Shortened Hydroxyacyl Chains on Lipid A of *Escherichia coli* Cells Expressing a Foreign UDP-N-Acetylglucosamine O-Acyltransferase*

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The first reaction of lipid A biosynthesis in Gram-negative bacteria is catalyzed by UDP-N-acetylglucosamine (UDP-GlcNAc) O-acetyltransferase, the product of the *lpxA* gene. The reaction involves the transfer of an acyl chain from hydroxyacyl-acyl carrier protein (ACP) to the glucosamine 3-OH position of UDP-GlcNAc. The lipid A isolated from *Escherichia coli* contains (R)-3-hydroxyacylmurate at the 3 and 3′ positions. Accordingly, LpxA of *E. coli* is highly selective for (R)-3-hydroxyacyl-ACP over ACP thioesters of longer or shorter acyl chains. We now demonstrate that the LpxA gene from *Neisseria meningitidis* encodes a similar acyltransferase that selectively utilizes 3-hydroxyacyl-ACP. Strains of *E. coli* harboring the temperature-sensitive *lpxA2* mutation make very little lipid A and lose viability rapidly at 42 °C. We have created an *E. coli* strain in which the chromosomal *lpxA2* mutation is complemented by the *N. meningitidis* LpxA gene introduced on a plasmid. This strain, RO138/pT06, grows similarly to wild type cells at 42 °C and produces wild type levels of lipid A. However, the lipid A isolated from RO138/pT06 contains mostly hydroxylaurate and hydroxydecanoate in the 3 and 3′ positions. The strain RO138/pT06 is more susceptible than wild type to certain antibiotics at 42 °C. This is the first report of an *E. coli* strain growing with shortened hydroxyacyl chains on its lipid A. The lipid A gene product appears to be a critical determinant of the length of the ester-linked hydroxyacyl chains found on lipid A in living cells.

Lipopolysaccharide (LPS)* is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria (1, 2). Intact LPS is known to be important in maintaining the permeability barrier of the outer membrane (1–3). The lipid anchor portion of LPS, lipid A, is required for bacterial growth and is responsible for some of the toxicity of Gram-negative bacteria to mammalian hosts (1, 4–6). Lipid A is a β-1,6-linked glucosamine disaccharide substituted with (R)-3-hydroxyacyl groups at the 2, 3, 3′, and 3′′ positions and phosphates at the 1 and 4′′ positions (1, 5, 7–9). Lipid A of *E. coli* is additionally esterified at the 2′ and 3′′ (R)-3-hydroxyacylthioesters with laurate and myristate, respectively (1). The first enzyme in the lipid A biosynthetic pathway, LpxA (1, 7, 10), catalyzes the transfer of an (R)-3-hydroxy fatty acid from hydroxyacyl-acyl carrier protein (ACP) to the glucosamine 3-OH position of UDP-N-acetylglucosamine (UDP-GlcNAc) (11–16). LpxA is thus responsible for attachment of the O-linked fatty acids located at the 3 and 3′ positions of mature lipid A (Fig. 1) (1, 7, 10). The lengths of these fatty acids differ among bacterial species (7, 9, 16); the lipid A molecules of *Pseudomonas aeruginosa* (17) and *Rhodobacter sphaeroides* (18) are substituted with hydroxydecanoate at the 3′′ position and with hydroxytetradecanoate at the 3′ position; the lipid A of *Neisseria* strains (19–21) is substituted with hydroxyoctadecanoate and with hydroxytetradecanoate. The UDP-GlcNAc acyltransferases found in cell extracts of some of these bacteria display high specificity for acyl-ACP thioesters of the same acyl chain lengths that predominate in their lipid As *in vivo* (16).

Thus far, only conditional mutants of genes encoding the early lipid A biosynthetic enzymes have been identified, indicating that lipid A is required for growth (1, 10). One *lpxA* mutation, *lpxA2*, has been extensively investigated (22, 23). Strains carrying this mutation are temperature-sensitive and have little measurable LpxA activity at any temperature, as judged by assays of cell extracts. Mutants harboring *lpxA2* also are characterized by a very low lipid A to glycerophospholipid ratio compared with wild type cells at 42 °C (22, 23). In addition, such mutants have been shown to be supersusceptible to many antibiotics, perhaps due to the effect of the low lipid A content on outer membrane integrity (24, 25). These features reinforce the idea that LpxA and certain other enzymes of LPS biosynthesis are targets for the design of new antibiotics (26).

*Escherichia coli* lipid A activates the synthesis of cytokines and various other inflammatory mediators in macrophages (1, 5, 27). However, the lipid A molecules from some other organisms, for instance *R. sphaeroides*, are potent antagonists of that response in mouse and human cells (28–30). One major difference in the lipid A of *R. sphaeroides*, as compared with *E. coli*, is that the 3′′-O-linked fatty acids are hydroxydecanoates rather than hydroxymyristates (18, 30). Thus, subtle features...
have introduced the *N. meningitidis* lpxA gene (31) on a plasmid into RO138, an *E. coli* strain harboring the lpxA2 mutation (23). The resulting strain, RO138/pTO6, has wild type growth characteristics. However, the lipid A isolated from RO138/pTO6 is extensively substituted with shorter than normal ester-linked hydroxy fatty acids. This new strain provides a system in which to investigate the effects of a specific lipid A modification on *E. coli* growth and outer membrane function.

