into the lymphatic system by colonizing phagocytic cells. Subsequently, the bacteria survive and multiply within modified vacuoles of macrophages that can ultimately produce macrophage apoptosis (3–6).

Lipopolysaccharide (LPS) is the principal component of the outer leaflet of the outer membrane of Gram-negative bacteria. Recognition of LPS by the mammalian innate immune system results in the production of cell adhesion proteins in endothelial cells and of pro-inflammatory molecules such as tumor necrosis factor-α and interleukin-1β in monocytes (7, 8). Lipid A, the hydrophobic anchor of LPS, produces most of these responses (9, 10) after its detection by Toll-like receptor 4 (TLR-4) (11–13). Lipid A of S. typhimurium and Escherichia coli is a β1’-6-linked disaccharide of glucosamine, phosphorylated at the 1 and 4’ positions and acylated at the 2, 3, 2’, and 3’ positions with R-3-hydroxyxymristate (Fig. 1A) (9, 14, 15). The hydroxyl groups of the R-3-hydroxyxymristate residues, attached at positions 2’ and 3’, are further acylated with laurate and myristate, respectively. Key features of the molecule shown to be important for TLR-4 activation include the phosphate groups and the secondary acyl chains (15–17). The biosynthetic enzymes that generate lipid A have been identified and their corresponding structural genes cloned, primarily from E. coli (15).

In response to certain environmental conditions, S. typhimurium synthesizes additional enzymes that covalently alter lipid A (Fig. 1B). The inner membrane enzymes 1,4-aminoarabinose transferase (ArnT) (18) and phosphoethanolamine transferase (EptA) (PmrC) (19, 20) can modify the phosphate moieties of lipid A with 4-amino-4-deoxy-l-arabinose or phosphoethanolamine groups, respectively. The addition of 4-amino-4-deoxy-l-arabinose and phosphoethanolamine groups to lipid A has been correlated with resistance to cationic antimicrobial peptides such as polymyxin (21–24). In addition, the inner membrane of S. typhimurium contains an Fe²⁺/α-ketoglutarate/O₂-dependent enzyme, designated LpxO, that hydroxylates position 2 of the 3’ secondary myristate chain (25). Two outer membrane enzymes, PagP (26) and PagL (27), remodel lipid A. PagP catalyzes the addi-

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tion of a phospholipid-derived palmitate chain to the hydroxyl of the R-3-hydroxy-myristate chain at the 2 position of lipid A. PagL catalyzes the removal of the ester-linked R-3-hydroxy-myristoyl chain at the 3 position of lipid A.

We now report a novel, Ca\(^{2+}\)-dependent 3'-O-deacylase present in the membranes of S. typhimurium (Fig. 2). The structural gene (lpxR) encoding the 3'-O-deacylase was identified and expressed in E. coli K-12. LpxR, like PagP and PagL, is localized to the outer membrane. Expression of LpxR in the E. coli K-12 strain W3110 results in a significant production of 3'-O-deacylated lipid A species but does not affect growth. Orthologues of S. typhimurium LpxR are found in the genomes of several other Salmonella species, including both typhi and paratyphi, as well as several other Gram-negative pathogens. The function of LpxR is not known; however, partial 3'-O-deacylation of lipid A might be advantageous during infection, allowing S. typhimurium to evade the innate immune response by remodeling its lipid A.

**EXPERIMENTAL PROCEDURES**

*Materials—*\( ^{32}P \) and \( [\gamma-^{32}P]ATP \) were obtained from PerkinElmer Life Sciences. Silica gel 60 (0.25 mm) thin layer
LpxR, a $\text{Ca}^{2+}$-dependent Lipid A-3′-O-deacylase

| Strain/Plasmid | Description | Source or reference |
|----------------|-------------|---------------------|
| E. coli strains | mcrABC recA1 endA1 pyrG196 relA1 supE44 thi-1 lac | Stratagene |
| XLI Blue-MR | Wild type, F-, λ | Novagen |
| W3110 | K-12 strain, DE3 lysogen | 41 |
| NovaBlue(DE3) | W3110 mtl, Δ(waaC-waaF)-tet,tet, heptose-deficient | 37 |
| WBB06 | 3′-O-deacylase-deficient | 36 |
| MKV15 | 3′-O-deacylase-deficient | 36 |

**TABLE 1**
Relevant bacterial strains and plasmids

| Strain/Plasmid | Description | Source or reference |
|----------------|-------------|---------------------|
| S. typhimurium strain | Virulent wild type | Salmonella Genetic Stock Center, University of Calgary, Canada |
| LT2 | | |

**Plasmids**

| PET23a | Expression vector, T7 promoter, amp' | Novagen |
| pWSK29 | Low copy expression vector, lac promoter, amp' | 33 |
| pLpxR1 | pET23a expressing lpxR (STM1328) | This work |
| pLpxR2 | pWSK29 expressing lpxR (STM1328) | This work |

chromatography (TLC) plates were from Merck. Chloroform, ammonium acetate, and sodium acetate were obtained from EM Science. Trypsone and yeast extract were from Difco. The bicinechonic acid protein determination kit and Triton X-100 were from Pierce. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

**Bacterial Strains**—The bacterial strains used in this study are described in Table 1. Typically, bacteria were grown at 37 °C in LB medium (28). When required for selection of plasmids, cells were grown in the presence of 100 μg/ml ampicillin, 12 μg/ml tetracycline, 25 μg/ml chloramphenicol, or 30 μg/ml kanamycin.

**Molecular Biology Applications**—Protocols for handling of DNA samples were those of Sambrook and Russell (29). Transformation-competent cells of *E. coli* were prepared by the method of Inoue et al. (30). When required, *E. coli* cells were prepared for electroporation by the method of Sambrook and Russell (29). Plasmids were isolated using the Qiagen Spin Prep kit. DNA fragments were isolated from agarose gels using the QIAquick gel extraction kit. Genomic DNA was isolated using the protocol for bacterial cultures in the Easy-DNA™ kit (Invitrogen). T4 DNA ligase (Invitrogen), restriction endonucleases (New England Biolabs), and shrimp alkaline phosphatase (U.S. Biochemical Corp.) were used according to the manufacturers’ instructions. Double-stranded DNA sequencing was performed with an ABI Prism 377 instrument at the Duke University DNA Analysis Facility. Primers were purchased from MWG-Biotech.

