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Enzymatic Synthesis of Lipid A Molecules with Four Amide-linked Acyl Chains

LpxA ACYLTRANSFERASES SELECTIVE FOR AN ANALOG OF UDP-*N*-ACETYLGLUCOSAMINE IN WHICH AN AMINE REPLACES THE 3″-HYDROXYL GROUP*

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LpxA of *Escherichia coli* catalyzes the acylation of the glucosamine 3-OH group of UDP-GlcNAc, using *R*-3-hydroxymyristoyl-acyl carrier protein (ACP) as the donor substrate. We now demonstrate that LpxA in cell extracts of *Mesorhizobium loti* and *Leptospira interrogans*, which synthesize lipid A molecules containing 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcN3N) units in place of glucosamine, do not acylate UDP-GlcNAc. Instead, these LpxA acyltransferases require a UDP-GlcNAc derivative (designated UDP 2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose or UDP-GlcNAc3N), characterized in the preceding paper (Sweet, C. R., Ribeiro, A. A., and Raetz, C. R. H. (2004) *J. Biol. Chem.* 279, 25400–25410), in which an amine replaces the glucosamine 3-OH group. *L. interrogans* LpxA furthermore displays absolute selectivity for 3-hydroxylauroyl-ACP as the donor, whereas *M. loti* LpxA functions almost equally well with 10-, 12-, and 14-carbon 3-hydroxyacyl-ACPs. The substrate selectivity of *L. interrogans* LpxA is consistent with the structure of *L. interrogans* lipid A. The mechanism of *L. interrogans* LpxA appears to be similar to that of *E. coli* LpxA, given that the essential His^{125} residue of *E. coli* LpxA is conserved and is also required for acyltransferase activity in *L. interrogans*. *Acidithiobacillus ferrooxidans* (an organism that makes lipid A molecules containing both GlcN and GlcN3N) has an ortholog of LpxA that is selective for UDP-GlcNAc3N, but the enzyme also catalyzes the acylation of UDP-GlcNAc at a slow rate. *E. coli* LpxA acylates UDP-GlcNAc and UDP-GlcNAc3N at comparable rates *in vitro*. However, UDP-GlcNAc3N is not synthesized *in vivo*, because *E. coli* lacks *gnnA* and *gnnB*. When the latter are supplied together with *A. ferrooxidans lpxA*, *E. coli* incorporates a significant amount of GlcN3N into its lipid A.

Many Gram-negative bacteria contain a 2,3-diamino-2,3-dideoxy-D-glucopyranose (GlcN3N)[1] unit in place of one or both glucosamine residues in their lipid A molecules (Fig. 1) (1–3). As explained in the preceding manuscript (4), these lipid A variants may be generated from the sugar nucleotide UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose (UDP-GlcNAc3N). The enzymes GnnA and GnnB are responsible for the biosynthesis of UDP-GlcNAc3N from UDP-GlcNAc in *Acidithiobacillus ferrooxidans* (4). GnnA catalyzes the oxidation and GnnB catalyzes the subsequent transamination of the glucosamine 3-OH group of UDP-GlcNAc to generate UDP-GlcNAc3N (4).

We now demonstrate that UDP-GlcNAc3N is selectively utilized by LpxA orthologs present in cell extracts of *Leptospira interrogans* and *Mesorhizobium loti* and by the cloned LpxA proteins of *A. ferrooxidans* (AfLpxA) and *L. interrogans* (LiLpxA). All three of these organisms synthesize lipid A molecules containing GlcN3N units (5–7). *L. interrogans* LpxA displays no measurable activity with UDP-GlcNAc, and it uses *R*-3-hydroxylauroyl-ACP in absolute preference to all other acyl-ACPs. These findings are consistent with the proposed structure of *L. interrogans* lipid A, which is presented in the following paper (7). AfLpxA can also acylate UDP-GlcNAc at a slow rate, which is consistent with the reported structure of lipid A in this organism (Fig. 1) (5). *Escherichia coli* LpxA (8–11) utilizes both UDP-GlcNAc and UDP-GlcNAc3N with equal efficiency under standard assay conditions. However, the latter sugar nucleotide is not synthesized in wild-type cells. We have therefore constructed a novel strain of *E. coli* harboring the temperature-sensitive chromosomal *lpxA2* mutation (12, 13) while simultaneously expressing the cloned *gnnA*, *gnnB*, and *lpxA* genes of *A. ferrooxidans*. The lipid A backbone of this organism is partially substituted with GlcN3N when the cells are grown at elevated temperatures, consistent with our proposed pathway for the biosynthesis of GlcN3N-containing lipid A molecules (4).