**EXPERIMENTAL PROCEDURES**

**Materials**—[α-32P]UTP and [3P] were purchased from NEN Life Science Products. Tryptophane, yeast extract, and agar were from Difco. Antibiotics, glucosamine 1-phosphate, acyl carrier protein, (R,S)-3-hydroxydecanonic acid, (R,S)-3-hydroxyauric acid, and (R,S)-3-hydroxymyristic acid were products of Sigma. Chloroform, methanol, acetic acid, pyridine, and 88% formic acid were from Mallinckrodt Chemical Works. All other chemicals were obtained from Sigma or Mallinckrodt. Silica gel-60 thin layer plates (0.25 mm) were purchased from E. Merck, Darmstadt, Germany. Restriction enzymes and T4 DNA ligase were from New England Biolabs or Boehringer Mannheim. Shrimp alkaline phosphatase was from U. S. Biochemical Corp. Primers for PCR and sequencing were custom-made by Life Technologies, Inc. The LpxC inhibitor L-573,655 (26) was provided by Dr. A. Patchett (Merck Research Laboratories). The FabA inhibitor 3-decyonyl-N-acetylcysteamine (33) was provided by Dr. John Cronan, University of Illinois, Urbana.

**Bacterial Strains and Growth Conditions**—The bacterial strains used in this study are all derivatives of *E. coli* K-12. DH5αF was from our laboratory collection; XL1 Blue MR was purchased from Stratagene, and BL21(DE3) and BL21(DE3)pLysS were from Novagen. SM105 (lpxA+, Strep') has been described previously (22). RO138 (lpxA2 recA, Strep') was obtained from Matt Anderson, Merck Research Laboratories. Cells were grown in LB medium (34), containing 10 μl/gl tryptone, 5 μl/gl yeast extract, and 10 μl/gl NaCl, or on LB plus 15 μl/gl agar, at either 30, 37, or 42 °C, as indicated. Antibiotics were used, when appropriate, at the following final concentrations: 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, and 30 μg/ml streptomycin.

**General Recombinant DNA Techniques**—Most recombinant DNA techniques were as described by Sambrook et al. (35). Plasmids were prepared using the Wizard Plus Miniprep DNA Purification System (Promega) or the Bigger Prep Plasmid DNA Preparation Kit (5 Prime, Inc., Boulder, CO). Restriction endonucleases, T4 DNA ligase, and shrimp alkaline phosphatase were used according to the manufacturer’s specifications. DNA was extracted from gels using the GeneClean kit (Bio 101), according to the manufacturer’s directions.

**Transformation of E. coli Cells**—Cells were made competent for transformation by CaCl2 treatment (35). Transformants were plated on LB agar containing appropriate antibiotics and incubated at 30 or 37 °C.

**Plasmid Constructions**—The plasmids used in this study are listed in Table I. We chose to use the pET expression system (Novagen), in which genes are driven by the T7 promoter, to obtain high levels of protein expression. The *N. meningitidis* lpxA gene (31) was cloned for expression in *E. coli* as follows. PCR primers were designed from the DNA sequence of *N. meningitidis* lpxA (31) to generate LpxA protein carrying a C-terminal fusion of six histidine residues (a His-Tag) to enable one-step purification of the expressed protein. *Nde*I and *Hind*III sites were incorporated into these oligonucleotide primers to facilitate cloning. The primer sequences were as follows: forward primer 5′ GAA AGG AAA CAT ATG ACC CTC 3′ and reverse primer 5′ GGG GTT TA CaG AGC TTC AGT GAT GGT GAT GAT GAT GAC GGA TGA TGC CGC CCG C 3′. *Nde*I and *Hind*III sites are underlined. Using *N. meningitidis* MC58 chromosomal DNA as template, a PCR reaction was carried out with forward and reverse primers, using Pfu polymerase (Stratagene). The cycle was 90 °C for 2 min, 55 °C for 2 min, and 72 °C for 2 min. This cycle was repeated 30 times. The PCR reaction was electrophoresed on a 0.8% agarose gel, and the expected DNA species of approximately 800 bp was identified. DNA from this fragment was eluted using GeneClean kit (Bio 101), according to the manufacturer's directions.

**FIG. 1. Role of LpxA in lipid A biosynthesis.** LpxA catalyzes the first step of lipid A biosynthesis (13). The reaction shown is for *E. coli*, in which the hydroxyacyl group is (R)-3-hydroxymyristate. LpxAs from diverse Gram-negative bacteria display specificity for acyl-ACPs of different lengths (16). Biosynthetic intermediates and genes encoding modification on lipid A structure (20, 21). To determine whether a foreign LpxA selective for a different fatty acid could substitute for the enzyme of wild type *E. coli*, we

![](https://example.com/figure1.png)

The *E. coli* lpxA gene (14, 36) was amplified using the following primers:
TABLE I
Plasmids

| Plasmid       | Description                              | Source            |
|---------------|------------------------------------------|-------------------|
| pET21a(+)     | T. expression vector, Amp'                | Novagen           |
| pET21a        | T. expression vector, Amp'                | This work         |
| pNMHT         | pET21a(+) containing N. meningitidis lpxA | This work         |
| pTO1          | pET23c containing E. coli lpxA           | This work         |
| pBluescriptKS(SK(+)) | Lac expression vector, Amp'            | Stratagene       |
| pTO4          | pBluescriptSK(+)+ containing N. meningitidis lpxA | This work         |
| pTO4          | pBluescriptSK(+)+ containing E. coli lpxA | This work         |
| pNGH1         | Low copy number Trp-Lac expression vector, Cm' | Nora Gardner Haigh, Duke University |
| pTO6          | pNGH1 containing N. meningitidis lpxA    | This work         |
| pTO7          | pNGH1 containing E. coli lpxA            | This work         |