**Screening of a S. typhimurium Genomic DNA Library for the 3′-O-Decylase**—A bacterial artificial chromosome (BAC) library generated from genomic DNA of the *S. typhimurium* strain LT2 and harbored in an *E. coli* K-12 background was obtained from the *Salmonella* Genetic Stock Center (University of Calgary). Each of the 71 BAC clones harbors the pBeloBAC11 vector (31) with inserts of *S. typhimurium* genomic DNA. The BAC clones were grown in 5 ml of LB medium with 25 μg/ml chloramphenicol to an $A_{600}$ of 1.0. The membrane fraction of each BAC clone was prepared as previously described (32) and assayed for 3′-O-deacylase activity. Protein concentrations were determined by the bicinechonic acid method using bovine serum albumin as the standard.

**Construction of lpxR Expression Vectors**—*S. typhimurium* lpxR, formerly STM1328, was cloned into pET23a (Novagen) behind the T7 promoter. The predicted coding region for lpxR was amplified by PCR from *S. typhimurium* strain LT2 genomic DNA using *Pfu* DNA polymerase (Stratagene), according to the manufacturer’s instructions. The forward primer contained a clamp region, an NdeI site (underlined), and the lpxr-coding region with its stop codon (bold). The reverse primer contained a clamp region, a BamHI site (underlined) and the coding region with its stop codon (bold). Sequences of the forward and reverse primers were 5′-GGCGGCAATATGACAAATAATACGCTATTGCGCAACG-3′ and 5′-GGCGGCGGATCCCATCTTGCAGATC-3′, respectively. The PCR product and the vector were both digested with NdeI and BamHI, ligated together, and transformed into XL-1 Blue cells (Stratagene) for propagation of the plasmid, designated pLpxR1. For some experiments, pLpxR1 was used directly for LpxR expression in *E. coli* NovaBlue(DE3) (Table 1). Alternatively, the *lpxr* gene was transferred from pET23a to pWSK29 (33), a lac-inducible, low-copy expression vector. The XbaI/BamHI-digested fragment, consisting of the *lpxr* gene as well as the pET23a-derived ribosome binding site, was ligated to the corresponding restriction sites of pWSK29. This plasmid, designated pLpxR2, was then transformed into competent *E. coli* W3110 or *S. typhimurium* LT2.

**Heterologous Expression of Salmonella LpxR**—*E. coli* NovaBlue(DE3) harboring either the pET23a vector or pLpxR1 were grown at 37 °C until the $A_{600}$ reached 0.4. Isopropyl-1-thio-β-D-galactopyranoside at 1 mM was then added, and the cultures were shifted to 25 °C for 4 h more. Washed membranes were prepared as previously described (32) and stored in 50 mM MES, pH 6.5, at −80 °C. Cultures of W3110 harboring either pWSK29 or pLpxR2 were grown at 37 °C with 1 mM isopropyl-1-thio-β-D-galactopyranoside present throughout the growth. Cells were harvested when the $A_{600}$ of the cultures reached 1.0, and washed membranes were prepared as previously described (32). Membranes were also prepared from LB-grown cultures of *S. typhimurium* LT2 grown without CaCl$_2$ or in the presence of 5 mM CaCl$_2$, using the methods described above.

**Preparation of Radiolabeled and Carrier Substrates**—The 4′-32P-labeled lipid IV$_A$ was generated from 100 μCi of [γ-32P]ATP and the disaccharide 1-phosphate lipid acceptor
using the overexpressed 4'-kinase present in membranes of E. coli BLR(DE3)/pLysS/pKJ2 (18, 34, 35). Kdo\textsubscript{2-4'\textsuperscript{32}P}-labeled lipid IV\textsubscript{A} was prepared by adding purified E. coli Kdo transferase (WaaA) immediately after the 4'-kinase reaction (35, 36). Kdo\textsubscript{2-4'\textsuperscript{32}P}-labeled lipid A was synthesized enzymatically from Kdo\textsubscript{2-4'\textsuperscript{32}P}-labeled lipid IV\textsubscript{A} (32). Carrier lipid IV\textsubscript{A} (5–10 mg) was isolated from E. coli strain MKV15 (37–39). Carrier Kdo\textsubscript{2-}lipid IV\textsubscript{A} was synthesized in vitro by WaaA-catalyzed Kdo additions to lipid IV\textsubscript{A} (40). Carrier Kdo\textsubscript{2-}lipid A was isolated from the heptose-deficient E. coli strain WBB06 (41), described previously (42).

**Assay of the 3'-O-Deacylase—**The 3'-O-deacylase activity of LpxR was assayed under optimized conditions in a 15-μL reaction mixture containing 50 mM MES, pH 6.5, 1% Triton X-100, 5 mM CaCl\textsubscript{2}, and either 10 μM Kdo\textsubscript{2-4'\textsuperscript{32}P}-labeled lipid A, Kdo\textsubscript{2-4'\textsuperscript{32}P}-labeled lipid IV\textsubscript{A}, or 4'-32P-labeled lipid IV\textsubscript{A} (each at 50,000 cpm/nmol) as the substrate. Washed membranes were employed as the enzyme source. Reactions were incubated at 30 °C for the indicated times. Reactions were stopped by the addition of a few drops of a saturated solution of 6-aza-2-thiothymine in 50% acetoni-trile and 10% tribasic ammonium citrate (9:1, v/v). After chromatography, the plates were dried, and radioactive substrates were detected using a GE Healthcare PhosphorImager (STORM 840) equipped with ImageQuant software. The enzyme units (nmol/min) were calculated by determining the percentage of the substrate converted to product.