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AY541060.

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[1] The abbreviations used are: GlcN3N, 2,3-diamino-2,3-dideoxy-D-glucopyranose; BisTris, 2,2-bis(hydroxymethyl)-2,2′,2″-nitrilotriethanol; ACP, acyl carrier protein; UDP-GlcNAc3N, UDP-2-acetamido-3-amino-2,3-dideoxy-α-D-glucopyranose; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

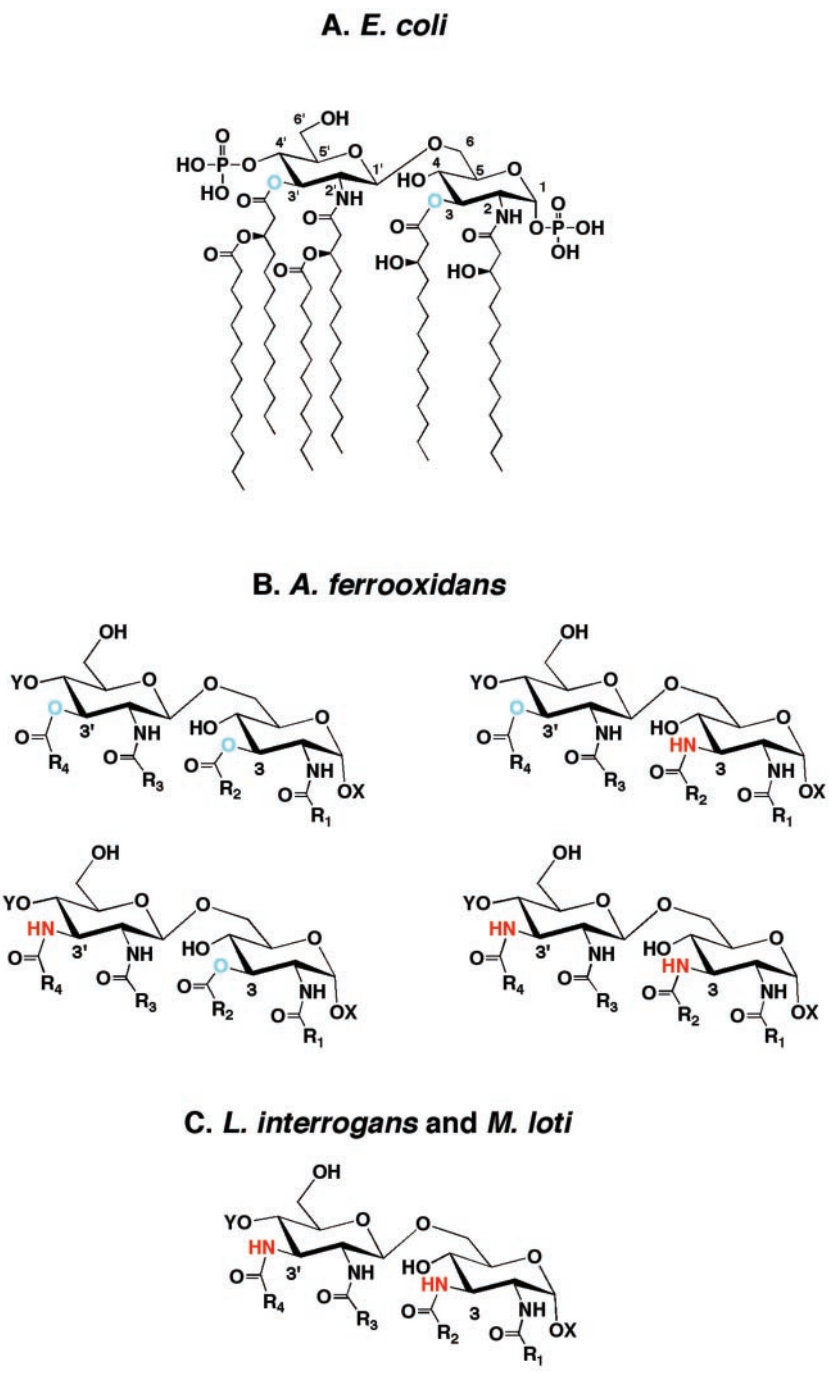

FIG. 1. **Structure of the lipid A backbone in *E. coli versus A. ferrooxidans*, *L. interrogans*, or *M. loti*.** *A*, this is the predominant *E. coli* lipid A molecule found in cells grown at pH 7.4 (3). The *cyan* O atom at positions 3 and 3′ are characteristic of a GlcN disaccharide. *B*, the *A. ferrooxidans* lipid A backbone contains both GlcN and GlcN3N (3 and/or 3′-nitrogen atoms of GlcN3N in *red* and corresponding oxygen atoms of GlcN in *cyan*). This lipid A is likely a mixture of four species. Limited structural data suggest that it contains five acyl chains (5). The 1- and 4′-positions (*X* and *Y*, respectively) lack phosphate moieties (5). *C*, *L. interrogans and M. loti* lipid A contain GlcN3N exclusively (3- and 3′-nitrogen atoms in *red*). The structure of *M. loti* lipid A is not fully characterized (6). That of *L. interrogans* is presented in the following paper (7). In lipopolysaccharide, 3-deoxy-D-*manno*-octulosonic acid would be attached at position 6′.

EXPERIMENTAL PROCEDURES

Materials—All growth media, materials, and fine chemicals were the same as in the preceding manuscript (4) or were purchased from Sigma-Aldrich. PerkinElmer Life Sciences was the source of [α-^{32}P]UTP. The LpxC inhibitor L-573,655 was kindly provided by Dr. A. Patchett (Merck Research Laboratories) (14).