Lipid A Modification by Expression of Neisseria lpxA in E. coli

Primers: 5′ CGG CGG GGA ATT CCA TAT GAT TGA TAA ATC CGC CT 3′ and 5′ CGG GGA TAC CTG TCG GAC GTA AAC GAA TCA GAC CG C 3′. The upstream and downstream primers introduced NdeI and BamHI sites (underlined), respectively. Plasmids pSR1 (15) was used as the template. The PCR reaction mixture included 5 ng of template, 0.5 μM each primer, 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-Cl (pH 8.75), 2 mM MgCl₂, 0.1% Triton X-100, 100 μg/ml bovine serum albumin, 4 mM MgCl₂, 200 μM each dNTP (Boehringer Mannheim), and 2.5 units of Pfu polymerase (Stratagene) in a final volume of 0.1 ml. This mixture was subjected to a 5-min denaturation at 94 °C and then 20 cycles of [94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s] plus a 7-min extension at 72 °C in a Perkin-Elmer GeneAmp PCR System 2400. The PCR product was purified by gel electrophoresis. Simultaneously, pBluescriptSK(+) was digested with PstI and was ligated with PstI- and SacI-tailed templates. These pieces were ligated together and transfected into DH5αF with selection on ampicillin. Plasmids from resultant colonies were screened for the presence of the correct insert by digestion with NdeI and BamHI. One correct plasmid preparation was digested with NdeI and BamHI, and the resulting insert (containing the E. coli lpxA gene) was ligated into the similarly cut and phosphatase-treated vector, pET21a. The ligation mixture was transformed into DH5αF with selection on ampicillin, and plasmids from resultant colonies were screened for the presence of the correct insert by digestion with NdeI and HindIII. One correct plasmid, designated pTO1, was re-transformed into DH5αF for amplification and preparation, and finally transformed into BL21(DE3)pLysS. The sequence of the entire lpxA gene in pTO1 was confirmed using primers based on the published sequence (36) and a DNA sequencing kit from U. S. Biochemical Corp.

The plasmid was then transformed into SM105 and RO138 as a vector control in the experiments involving pTO6 and pTO7. The first step in constructing pTO6 was to ligate the 800-bp XbaI-HindIII fragment from pNMHT into the similarly cut and phosphatase-treated vector pBluescriptSK(+), to give the plasmid pTO4. This plasmid was constructed to obtain flanking restriction sites suitable for subcloning into pNGH1. Subsequently, the 989-bp SacI-KpnI fragment from pTO4 was ligated into the similarly cut and phosphatase-treated vector pNGH1. The ligation mixture was transformed into XL1-Blue MR with selection on chloramphenicol, and plasmids from resultant colonies were screened for the presence of the correct insert by digestion with SacI and KpnI. One correct plasmid, designated pTO6, was re-transformed into XL1-Blue MR for amplification and preparation. This plasmid was then transformed into SM105 and RO138.

Plasmid pTO7 was constructed similarly. The 800-bp XbaI-HindIII fragment from pTO1 was ligated into the similarly cut and phosphatase-treated vector pBluescriptSK(+), to give the plasmid pTO5. Then the 877-bp SacI-SacI fragment of pTO5 was ligated into the similarly cut and phosphatase-treated vector pNGH1. The ligation mixture was transformed into XL1-Blue MR with selection on chloramphenicol, and plasmids from the resultant colonies were screened for the presence of the correct insert by digestion with SacI. One correct plasmid, designated pTO7, was transformed into XL1-Blue MR for amplification and preparation. This plasmid was then transformed into SM105 and RO138.

Preparation of Cell Extracts—Strains BL21(DE3)pNMHT and BL21(DE3)pLYsS/pTO1 were grown to an OD₆₀₀ of 0.6, induced with a final concentration of 1 mM IPTG, and grown for 3 more h. Strains SM105/pNGH1, SM105/pTO6, SM105/pTO7, RO138/pNGH1, RO138/pTO6, and RO138/pTO7 were grown to an OD₆₀₀ of 0.1, grown at 30 °C to an OD₆₀₀ of 0.6, shifted to 42 °C, and grown for 3 more h. The cultures were not induced with IPTG because there is sufficient LpxA expression without induction. Cultures were harvested by centrifugation at 4000 × g for 15 min. Cells were washed once with 10 ml of 10 mM potassium phosphate (pH 7.0), 2 mM NaCl, and 20% glycerol, and resuspended in 3–5 ml of the same buffer. Cells were broken by one passage through a French pressure cell at 18,000 p.s.i. This suspension was then centrifuged at 10,000 × g for 20 min, and the supernatant was stored at −80 °C. Protein concentration was determined using the bicinchoninic acid assay (Pierce) (37).

Preparations of Substrates for LpxA Assay—[α-32P]UDP-GlcNAc was prepared as described previously (38) with minor modifications. The initial reaction was allowed to proceed for only 1 h at room temperature, and the acetylation reaction was not quenched by boiling. In addition, the triethyl ammonium bicarbonate buffer was used at pH 8.5, and the final product was dried in a SpeedVac (Savant), rather than being lyophilized (39). None of these modifications had any effect on yield. (R,S)-3-Hydroxyauracil-ACP and (R,S)-3-hydroxyauracil-ACP were prepared as described previously (40) with a few modifications. The acyl substrates for the reaction were (R,S)-3-hydroxyauracil acid and (R,S)-3-hydroxyauracil-ACP. The acylation reactions were allowed to proceed for 4 h at room temperature. In addition, 0.7 mM 3-decynoyl-β-n-acetyl-cysteamine, an inhibitor of FabA (33), was added to the hydroxyauracil-ACP acylation reaction. (R,S)-3-Hydroxydecanoyl-ACP was prepared similarly and was a kind gift from Dr. Gary Dotson.