**Purification of the Salmonella 3'-O-Deacylase in Vitro Reaction Product—**A large scale 3'-O-deacylase reaction mixture was prepared by assembling the following components: 50 mM MES, pH 6.5, 1% Triton X-100, 5 mM CaCl\textsubscript{2}, and either 1 mg/ml S. typhimurium strain LT2 membranes or 0.1 mg/ml NovaBlue(DE3)/pLpxR1 membranes, as described below. The reaction was initiated by the addition of the membranes and incubated at 30 °C for 12 h. The reaction mixture was then converted into a two-phase Bligh/Dyer (43) system consisting of chloroform, methanol, and 0.1 M HCl (2:2:1.8, v/v). The phases were separated by centrifugation at 4000 × g for 8 min. The organic phase was removed, and the resulting aqueous phase was extracted a second time by the addition of pre-equilibrated acidic organic phase. The organic phases were pooled and neutralized by the addition of a few drops of pyri-dine. The sample was dried by rotary evaporation, and lipids were fractionated using a DEAE cellulose anion-exchange column as previously described (44). Fractions containing the desired lipid species were pooled and converted to an acidic two-phase Bligh/Dyer mixture by the addition of appropriate amounts of chloroform and HCl. The organic phase was dried under a stream of N\textsubscript{2} and stored at −80 °C.

**Separation of Inner and Outer Membranes—**Membranes isolated from either W3110/pWSK29 or W3110/pLpxR2 were separated by isopycnic sucrose gradient centrifugation using previously described methods (45, 46). Each gradient fraction (0.5 ml) was assayed for NADH oxidase (inner membrane marker) and phospholipase A (outer membrane marker) (47). The amount of protein in each fraction was determined using the bicinchoninic acid assay (48). Each fraction was also assayed for 3'-O-deacylase activity using the standard conditions described above.

**Protein Microsequencing of LpxR—**Inner and outer membranes of W3110/pWSK29 and W3110/pLpxR2 were analyzed by SDS-PAGE on a Bio-Rad Protein II XI apparatus with a 12% polyacrylamide, 1.5 mm thick gel (49). A piece of the gel containing LpxR was excised, rinsed three times with distilled water, and analyzed by high sensitivity protein microsequencing at the Harvard Microchemistry and Proteomics Facility (Cambridge, MA).

**Large Scale Isolation of Lipid A from LpxR-expressing Cultures—**Cultures of E. coli strain W3110 harboring either pWSK29 or pLpxR2 were grown in 1 liter of LB medium as described above. The cultures were harvested by centrifugation at 5000 × g for 20 min when the A\textsubscript{600} reached 1.0. The cells were washed once with 40 ml of phosphate-buffered saline, pH 7.4. The final cell pellet was resuspended in 40 ml of phosphate-buffered saline, pH 7.4. Lipid A was released from the LPS, purified as described previously (38, 39), and stored frozen at −80 °C. Lipid A was also prepared from CaCl\textsubscript{2} (10 mM)-treated cultures of S. typhimurium strain LT2 harboring either pWSK29 or pLpxR2, as just described.

**Mass Spectrometry of Kdo\textsubscript{2-}-Lipid A or Lipid A Samples—**Spectra were acquired in the negative-ion linear mode using an AXIMA-CFR (Kratos Analytical, Manchester, UK) matrix-assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometer with a 337-nm nitrogen laser. The instrument was operated with a 20-kV extraction voltage and time-delayed extraction, providing a mass resolution of about ±1 atomic mass unit for compounds with a M\textsubscript{r} of ~2,000. Each spectrum was the average of 100 laser shots. Samples were prepared for MALDI-TOF analysis by depositing 0.3 μl of the lipid dissolved in chloroform/methanol (4:1, v/v) followed by 0.3 μl of a saturated solution of 6-aza-2-thiothymine in 50% acetonitrile and 10% tribasic ammonium citrate (9:1, v/v) as the matrix. The samples were dried at room temperature before the spectra were acquired.

**Mass Spectrometry of the Released 3’-AcOxyacyl Moiety—**Spectra were acquired on a QSTAR XL quadrupole TOF tandem mass spectrometer (ABI/MDS-Sciex, Toronto, Canada) equipped with an electrospray ionization (ESI) source. Spectra were acquired in the negative-ion mode and typically were the accumulation of 60 scans collected from 200–2000 atomic mass units. For mass spectrometry (MS) analysis, the extracted lipids were dissolved in 200 μl of chloroform/methanol (2:1, v/v) and infused into the ion source at 5–10 μl/min. The nega-
Detection of a novel 3'-O-deacylase in membranes of wild type *S. typhimurium* strain LT2.

**A.** Kdo₂-[4'-³²P]-lipid A

- **Lane 1**: no enzyme
- **Lane 2**: *S. typhimurium* grown without added Ca²⁺
- **Lane 3**: *S. typhimurium* grown with added Ca²⁺ (5 mM)
- **Lane 4**: *S. typhimurium* grown without added Ca²⁺
- **Lane 5**: *S. typhimurium* grown with added Ca²⁺ (5 mM)

5 mM Ca²⁺ in Medium: - - + - +
5 mM Ca²⁺ in Assay: - - - + +

**B.** % of Conversion to Product A

Time, min

**C.** Kdo₂-lipid A and its deacylated product were partially purified from a large-scale reaction mixture, prepared with 1 mg/ml *S. typhimurium* LT2 membranes. The lipids were analyzed by negative-ion mode MALDI-TOF mass spectrometry. amu, atomic mass units.