Bacterial Strains and Plasmids—*A. ferrooxidans* ATCC 23270 and *M. loti* ATCC 700743 were purchased from the American Type Culture Collection. *A. ferrooxidans* cells were grown on modified 9K medium (15). *M. loti* cells were grown on yeast mannitol agar or liquid medium (6). *E. coli* XL1-Blue (Stratagene) was used for cloning and maintenance of plasmids. *E. coli* BL21(DE3)/pLysS (Stratagene) was used for overexpression of cloned genes, typically using the Novagen plasmids pET23c+ and pET30a+. The *E. coli* temperature-sensitive mutant RO138 (*lpxA2 recA rpsL Tet*^{r}), a *recA* derivative of SM101 (12), was provided by Dr. M. Anderson (Merck Research Laboratories). The vector pBluescript II SK(+) (Stratagene) was used to express foreign genes in R0138, as described below. Plasmid constructs are summarized in Table I.

E. coli cells were generally grown on LB agar or in LB broth, adjusted to pH 7.4 (16). Bacteria harboring hybrid plasmids were selected using ampicillin at 100 μg/ml. The recombinant plasmids pTO1 (17), pTO5 (17), and pCS355 (4) have been previously described. The plasmid pCS61 was constructed by subcloning the *E. coli lpxA* gene from pTO5 (17) into the low copy vector pNGHamp (18), using the SacI restriction site.

Recombinant DNA Techniques—Transformation of competent cells, nucleic acid purification, and electrophoresis were carried out according to established procedures (19, 20). The plasmids were purified using the Qiaprep miniprep spin column kit (Qiagen). Digested PCR products and plasmid DNA were purified by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation (19, 20) or by agarose gel electrophoresis in conjunction with the Qiagen Qiaquick gel extraction kit (4).