Assay of LpxA Activity—This assay monitors the conversion of [α-32P]UDP-GlcNAc to [α-32P]UDP-GlcNAc by LpxA, leading to the formation of the acylated 3-hydroxy auracil-ACP. The reaction mixture was incubated at 30 °C for 2 to 10 min (12, 13), and reactions were terminated by spotting 2.5 μl on a silica thin layer chromatography plate. The plates were developed in a solvent system containing chloroform/methanol/water/acetic acid (25:15:4:2, v/v/v). The plates were then exposed to imaging screens overnight at room temperature. The plates were visualized, and the extent of the reaction was quantified using a Molecular Dynamics PhosphorImager, operated with ImageQuant software.

Determination of the Lipid A to Glycerophospholipid Ratio—Bacteria from an overnight culture were inoculated into 3–5 ml of fresh LB medium to an OD₆₀₀ of 0.1, grown at 30 °C to an OD₆₀₀ of 0.6, and shifted to 42 °C. After 1 h, 5 μg/ml LPS was added to the cultures, and the cultures were grown at 42 °C for 30 min. At this point 0.8-ml samples were removed, and lipid A and glycerophospholipids were prepared and quantified by the method of Galloway and Raetz (22) with the following modifications. Lipid A and glycerophospholipids were counted in Econoscan scintillation fluid and analyzed by TLC in a system containing chloroform/pyridine/formic acid/water (50:50:16.5, v/v). The plates were visualized, and the percent of the total counts attributable to the desired material was determined using a PhosphorImager.
Preparation of bis-Phospho-lipid A— Cultures (250 ml) of RO138/pTO6 and RO138/pTO7 were grown with shaking (250 rpm) in LB at 30 °C to an A_{600} of 0.6, shifted to 42 °C, and grown for 3 more h. Cells were harvested by centrifugation at 4000 × g for 20 min, washed once with 40 ml of phosphate-buffered saline (35), resuspended in 80 ml of phosphate-buffered saline, and frozen at −80 °C. Glycerophospholipids were extracted for 1 h at room temperature using a single phase Bligh-Dyer mixture (chloroform/methanol/aqueous solution, 1:2:0.8, v/v), generated by addition of 100 ml of chloroform and 200 ml of methanol to 80 ml of washed cells in phosphate-buffered saline. The precipitated LPS, protein, and nucleic acids were collected by centrifugation at 4000 × g for 15 min and washed once with 250 ml of a fresh single-phase Bligh-Dyer mixture (chloroform/methanol/water, 1:2:0.8, v/v). Pellets were then resuspended in 80 ml of 12.5 mM ammonium (or sodium) acetate (pH 4.5) and 1% SDS and incubated at 100 °C for 30 min to cleave the glycosidic linkage between D3-deoxy-D-manno-octulonosonic acid (Kdo) and lipid A without loss of the 1 or 4′ phosphates (41, 42). This solution was converted to a two-phase Bligh-Dyer mixture (having the proportions chloroform/methanol/water, 2:2:1.8, v/v) by addition of 89 ml of chloroform and 89 ml of methanol. The phases were separated by centrifugation at 4000 × g for 15 min. The upper phase was washed once with a fresh neutral pre-equilibrated lower phase, and then the combined lower phases, containing the lipid A and a few impurities, were washed once with fresh pre-equilibrated upper phase. Half of the lower phase was dried by rotary evaporation, resuspended in 10 ml of chloroform/methanol/water (2:2:1.8, v/v), and applied to a 1-ml DEAE-cellulose (Whatman DE52) column, equilibrated as the acetate form in the same solvent mixture (43). The column was washed with 4 ml of chloroform/methanol/water (2:2:1.8, v/v) and eluted with 4 ml each of chloroform/methanol/ammonium acetate (60, 120, 240, or 480 mM) in water (2:2:1.8, v/v), while collecting 1-ml fractions throughout the elution. The bis-phospho-lipid A eluted with the 240 mM ammonium acetate. These fractions were combined and converted to neutral two-phase Bligh-Dyer proportions (chloroform/methanol/water, 2:2:1.8, v/v) by addition of chloroform and water. The lower phase was dried under a stream of N2 and then frozen at −80 °C until being prepared for mass spectrometry. The preparation was monitored at various stages by spotting samples onto silica TLC plates, developing the plates in chloroform/pyridine/88% formic acid/water (50:50:16.5:5, v/v), and visualizing by sulfuric acid charring.

**RESULTS**

Catalytic Activity and Acyl Chain Specificity of N. meningitidis LpxA—The lipid A gene of N. meningitidis was recently cloned and found to code for a 258-amino acid (28 kDa) protein with high similarity to E. coli lipA (31). Since the O-linked hydroxy fatty acids in the meningococcal lipid A are 3-hydroxy-laurate (20, 21), it was postulated that the N. meningitidis LpxA would catalyze the transfer of 3-hydroxylaurate from 3-hydroxylauroyl-ACP to UDP-GlcNAc (31).