**FIGURE 3.** Detection of a novel 3'-O-deacylase in membranes of wild type *S. typhimurium* strain LT2. A. membranes (0.02 mg/ml) from wild type *S. typhimurium* LT2 grown with or without 5 mM CaCl₂ were assayed with 10 μM Kdo₂-[4'-³²P]-labeled lipid A in the absence or presence of 5 mM CaCl₂. The reactions were incubated at 30 °C for 60 min. Products were separated by TLC in the solvent chloroform/methanol/water/acetic acid (25:15:4:4, v/v) and analyzed with a PhosphorImager. Lane 1, no enzyme; lane 2, *S. typhimurium* grown without added Ca²⁺; lane 3, *S. typhimurium* grown with added Ca²⁺ (5 mM); lane 4, *S. typhimurium* grown without added Ca²⁺; lane 5, *S. typhimurium* grown with added Ca²⁺ (5 mM). B, *S. typhimurium* LT2 membranes (0.1 mg/ml) from the "no Ca²⁺" culture were assayed with 10 μM Kdo₂-[4'-³²P]-labeled lipid A for the indicated times. C, Kdo₂-lipid A and its deacylated product were partially purified from a large-scale reaction mixture, prepared with 1 mg/ml *S. typhimurium* LT2 membranes. The lipids were analyzed by negative-ion mode MALDI-TOF mass spectrometry. amu, atomic mass units.
tive-ion ESI was carried out at −4200 V. Data acquisition and analysis was performed using the Analyst QS software.

**NMR Spectroscopy of 3′-O-Deacylated Lipid A**—The lipid A of *E. coli* W3110 harboring plpxR2 was isolated as described above and further purified by DEAE cellulose anion exchange chromatography (44). Fractions containing 3′-O-deacylated lipid A were pooled, and the lipid was extracted by conversion into a two-phase Bligh/Dyer system (43). The lower phase was dried under a stream of N₂, and 0.5 mg of the dried lipid was dissolved in 0.35 ml of CDCl₃/CD₃OD/D₂O (2:3:1, v/v) in a 3-mm NMR tube. NMR spectroscopy was carried out at the Duke University NMR Spectroscopy Center. Proton chemical shifts are reported relative to tetramethylsilane at 0.00 ppm.

**RESULTS**

**Ca²⁺-dependent Conversion of Kdo₂-lipid A to a 3′-O-Deacylated Product**—As shown in Figs. 3, A and B, membranes of *S. typhimurium* LT2 efficiently convert Kdo₂-4′-3₂P-labeled lipid A to an unknown product, designated A, when 5 mM Ca²⁺ is included in the assay (lanes 4 and 5 versus lanes 2 and 3). Formation of A is not strictly dependent upon the addition of Ca²⁺ to the growth medium (lane 4 versus lane 5). The activity is localized primarily to *S. typhimurium* outer membranes, as discussed in detail below (data not shown).

Product A (Fig. 3A), generated in vitro with *S. typhimurium* membranes and Kdo₂-lipid A, was partially purified by chromatography on DEAE cellulose, as described under “Experimental Procedures” and was analyzed by low resolution MALDI-TOF mass spectrometry in the negative ion mode. As shown in Fig. 3C, the predominant peak at m/z 1801.7 atomic mass units can be interpreted as [M − H]⁻ of a 3′-O-deacylated Kdo₂-lipid A derivative, which has a molecular weight of 1802.01. The smaller peak at m/z 1361.5 atomic mass units is due to loss of both Kdo residues from 3′-O-deacylated Kdo₂-lipid A during MALDI-TOF mass spectrometry (44). However, the minor peak at m/z 2040.3 atomic mass units is attributed to [M − H]⁻ of a 3′-O-deacylated Kdo₂-lipid A derivative to which an additional palmitate residue was attached in vitro by the outer membrane enzyme PagP (26).

Positive-ion MALDI-TOF mass spectrometry of product A (not shown) confirmed the loss of the 3′-acyloxyacyl group. The spectrum revealed peaks at m/z 1825.1 atomic mass units, interpreted as [M + Na]⁺ of 3′-O-deacylated Kdo₂-lipid A, m/z 1704.8 atomic mass units, interpreted as the B₁⁺ ion of 3′-O-deacylated Kdo₂-lipid A, and m/z 1091.1 atomic mass units, interpreted as the B₂⁺ ion of 3′-O-deacylated Kdo₂-lipid A. The mass of the B₁⁺ oxonium ion, derived from the distal lipid A unit by cleavage of the β₁-6 glycosidic linkage (52), provides additional direct support for the proposed 3′-O-deacylation.

**Identification of the S. typhimurium 3′-O-Deacylase Structural Gene**—To find the gene encoding the *S. typhimurium* 3′-O-deacylase, an expression-cloning strategy was employed. Extracts of *E. coli* K-12 do not exhibit detectable 3′-O-deacylase activity, allowing us to utilize *E. coli* K-12 as the host. A BAC library of *S. typhimurium* LT2 genomic DNA harbored in *E. coli* K-12 was acquired from the *Salmonella* Genetic Stock Center (Calgary, Canada). Assays of the membranes from each of the 71 BAC clones revealed only one (BAC 23) that exhibited...
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3′-O-deacylase activity (data not shown). BAC 23 contained a DNA fragment encompassing bases 1379597–1496842 of the S. typhimurium chromosome (53).

To identify the gene encoding the enzyme, we assumed that an orthologue of the S. typhimurium 3′-O-deacylase was not present in E. coli K-12 and that it was annotated as an outer membrane protein in the S. typhimurium data base. Only one open reading frame, STM1328, within the relevant segment of the S. typhimurium genome met both criteria. The product of STM1328 has a predicted N-terminal signal peptide, consistent with outer membrane targeting (54). A tBLASTn (55) search with the predicted protein product of STM1328 also did not reveal any significant orthologues in E. coli K-12.

STM1328 (renamed lpxR) was subcloned into pET23a to create pLpxR1 and expressed in E. coli NovaBlue(DE3). As shown in Fig. 4, membranes derived from LpxR-expressing E. coli NovaBlue(DE3) exhibited robust in vitro 3′-O-deacylase activity (lane 4) qualitatively similar to that seen with membranes of S. typhimurium LT2 (lane 2). Membranes of E. coli NovaBlue(DE3) harboring the vector control showed no activity (lane 3). The specific activity of S. typhimurium LT2 membranes (0.48 nmol/min/mg) and of the LpxR-expressing E. coli NovaBlue(DE3) membranes (434 nmol/min/mg) were determined using 10 \( \mu \)M Kdo\(_2\)-4′,25′P-labeled lipid A as the substrate.