To test this hypothesis, we constructed pNMHT, which contains the N. meningitidis lipA gene under control of the T7 promoter for use in the pET expression system (see “Experimental Procedures”). A similar construction, pTO1, was made containing the E. coli lipA gene. After growth and induction with IPTG, the strains BL21(DE3)/pNMHT and BL21(DE3)-pLysS/pTO1 both overexpress a 28-kDa protein, as judged by SDS-polyaerylamide gel electrophoresis and Coomassie staining (data not shown). Strain BL21(DE3), containing the pET23c vector alone, when grown and induced similarly, does not display overexpression of a protein of 28 kDa.

Extracts of the three strains were assayed for LpxA activity with each of the following acyl donor substrates: (R,S)-3-hydroxydecanoyl-ACP, (R,S)-3-hydroxy-myristoyl-ACP, and (R,S)-3-hydroxy-myristoyl-ACP. The results from these assays are shown in Table II. The extract from the strain containing pET23c alone shows typical wild type levels of LpxA activity with (R,S)-3-hydroxy-myristoyl-ACP at the substrate concentrations employed (12, 13) and very little activity with the other acyl donors. As expected, the preferred acyl donor for the overexpressed N. meningitidis LpxA is clearly 3-hydroxylaurate (Table II). As reported previously (16), E. coli LpxA prefers 3-hydroxy-myrystate, but it utilizes 3-hydroxylaurate at about 2% relative rate. The N. meningitidis LpxA shows slightly less specificity, utilizing 3-hydroxy-decanate at about 8% of the rate of 3-hydroxylaurate under standard assay conditions (Table II). N. meningitidis LpxA utilizes 3-hydroxy-myrystate at less than 2% of the rate of 3-hydroxylaurate.

**Effect of the N. meningitidis LpxA Gene on the Temperature-sensitive Growth of RO138**—We are interested in developing systems that permit the modification of the structure of lipid A in living cells of E. coli to understand the biological functions of lipid A. We therefore wanted to construct a strain of E. coli in which N. meningitidis LpxA, rather than E. coli LpxA, would be responsible for incorporation of the 3 and 3′ O-linked hydroxy fatty acids of lipid A. As an approach to this problem, we used strain RO138, a recA− derivative of SM101. The temperature-sensitive E. coli mutant SM101 (bearing lpxA2) has very low levels of LpxA activity in cell extracts and a nearly complete defect in lipid A biosynthesis at 42 °C in cells (22, 23). The recA− nature of RO138 prevents recombination between plasmid-borne and chromosomal lpxA genes. SM105, the isogenic wild type strain of SM101, was also used as a control in some experiments (22, 23).

Plasmids pTO6 and pTO7 were constructed as described
under "Experimental Procedures" to achieve modest overexpression of the N. meningitidis and E. coli LpxA genes, respectively, for use in complementation studies. The parent vector, pNGH1, was used as a control. We transformed SM105 and RO138 with pNGH1, pTO6, and pTO7 and first incubated the cells at 30 °C with chloramphenicol selection for the plasmids. Following purification at 30 °C, the transformants were restreaked and incubated at 42 °C. All strains grew rapidly and formed single colonies at 42 °C, except for RO138/pNGH1, which did not grow at all. The LpxA genes of both pTO6 and pTO7 therefore complement the temperature-sensitive phenotype of RO138 on plates (data not shown).

To determine whether the plasmids also prevent the loss of cell viability observed in liquid cultures of SM101 at 42 °C (22, 23), exponentially growing cells of each construct were shifted from 30 to 42 °C. RO138/pTO6 and RO138/pTO7 continued to grow after the temperature shift, just like SM105/pNGH1, but the optical density of the culture of RO138/pNGH1 began to decrease after 1 h at 42 °C (Fig. 2), as reported previously for SM101 (22, 23). Thus, the presence of the N. meningitidis lpxA gene allows E. coli cells containing the lpxA2 mutation to grow normally at high temperatures in liquid culture.

Restoration of LpxA Activity in RO138/pTO6 and RO138/pTO7—To demonstrate that the N. meningitidis and E. coli LpxA genes carried on pTO6 and pTO7 are expressed and generate catalytically active LpxA, cell extracts of various strains were prepared and assayed (Table III). As expected, SM105/pNGH1 shows wild type levels of LpxA activity with the indicated acyl donor substrates. RO138/pNGH1 displays no measurable 3-hydroxylauroyl-ACP and very little activity with 3-hydroxymyristoyl-ACP. RO138/pTO6—containing the E. coli lpxA2 gene at 42 °C) would be derivatized with [32P]-3-Hydroxylauroyl-ACP (Table III). The results demonstrate that functional N. meningitidis and E. coli LpxA genes, respectively, in the pNGH1 vector.

Mass Spectrometry of Lipid A from RO138/pTO7 and RO138/pNGH1—The lipid A from wild type E. coli LpxA with that substrate (12, 16). Interestingly, strain SM105/pTO6 displays consistently lower 3-hydroxymyristoyl-ACP-dependent acyltransferase activity than does the SM105/pNGH1 control (Table III). Since LpxA is a trimer (36), this finding could be explained by the formation of inactive heterotrimers between N. meningitidis and E. coli LpxA monomers.

Lipid A to Glycerophospholipid Ratios in SM105- and RO138-derived Strains—The death of strains carrying the lpxA2 mutation at 42 °C has been attributed to their reduced ability to make lipid A, as reflected by the lipid A to glycerophospholipid ratio in pulse-labeling studies with intact cells (22). This defect presumably reflects the low LpxA activity in these strains. Since the LpxA specific activity in RO138/pTO6 is comparable to that in SM105/pNGH1, but is directed toward 3-hydroxyaurate rather than 3-hydroxyaurate (Table III), we wanted to determine the lipid A to glycerophospholipid ratio in RO138/pTO6 grown at 42 °C.

Table IV shows that the lipid A to glycerophospholipid ratios of SM105/pNGH1 and RO138/pNGH1 match those reported previously (22). RO138/pTO6 displays a ratio very similar to wild type (SM105/pNGH1), whereas the ratio for RO138/pTO7 is slightly higher than normal. This may be due to a higher degree of overexpression of LpxA activity in that strain (Table II). The three constructs with normal or slightly elevated ratios (Table IV) also display similar levels of total [32P] incorporation into lipid A, as normalized to the optical density of the culture (data not shown). Therefore, the absolute amount of lipid A made in cells of RO138/pTO6 is similar to that found for SM105/pNGH1 (wild type).

Samples of bis-phospho-lipid A were prepared using pH 4.5 hydrolysis (41, 42) of cells of RO138/pTO6 and RO138/pTO7, as described under "Experimental Procedures." The lipid A isolated by direct hydrolysis of cells appears to be the same as lipid A obtained by hydrolysis of LPS (21, 22, 45, 46). The most abundant negative ion peaks in the LSIMS spectrum of the RO138/pTO7 lipid A are those at m/z 1796.5, [M – H]− and 1818.5, [M – 2H + Na]− (Fig. 4A). These masses are consistent with two different acyl donors.

| Strain          | Specific activity (pmol/min/mg) | (R,S)-3-Hydroxylauroyl-ACP | (R,S)-3-Hydroxymyristoyl-ACP |
|-----------------|--------------------------------|-----------------------------|-------------------------------|
| SM105/pNGH1     | <0.5                           | 20                          |                               |
| SM105/pTO6      | 28                             | 9.6                         |                               |
| SM105/pTO7      | 4.5                            | 210                         |                               |
| RO138/pNGH1     | <0.5                           | <0.5                        |                               |
| RO138/pTO6      | 17                             | <0.5                        |                               |
| RO138/pTO7      | 2.9                            | 200                         |                               |

a SM105 is a wild type E. coli strain. RO138 harbors the lpxA2 mutation. Plasmid pNGH1 is a low copy number vector. pTO6 and pTO7 contain the N. meningitidis and E. coli lpxA genes, respectively.
TABLE IV  
Ratio of newly made lipid A to glycerophospholipids  
after 1 h of growth at 42 °C in various strains

| Strain       | Lipid A to glycerophospholipid ratio |
|--------------|--------------------------------------|
| SM105/pNGH1  | 0.106 ± 0.004*                       |
| RO138/pNGH1  | 0.008 ± 0.004*                       |
| RO138/pTO6   | 0.124 ± 0.003                        |
| RO138/pTO7   | 0.157 ± 0.011                        |

*Strains are as described in the legend to Table III.

Fig. 3. *bis*-Phospholipid A isolated from *E. coli* cells by pH 4.5 hydrolysis and prominent fragments observed during mass spectrometry. The nomenclature of Costello and Vath (47) is used for each of the fragments.

with the known chemical structure of wild type *E. coli* *bis*-phospholipid A (monoisotopic mass of free acid 1797.2) (1, 9). Other minor ion peaks clustered around m/z 1570.4 and 1360.3 correspond to the loss of the hydroxyxymyristoyl (R₁) or the myristoxygenyristoyl (R₁OR₁⁻) moieties (see Fig. 3 for cleavages and terminology), respectively, consistent with the fragmentation pattern expected for *Escherichia*-type lipid A. The fragment ion peaks at m/z 738.1, 710.1, 708.1, and 692.1 are the reducing-end fragments 0.092X₁⁻-, Y₁⁻, Y₁⁻-H₂O, and Z₁⁻, which are typical low mass fragment anions of lipid A (Fig. 3) (48).

Negative ion LSIMS analysis of the RO138/pTO6 lipid A reveals a significantly different pattern (Fig. 4B). A very weak ionic signal is found at the wild type [M – H]⁻ (m/z 1796.5). Another minor peak is found at m/z 1768.6, which is 28 a.m.u. smaller than the mass of the wild type molecular anion. However, the most abundant anion peaks (interpreted here as molecular anions [M – H]⁻ of the RO138/pTO6 lipid A) are those at m/z 1740.6, 1712.6, and 1684.4. These correspond to lipid A molecules that are 56, 84, and 112 a.m.u. smaller than the wild type species, respectively. In addition, the measured masses of the fragment ions resulting from the de-O-acylation of lipid A ([(M – R₃H)]⁻ at m/z 1513.4 and 1541.6, and [(M – R₉OR₁⁻H)]⁻ at m/z 1304.2 and 1332.4) are 28 and 56 a.m.u. smaller than the Y₁⁻ fragment ion peak in the spectrum of the wild type lipid A. These results are consistent with the hypothesis that *bis*-phospho-lipid A of RO138/pTO6 is a mixture of four major molecular species, one with hydroxylaurates at R₁ and R₃ (as defined in Fig. 3), one with hydroxydecanoates at R₁ and R₉, and two species of lipid A with one hydroxyaurate and one hydroxydecanoate at either position. These findings are consistent with the acyl chain length specificity of *N. meningitidis* LpxA, as determined by assays of cell extracts (Table II). The small peaks (interpreted as [M – H]⁻) at m/z 1768.6 and 1796.5 (Fig. 4B) correspond to lipid A molecules containing one or two hydroxyxymyristates, respectively, and these species probably result from residual *E. coli* LpxA activity.