Table 2 lists some of the closest orthologues of LpxR present in the non-redundant data base. Helicobacter pylori and Yersinia enterocolitica, which have orthologues of LpxR, have been previously reported to contain lipid A species that lack the 3′-acyloxyacyl moiety (56, 57). Ca\(^{2+}\)-dependent 3′-O-deacylase activity is present in the membranes of several Gram-negative pathogens that contain LpxR orthologues, including E. coli O157:H7, Y. enterocolitica, and Vibrio cholerae. The complete nucleotide sequence of lpxR has been submitted to GenBank under accession number DQ272513.

LpxR-catalyzed Production of 3′-O-deacylated Kdo\(_2\)-lipid A and Release of the Intact 3′-Acyloxyacyl Moiety—To confirm that the recombinant LpxR catalyzes the same 3′-O-deacylation of Kdo\(_2\)-lipid A seen with Salmonella membranes (Fig. 3), a large-scale in vitro 3′-O-deacylase reaction mixture was prepared using E. coli NovaBlue(DE3)/pLpxR1 membranes as the enzyme source. After purification over DEAE-cellulose, negative-ion MALDI-TOF mass spectrometry was used to analyze the products eluting with 240 to 480 mM ammonium acetate. The major peak, interpreted as [M−H]− of 3′-O-deacylated Kdo\(_2\)-lipid A, at m/z 1801.9 atomic mass units (data not shown) was the same within experimental error as that seen with wild-type Salmonella membranes (Fig. 3C).

High-resolution electrospray ionization (ESI) mass spectrometry was used to determine whether or not the 3′-acyloxyacyl moiety was released intact from Kdo\(_2\)-lipid A by recombinant LpxR. An analysis of the LpxR-derived fatty acids, which elute with 30 mM ammonium acetate from the DEAE cellulose column, revealed a major species at m/z 453.387 atomic mass units, consistent with the predicted [M−H]− of the cleaved 3′-acyloxyacyl moiety of E. coli lipid A (exact mass of 453.394 atomic mass units). The peak at m/z 453.387 atomic mass units was then subjected to tandem ESI-MS/MS analysis to confirm the structural assignment. Collisional activation of the parent ion at m/z 453.387 generated a product ion at m/z 227.193 atomic mass units (Fig. 5B), as expected for the [M−H]− ion of myristic acid (predicted exact mass of 227.201).

Substrate Specificity of S. typhimurium LpxR—In vitro 3′-O-deacylation of Kdo\(_2\)-4′,3′P-labeled lipid A by LpxR required the presence of the nonionic detergent Triton X-100 with optimal activity at 1%. LpxR also required Ca\(^{2+}\) for activity, with an optimum at 5 mM (data not shown). Production form E. coli NovaBlue(DE3) membranes over-expressing LpxR, assayed with 10 \( \mu \)M Kdo\(_2\)-4′,3′P-labeled lipid A under optimized conditions, was linearly dependent upon both protein concentration and time (data not shown). The ability of LpxR to hydrolyze (Kdo\(_2\)-4′,3′-P-labeled lipid A, Kdo\(_2\)-4′,3′P-labeled lipid IV\(_A\), and 4′,3′P-labeled lipid IV\(_A\)) in vitro was compared. Assays with each of these substrates at 10 \( \mu \)M revealed that 4′,3′P-labeled lipid IV\(_A\) was a relatively poor substrate (1 nmol/min/mg of membrane protein) when compared with Kdo\(_2\)-4′,3′P-labeled lipid IV\(_A\) (132 nmol/min/mg) or Kdo\(_2\)-4′,3′P-labeled lipid A (434 nmol/min/mg). The data show that the 3′-O-deacylase activity is strongly Kdo-dependent.

Divalent Cation Dependence and Thermal Stability of the 3′-O-Deacylase—Membranes of E. coli strain NovaBlue(DE3)/pLpxR1 were assayed for LpxR activity in the presence of various divalent cations, each held at 5 mM (Fig. 6). Ca\(^{2+}\) (lane 5) and Sr\(^{2+}\) (lane 9) produced the greatest stimulation, Cd\(^{2+}\) (lane 15) showed partial stimulation, whereas Mg\(^{2+}\), Zn\(^{2+}\), and Ba\(^{2+}\) were inactive.

The outer membrane enzymes PagP and PagL have been previously shown to resist heat denaturation (26, 27). Similarly, E. coli membranes over-expressing LpxR retained significant activity (~67%) after a 10-min pre-incubation at 100°C (data not shown).

Outer Membrane Localization of Recombinant LpxR—Inner and outer membranes of E. coli W3110/pLpxR2 were separated by isopycnic sucrose gradient centrifugation (Fig. 7). The heavier outer membranes were detected by their phospholipase A activity, whereas the lighter inner membranes were detected by assaying for NADH oxidase (Fig. 7B). LpxR activity was pre-

\[\text{TABLE 2} \]

| Organism  | Homology (gaps)* | Approximate E values |
|-----------|------------------|---------------------|
| S. typhi  | 318/318/319      | 8 × 10⁻⁷⁷           |
| S. paratyphi | 308/308/310   | 2 × 10⁻⁷⁷           |
| E. coli O157:H7 | 228/272/318 (1) | 1 × 10⁻³³           |
| Y. enterocolitica | 230/269/320 (1) | 2 × 10⁻¹²           |
| Rhodospirillum rubrum | 76/117/254 (20) | 8 × 10⁻⁵            |
| Glucobacter oxydans | 60/102/236 (9) | 2 × 10⁻¹¹           |
| Xanthomonas campestris | 59/94/254 (35) | 3 × 10⁻¹¹           |
| V. cholerae V51 | 74/130/338 (61) | 5 × 10⁻¹⁰           |
| H. pylori J99 | 46/103/250 (15) | 7 × 10⁻⁸            |

*Homology is given as the number of identities/number of positives/number of residues (including gaps) in the related segment when compare with S. typhimurium LpxR, a hypothetical protein of 319 amino acid residues.
dominantly localized to the outer membrane (Fig. 7A), although a portion was also recovered in the intermediate region (fractions 9 to 13), which was depleted of both marker enzymes (Fig. 7B).