Mass Spectrometry of Mild Base-treated Lipid A from RO138/pTO7 and RO138/pTO6—To confirm our hypothesis, mass spectral analyses were performed on the above lipid A samples after mild base hydrolysis with ammonium hydroxide (see “Experimental Procedures”). Base treatment is known to remove preferentially the O-linked fatty acyl and hydroxy fatty acyl chains from lipid A (49). The rate of hydrolysis of the hydroxy fatty acyl chains is more rapid (50, 51). The mass spectra of the NH₄OH-treated lipid A samples are shown in Fig. 5. The mass spectrum of incompletely degraded lipid A from RO138/pTO7 (Fig. 5A), acquired after a relatively short treatment with base (25 min), shows ion peaks corresponding to intact lipid A (m/z 1796.6), as well as ion peaks correspond-
The enzyme LpxA is responsible for the incorporation of the 3 and 3'-O-linked hydroxy fatty acids found in the lipid A of Gram-negative bacteria (1). In E. coli these ester-linked fatty acids are (R)-3-hydroxymyristate (8, 9, 16), and in N. meningitidis, they are (R)-3-hydroxydecanoate (20, 21). The LpxA enzymes from E. coli and other organisms have been shown to have a high specificity for the ACP thioester of the fatty acid found in the 3 and 3' positions of their lipid A, compared with ACP thioesters of longer or shorter hydroxyacyl chains (12, 16). We have shown in this study that, as expected, the N. meningitidis LpxA is specific for 3-hydroxylauroyl-ACP as the acyl

The ion peaks Y₁⁻-R₃ and [M - R₉OR₁⁻ - R₉ - H]⁻ are at m/z 484.3 and 1133.5, respectively, exactly the same as for RO138/pTO7 (Fig. 5A)! The peak at m/z 1133.5 dominates the mass spectrum after an hour of NH₄OH treatment (Fig. 5C), suggesting that it is the final product of this hydrolysis treatment. Since the masses of Y₁⁻-R₃ and [M - R₉OR₁⁻ - R₉ - H]⁻ are conserved in the lipid A samples from RO138/pTO6 and RO138/pTO7, the lipid A from the two strains must have identical R₂, R₇', and R₉ groups. Furthermore, since both the Y₁⁻ and [M - R₁OR₁⁻ - H]⁻ ions in the spectrum of hydrolyzed RO138/pTO6 lipid A are 28 and 56 a.m.u. less than their analogs in the spectrum of the RO138/pTO7 lipid A, the R₂ group of RO138/pTO6 must be either hydroxyaurate or hydroxydecanoate. Similarly, since the [M - R₉ - H]⁻ ions of the RO138/pTO6 lipid A are 28 and 56 a.m.u. smaller than those of the wild type species, the R₉ (or R₉') groups of the RO138/pTO6 lipid A must be two or four methylenes shorter than on the lipid A from RO138/pTO7.

The only ambiguity in the RO138/pTO6 lipid A structure, therefore, is whether the R₉ or the R₉' group is actually shortened. Given that the N. meningitidis lpxA does appear to cause the modification of the R₉ group and that the R₂ and R₉ groups are equivalent in the early biosynthetic pathway (see Fig. 1), it seems probable that the R₁ group is the one that is modified. In addition, there is no enzymological reason to expect that the R₉ group would be modified in this strain. Taken together, these data strongly suggest that RO138/pTO6 lipid A is a mixture of species substituted at the 3 and 3' positions with hydroxyaurate and/or hydroxydecanoate. Thus, in RO138/pTO6, we have created a viable E. coli strain that synthesizes and tolerates a modified lipid A with shortened hydroxyacyl chains.

**DISCUSSION**

The enzyme LpxA is responsible for the incorporation of the 3 and 3'-O-linked hydroxy fatty acids found in the lipid A of Gram-negative bacteria (1). In E. coli these ester-linked fatty acids are (R)-3-hydroxymyristate (8, 9, 16), and in N. meningitidis, they are (R)-3-hydroxydecanoate (20, 21). The LpxA enzymes from E. coli and other organisms have been shown to have a high specificity for the ACP thioester of the fatty acid found in the 3 and 3' positions of their lipid A, compared with ACP thioesters of longer or shorter hydroxyacyl chains (12, 16). We have shown in this study that, as expected, the N. meningitidis LpxA is specific for 3-hydroxylauroyl-ACP as the acyl...
donor substrate. How lpxA-encoded acyltransferases achieve their remarkable acyl chain selectivity is unknown. However, the crystal structure of E. coli LpxA was recently solved (36), and further structural studies of LpxA enzymes having different selectivity may lead to the identification of the acyl chain measuring device in these unusual enzymes.

The availability of N. meningitidis LpxA has made possible in vivo investigations of lipid A molecules with different length acyl chains. The construction of an lpxA mutant strain complemented by N. meningitidis lpxA on a plasmid (RO138/pTO6) creates a system in which an E. coli strain produces lipid A molecules with hydroxydecanoyl and hydroxylauroyl chains at the O-linked 3 and 3′ positions. In wild type E. coli and Salmonella, over 95% of the lipid A contains (R)-3-hydroxymyristate at the 3 and 3′ positions (7, 9, 43). Studies of N. meningitidis have revealed lipid A molecules with predominantly 3-hydroxylauroyl (20, 21, 54), although some investigators note the presence of some 3-hydroxydecanoyl in lipid A from strains of Neisseria (21, 55).