Total membranes, outer membranes, and inner membranes of W3110, harboring either the vector pWSK29 or pLpxR2, were analyzed by 12% SDS-PAGE. As shown in Fig. 8, a band corresponding to processed LpxR (32.4 kDa) was present in the un-fractionated membranes of W3110/pLpxR2 (lane 3) and absent in the vector control (lane 2). The LpxR band was greatly enriched in the outer membranes (lane 5) versus the inner membranes (lane 7).

To verify the presence of the signal peptide predicted by Signal-P (54) (http://www.cbs.dtu.dk/services/SignalP/) and to establish the exact cleavage site, LpxR from the outer membranes of W3110/pLpxR2 was blotted to a polyvinylidene difluoride membrane and subjected to N-terminal micro-sequencing. The first ten amino acid residues were SSLAISVAND, indicating that the cleavage of the signal peptide occurred between residues 22 and 23 of the full-length protein, consistent with the prediction of the Signal-P program. Thus, mature LpxR is comprised of 296 amino acids with a molecular weight of 32,400.

Decaylation of Lipid A by LpxR in Living Cells—Although wild-type S. typhimurium membranes contain measurable LpxR activity (Fig. 3A), the lipid A of Salmonella is not appreciably 3'-O-deacylated under the commonly used growth conditions (58–61). In contrast, heterologous over-expression of Salmonella LpxR in E. coli K-12 resulted in extensive 3'-O-deacetylation of endogenous lipid A (Fig. 9). The lipid A species, released from cells by hydrolysis at pH 4.5, were purified and analyzed by low-resolution MALDI-TOF mass spectrometry. The endogenous lipid A of W3110/pWSK29 consisted primarily of the hexa-acylated bis-phosphate form, typically seen with wild-type E. coli K-12 strains. The peak at m/z 1798.4 atomic mass units in the negative-ion mode was interpreted as [M – H]⁻ of lipid A (Fig. 9A). Upon over-expression of LpxR, an additional peak was seen at m/z 1361.6 atomic mass units in the negative-ion mode (Fig. 9B), interpreted as [M – H]⁻ of 3'-O-deacylated lipid A. Heterologous expression of LpxR in E. coli did not slow the growth rate (data not shown).

A similar result was seen upon overexpression of the 3'-O-deacylase in S. typhimurium strain LT2. As shown in Fig. 10A, analysis of the lipid A species from S. typhimurium/pWSK29 grown in the presence of 10 mM CaCl₂ yielded a major peak at m/z 1798.3 atomic mass units, interpreted as [M – H]⁻ of lipid A. Calcium was included in the growth media to suppress the PhoP/PhoQ-and/or PmrA/PmrB-regulated lipid A modifications (62). Over-expression of LpxR resulted in an additional

FIGURE 5. LpxR releases the intact 3'-acyloxyacyl moiety from Kdo₂-lipid A. A large scale 3'-O-deacylase reaction using E. coli NovaBlue(DE3)/pLpxR1 membranes (0.1 mg/ml) as enzyme was carried out as described under “Experimental Procedures.” Lipids were fractionated by DEAE cellulose chromatography (44). A, negative-mode ESI-MS analysis of the 30 mM ammonium acetate fraction revealed a major species (453.387 atomic mass units) that could be interpreted as [M – H]⁻ of the intact 3'-acyloxyacyl moiety of lipid A (exact molecular mass = 454.402 atomic mass units). Most of the minor species in this fraction are contaminating phospholipids from the membranes of the LpxR over-producing strain. B, the peak at m/z 453.387 atomic mass units was subjected to tandem MS/MS in the negative mode to confirm the structural assignment. The product ion (227.193 atomic mass units) is interpreted as [myristic acid – H]⁻ (the exact mass of myristic acid = 228.209 atomic mass units). Mass spectrometry of the Kdo₂-lipid A present in the 240 mM ammonium acetate step confirmed the production of 3'-O-deacylated Kdo₂-lipid A by LpxR-expressing E. coli NovaBlue(DE3) membranes (data not shown).
peak at m/z 1361.5 atomic mass units, interpreted as \([M-H]^-\) of the 3’-O-deacylated variant (Fig. 10B).

**NMR Spectroscopy of 3’-O-deacylated Lipid A** Isolated from W3110/pLpxR2—The partial 800 MHz \(^1\)H NMR spectrum of 3’-O-deacylated lipid A (Fig. 11), purified from W3110/pLpxR2, revealed well resolved resonances in the sugar and acyl chain regions (Table 3), similar to those previously reported for wild-type E. coli K-12 lipid A dissolved in CDCl\(_3/CD_3OD/D_2O\) (2:3:1 v/v) (50, 51). The sugar region was comprised of eight resolved single-proton resonances, three 2-proton envelopes (5.2, 3.97 and 3.65 ppm) and one 3-proton envelope (3.82 ppm), consistent with fourteen sugar and three \(\beta\)-hydroxymethine protons (Fig. 11A). The \(\alpha\)CH\(_2\) resonances integrated to eight protons (Fig. 11B), indicating the existence of four acyl chains. Comparison with the \(\alpha\)CH\(_2\) resonances of wild-type E. coli lipid A (50) showed the lack of the 2.7 ppm multiplet, previously assigned to the 3’-acyl chain, and the loss of one \(\alpha\)CH\(_2\) resonance near 2.3 ppm. The NMR data are therefore consistent with the removal of the 3’-acyloxyacyl moiety by LpxR.