The lipid A from RO138/pTO6 contains approximately equal amounts of 3-hydroxydecanoate and 3-hydroxylauroyl in the 3 and 3′ positions. This may be due to the relaxed selectivity toward acyl donor substrates of N. meningitidis LpxA, which incorporates 3-hydroxydecanoyl and 3-hydroxylauroyl chains at the O-linked 3 and 3′ positions. In wild type E. coli and Salmonella, over 95% of the lipid A contains (R)-3-hydroxymyristate at the 3 and 3′ positions (7, 9, 43). Studies of N. meningitidis have revealed lipid A molecules with predominantly 3-hydroxylauroyl (20, 21, 54), although some investigators note the presence of some 3-hydroxydecanoyl in lipid A from strains of Neisseria (21, 55).

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On plates and in liquid media, at either 30 or 42 °C, the growth of RO138/pTO6 is indistinguishable from the equivalent strain carrying the E. coli lpxA gene (RO138/pTO7). In addition, RO138/pTO6 produces wild type levels of lipid A. These data demonstrate that all the lipid A biosynthetic enzymes (Fig. 1) can effectively utilize substrate analogs containing 3-hydroxylauroyl and/or 3-hydroxydecanoate at the 3 and 3′ positions. Only one other E. coli mutant (msbB−) has been reported to grow with a modified lipid A structure, in this case with a penta-acylated lipid A (27, 56), rather than the usual hexa-acylated species (Fig. 1). Ours is the first description of an E. coli mutant with a modified lipid A that contains shorter, non-native hydroxacycl chains.

To date the only biological difference that we have found between RO138/pTO6 and RO138/pTO7 is the higher sensitivity of RO138/pTO6 to certain antibiotics at 42 °C. These types of antibiotics apparently cannot penetrate the outer membrane of wild type E. coli (24, 25). Strains like RO138, with mutations in early lipid A biosynthetic genes, are hypersensitive to these antibiotics (24, 25). At 30 °C, the lipid A molecules of RO138/pTO6 are sufficient to confer wild type levels of resistance, probably because some hydroxymyristate is incorporated (Table V). However, at 42 °C, RO138/pTO6 is significantly more susceptible to these antibiotics than are wild type strains, possibly because the shorter acyl chains at the 3 and 3′ positions of the lipid A result in a higher fluidity of the outer membrane of RO138/pTO6, as compared with wild type. This characteristic of RO138/pTO6 suggests that the acyl chain length of E. coli lipid A is optimized to contribute to the protective function of the outer membrane. It will be interesting to investigate E. coli constructs expressing acyltransferases specific for even shorter acyl chains, such as those of Pseudomonas or Rhodobacter. Conversely, the effects of replacing N. meningitidis lpxA with E. coli lpxA will also be of interest, especially with regard to N. meningitidis pathogenesis.

A possible application of E. coli strains producing modified lipid A molecules is in the biological production of LPS antagonists. Currently, such antagonists are chemically synthesized (20, 57) or are isolated from bacteria like R. sphaeroides and Rhodobacter capsulatus (5, 28, 29). Enzymatic and/or enzymatic synthesis might increase efficiency, yield, and accessibility. One antagonist currently in clinical trials (57) is modeled after R. capsulatus lipid A, which contains 3-hydroxydecanoate moieties at the 3 and 3′ positions, and only one acyloxyacyl unit. The recently cloned P. aeruginosa lpxA encodes an acyltransferase that is specific for 3-hydroxydecanoyl-ACP as the acyl donor. This gene, introduced on a plasmid, has been shown to correct the temperature sensitivity of RO138. It seems likely that the RO138 strain carrying the P. aeruginosa lpxA will be found to contain lipid A molecules with only 3-hydroxydecanoate in the 3 and 3′ positions. In combination with an msbB mutation, such strains (if viable) would have a lipid A structure almost identical to that of R. sphaeroides. The determination of whether such engineered lipid A are agonists or antagonists of LPS-induced responses in mammalian immune cells will be useful in defining the subtle structural features that are responsible for the bioactivity of lipid A-like molecules (5, 28–30, 32, 57, 58).

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TABLE V
Antibiotic susceptibility of selected strains determined by zones of inhibition (diameter in mm)

| Strain* | 30 °C | Novobiocin | Erythromycin | Rifampicin | Bacitracin | 42 °C | Novobiocin | Erythromycin | Rifampicin | Bacitracin |
|---------|-------|-----------|--------------|------------|------------|-------|-----------|--------------|------------|------------|
| SM105/pNGH1 | 6     | 13        | 20           | 6          | 6          | 42 °C | 6         | 6            | 16         | 6          |
| SM105/pTO6  | 6     | 13        | 22           | 6          | 6          |       | 6         | 6            | 17         | 6          |
| SM105/pTO7  | 6     | 13        | 20           | 6          | 6          |       | 6         | 6            | 17         | 6          |
| RO138/pNGH1 | 16    | 30        | 37           | 15         |           |       |           |              |            |            |
| RO138/pTO6  | 6     | 15        | 24           | 6          | 9          | 42 °C | 9         | 12           | 20         | 16         |
| RO138/pTO7  | 6     | 13        | 24           | 6          | 6          |       | 6         | 6            | 17         | 6          |

* Strains are as described in the legend to Table III.

b This strain does not grow at 42 °C.