The anomeric H-1 of the proximal sugar (5.45 ppm) and the H-1 of the distal sugar (4.54 ppm) served as convenient entry points for full 2D NMR assignments (Table 3), which were based on correlation spectroscopy and total correlation spectroscopy analysis (not shown). The proximal sugar protons (H-1 to H-6) of the 3’-O-deacylated lipid A showed similar chemical shifts to those of wild-type E. coli lipid A (50, 51) (Table 3). Notably, H-3 of the 3’-O-deacylated lipid A at 3.75 ppm (Table 3) was 1.44 ppm upfield compared with H-3 of wild-type E. coli lipid A (5.18 ppm). Other protons of the distal unit were not shifted by more than several tenth of a ppm (Table 3), thus confirming the selective deacylation of the 3’-position by LpxR.
DISCUSSION

Lipid A is present in virtually all Gram-negative bacteria and is detected by the innate immune system receptor TLR-4 (9, 10). Nine constitutive enzymes of lipid A biosynthesis have been identified in *E. coli* (15), and with few exceptions, single copies of the corresponding structural genes are present in all Gram-negative organisms. Despite the conservation of the biosynthetic pathway, which generates the lipid A 1,4'-bisphosphate shown in Fig. 1A, the lipid A moiety of LPS displays significant structural diversity in many important pathogens. For instance, in *Salmonella* lipid A is covalently modified in response to environmental stimuli that activates the two-component regulatory systems, PhoP/PhoQ (63, 64) and PmrA/PmrB (59, 65, 66) (Fig. 1B). Modification of lipid A is correlated with bacterial virulence, antimicrobial peptide resistance, and attenuation of TLR-4 activation (21–24, 66–70). The enzymes catalyzing the covalent modifications of *Salmonella* lipid A (Fig. 1B) have recently been identified and characterized (18–20, 25–27).

Here we report the discovery of LpxR, an outer membrane enzyme catalyzing a novel deacylation of lipid A at the 3'-position. Utilizing an in vitro assay system, we determined that membranes of wild-type *S. typhimurium* convert Kdo2-lipid A to 3'-O-deacylated Kdo2-lipid A (Fig. 3) in the presence of Ca2+. That membranes of *S. typhimurium* could catalyze 3'-O-deacylation of Kdo2-lipid A was surprising given that 3'-O-deacylated lipid A species have not been reported for this organism. However, 3'-O-deacylated lipid A species have been isolated from *H. pylori* (56), *Y. enterocolitica* (57), *Francisella tularensis* (71), and *Porphyromonas gingivalis* (72). Our data demonstrate that a lipid A-3'-O-deacylase is present in the outer membrane of *S. typhimurium* but that it is latent in vivo under all growth conditions examined to date. The previously reported 3'-O-deacylase PagL, which is also found in the outer membrane, displays a similar latency (73). Endogenous inhibitors present in stoichiometric quantities relative to LpxR and PagL might account for these findings. We found that it is possible to generate significant amounts of 3'-O-deacylated lipid A species in *S. typhimurium* LT2 through pWSK29-mediated overexpression of LpxR (Fig. 10B).

We can, thus, hypothesize that an up-regulation of lpxR expression in wild type *Salmonella* would lead to detectable 3'-O-deacylated lipid A species.

LpxR-catalyzed 3'-O-deacylase activity is absolutely dependent upon the presence of Ca2+ in our in vitro system (Fig. 6). Sr2+ and to a lesser extent Cd2+ could also support LpxR activity. LpxR is the second example of an LPS-modifying enzyme that exhibits a requirement for Ca2+. Recently, our laboratory reported that EptB, a phosphoethanolamine transferase specific for the outer Kdo moiety of LPS, is a Ca2+-dependent enzyme (32). LpxR also shares some characteristics with the outer membrane phospholipase A. Outer membrane phospholipase A is an integral Ca2+-dependent membrane enzyme that
participates in secretion of colicins and has been implicated in bacterial virulence (74–76). The activity of outer membrane phospholipase A is regulated by a Ca\(^{2+}\)-induced, reversible dimerization (74). It is possible that Ca\(^{2+}\) may likewise be required to facilitate LpxR dimerization. Interestingly, PagL, which catalyzes a related hydrolysis reaction (3'-O-deacylation), does not require Ca\(^{2+}\) or other divalent cations in vitro (27).

PagL and LpxR may utilize different mechanisms to deacylate the lipid A 3' or 3' positions, respectively. Studies of purified LpxR should provide insights into how Ca\(^{2+}\) modulates the activity of this enzyme.

Mass spectrometry of the reaction products generated during the \textit{in vitro} assay revealed that LpxR selectively cleaves the ester linkage at the 3' position of lipid A, as no 3'-O-deacylated species was detected. NMR spectroscopy of the LpxR-modified lipid A confirmed the selective loss of the 3'-acyloxyacyl group (Fig. 11 and Table 3). Our data further demonstrate that LpxR releases the intact 3'-acyloxyacyl moiety (Fig. 5) and does not further cleave the acyloxyacyl-linkage. Accordingly, it is reasonable that LpxR does not share any sequence similarity with the acyloxyacyl hydrolase (77, 78) of mammalian cells, which removes only the secondary acyl chains of lipid A.

Like the palmitoyltransferase PagP (26) and the 3'-O-deacylase PagL (27), LpxR is associated with the outer membrane. Subcellular fractionation studies of membranes derived from LpxR-expressing \textit{E. coli} W3110 cultures revealed that 3'-O-deacylase activity was present mostly in the outer membrane fractions (Fig. 7). SDS-polyacrylamide gel electrophoresis provided further evidence of LpxR localization within the outer membrane (Fig. 8). The overexpressed LpxR protein was missing its type I signal peptide, as shown by N-terminal sequencing of the outer membrane-associated band. LpxR (~32 kDa) is larger than either PagP (~20 kDa) (26) or PagL (~20 kDa) (27). The crystal structure of PagP revealed that this protein is an eight-stranded, inside-out \(\beta\)-barrel (79). Topology analysis predicts a 14-stranded \(\beta\)-barrel structure for LpxR.

All \textit{S. typhimurium} lipid A-modifying enzymes, with the exception of LpxO, are transcriptionally regulated by PhoP/PhoQ and/or PmrA/PmrB. Recently, SlyA (80, 81), a transcription factor that is under the control of PhoP, was shown to regulate \textit{STM1328} (lpxR) expression. Microarray analysis of a \textit{slyA} deletion mutant of \textit{S. typhimurium} revealed that the expression of \textit{STM1328} was reduced 3-fold (82). However, it is unclear whether or not SlyA regulation of \textit{lpxR} via PhoP is functionally significant at the level of protein synthesis, because membranes isolated from a \textit{Salmonella} phoP null mutant (83) display similar levels of 3'-O-deacylase activity as wild type (data not shown). Other SlyA-regu-

\begin{figure}[h]
\centering
\includegraphics[width=\columnwidth]{figure10}
\caption{Presence of 3'-O-deacylated lipid A in living cells of \textit{S. typhimurium} LT2/pLpxR2. A, lipid A isolated from CaCl\(_2\) (10 mM)-treated \textit{S. typhimurium} strain LT2 harboring the empty vector pWSK29 consists mainly of hexa-acylated lipid A. B, lipid A of CaCl\(_2\)-treated \textit{S. typhimurium} LT2/pLpxR2 contains a significant portion of an additional component with the mass expected for a 3'-O-deacylated lipid A species, as indicated. amu, atomic mass units.}
\end{figure}
LpxR, a Ca\(^{2+}\)-dependent Lipid A-3\(^\prime\)-O-deacylase

![Image](image.png)

FIGURE 11. \(^{1}H\) NMR spectroscopy of 3\(^\prime\)-O-deacylated lipid A. A, partial 800 MHz \(^{1}H\) NMR spectrum of 3\(^\prime\)-O-deacylated lipid A in CDCl\(_3\)/CD\(_3\)OD:D\(_2\)O (2:3:1, v/v) showing the sugar proton region. The sugar proton resonances integrate to 14 sugar and three \(\beta\)-hydroxymethines, consistent with the presence of two hexoses and three \(\beta\)-hydroxacyl chains. The intensity of the H-1\(^\prime\) doublet is reduced due to a presaturation pulse used to eliminate the water solvent signal. The sharp signal at 4.2 ppm (-1.2 Hz line width) arises from the butyl group of a solvent impurity in the sample preparation. The broadened lines of the lipid A resonances (3-8 Hz) are consistent with lipid self-aggregation. B, partial 800 MHz \(^{1}H\) NMR spectrum of 3\(^\prime\)-O-deacylated lipid A showing the acyl \(\alpha\)-methylene proton resonances, with resolution of the \(\alpha_{2}\) and \(\alpha_{3}\) methylene signals as eight-line multiplets. The \(\alpha\)-methylene region integrates to eight protons, indicating the existence of four acyl chains.

TABLE 3

\(^{1}H\) NMR chemical shifts of 3\(^\prime\)-O-deacylated lipid A versus E. coli lipid A

| Position | 3\(^\prime\)-O-deacylated lipid A | E. coli lipid A |
|----------|-------------------------------|------------------|
| 1        | \(\delta H, [\text{multi}, J(\text{Hz})]\) | \(\delta H, [\text{multi}, J(\text{Hz})]\) |
| 2        | 5.449 [dd, 3.3, 7.0]           | 5.450 [dd, 3.3, 6.8] |
| 3        | 4.137 [dd, 3.3, 10.9]          | 4.147 [dd, 3.3, 10.6] |
| 4        | 5.192 [dd, 10.9, 9.3]          | 5.213 [dd, 10.9, 9.3] |
| 5        | 3.650 [dd, 9.3]               | 3.679 [dd, 9.3] |
| 6\(_a\)  | 4.071 [m, 12.0]               | 4.103 [dd, 2.0, 12.3] |
| 6\(_b\)  | -3.82 [m]                     | 3.897 [dd, 5.1, 12.3] |
| 7\(_a\)  | 4.594 [d, 8.4]               | 4.613 [d, 8.8] |
| 7\(_b\)  | 3.674 [m]                     | -3.83 [dd, 8.8, 10.8] |
| 8        | 3.745 [m]                     | 5.18 [dd, 10.9, 9.3] |
| 9\(_a\)  | 3.958 [m]                     | 4.166 [dd, 9.3] |
| 9\(_b\)  | 3.415 [m]                     | 3.460 [m] |
| 10\(_a\) | -3.82 [m]                     | -3.94 [dd, 2.0, 12.3] |
| 10\(_b\) | -3.82 [m]                     | 3.774 [dd, 5.1, 12.3] |
| \(\alpha_{2}\) | 2.29 [m]                     | -2.33 |
| \(\beta_{2}\) | -3.92 [m]                     | -3.95 [m] |
| \(\gamma_{2}\) | 1.42 [m]                     | -1.45 [m] |
| \(\alpha_{3}\) | 2.472 [dd, 2.3, 15.3]        | -2.45 |
| \(\beta_{3}\) | -3.98 [m]                     | -4.03 [m] |
| \(\gamma_{3}\) | -1.45 [m]                     | -1.50 [m] |
| \(\alpha_{2}\) | 2.618 [dd, 7.4, 14.7]        | -2.561 |
| \(\beta_{2}\) | 2.526 [dd, 6.4, 14.7]        | -2.561 |
| \(\beta_{2}\) | -5.23 [m]                     | -5.18 [m] |
| \(\gamma_{2}\) | -1.62 [m]                     | -1.61 [m] |
| \(\sigma_{2}\) | 2.727 [dd, 7.2, 16.3]        | 2.665 [dd, 5.5, 16.3] |
| \(\beta_{3}\) | -5.25 [m]                     | -5.25 [m] |
| \(\gamma_{3}\) | -1.61 [m]                     | -1.61 [m] |
| \(\sigma_{3}\) | -2.32 [m]                     | -2.34 [m] |
| \(\beta_{3}\) | -3.82 [m]                     | -2.34 [m] |
| (CH\(_2\))\(_{in}\) | -1.25 [m]                     | -1.24 [m] |
| \(\omega-2\) \(\text{CH}_{3}\) | -1.87 [m]                     | -1.87 [m] |
| \(\omega-1\) \(\text{CH}_{3}\) | 1.279 [m]                     | 1.279 [m] |
| \(\omega_{2}\) \(\text{CH}_{3}\) | 0.886 [t, -7]                 | 0.891 [t, -7] |

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