Isolation and Cloning of the lpxA Gene from A. ferrooxidans—Genomic DNA was prepared from *A. ferrooxidans* as described (4). The *lpxA* gene was PCR-amplified with the *Pfu* polymerase using the oligonucleotide primers 5′-GGA ATT CCA TAT GAC GGT GCA GAT TCA TCC GCT GG-3′ (N-terminal primer) and 5′-CGG GAT CCC CCC GAT GAC CCC GGT TCT CAT ATG G-3′ (C-terminal primer). The NdeI and

TABLE I
Bacterial strains and plasmids

	Description	Source
M. loti 700743		ATCC
A. ferrooxidans 23270		ATCC
E. coli BL21(DE3)/pLysS	bears DE3 lysogen and pLysS plasmid	Stratagene
E. coli RO138	*lpxA2 recA Tetr*	M. Anderson, Merck & Co, Inc.
E. coli XL1-Blue		Stratagene
pET23c+	T7 expression vector, Amp^r	Novagen
pET30a+	T7*lac* expression vector, Kan^r	Novagen
pBluescript II SK+	*Lac* expression vector, Amp^r	Stratagene
pNGH100	*Trp/lac* expression vector, Cam^r	Ref. 17
pNGHamp	*Trp/lac* expression vector, Amp^r	Ref. 18
pLP3	pET30a+ containing *L. interrogans lpxA*	This work
pLP3–1	pET30a+ containing *L. interrogans lpxAH120A*	This work
pTO1	pET23c+ containing *E. coli lpxA*	Ref. 17
pTO5	pBluescript II SK+ containing *E. coli lpxA*	Ref. 17
pCS61	pNGHamp containing *E. coli lpxA*	This work
pCS311	pET23c+ containing *A. ferrooxidans lpxA*	This work
pCS355	pET23c+ containing *A. ferrooxidans gnnA* and *gnnB*	Ref. 4
pCS411	pBluescript II SK+ containing *A. ferrooxidans gnnA* and *gnnB*	This work
pCS421	pBluescript II SK+ containing *A. ferrooxidans lpxA*	This work
PCS439	pBluescript II SK+ containing *A. ferrooxidans gnnA*, *gnnB*, and *lpxA*	This work
pCS449	pNGH100 containing *A. ferrooxidans lpxA*	This work
pCS611	pBluescript II SK+ containing *L. interrogans lpxA*	This work
pCS623	pNGH100 containing *L. interrogans lpxA*	This work

BamHI sites, respectively, are underlined. The PCR product of *AflpxA* DNA was purified and digested with NdeI and BamHI in preparation for ligation into similarly treated pET23c+ vector. The desired derivative of pET23c+ bearing *AflpxA* was designated pCS311. The AfLpxA amino acid sequence is MTVQIHPLAI VDSSVQIGEG CTIGPFAVIG AGVEIGDHCR IGANTVIEGP CRLGAHNQIF QFASVGTAPQ DLGYAGEPTT LEIGSHNTIR EFVTINRGTV KGGGTTRIGH HNLLMAYCHV AHDCSIGDQV VMANAATLAG HVSVEDHAIL GGLSAVHQYA RVGAHAILGG GTMAPLDIPP FMMAAGNHAS LHGINVRGLA RRGIPRETIL QIKRAYRLLF RSGLRLEDAM DEVSQRGLNA PEVAYLLDFI RNSRRGITRP.

Generation of Plasmid Constructs Containing lpxA, gnnA, and gnnB from A. ferrooxidans—The *AflpxA* gene was subcloned from pCS311 along with the pET23c+ ribosome-binding site into pBluescript II SK(+) using XbaI and HindIII to generate pCS421 and then from this construct into pNGH100 (17) using SacI and BamHI to generate pCS449. The *gnnA* and *gnnB* genes were likewise cloned into pBluescript II SK(+) by insertion of the XbaI/HindIII fragment of the bicistronic construct pCS355 (4) into similarly treated pBluescript vector. The desired hybrid plasmid expressing the two genes was designated pCS411. In addition, a pBluescript II SK(+)-based construct was constructed bearing the *A. ferrooxidans gnnA*, *gnnB*, and *lpxA* genes. For this purpose, a PCR fragment bearing *AflpxA* was first generated using pCS311 as the template, and primers were designed to create a PCR product bearing the entire *lpxA* gene preceded by the pET23c+ ribosome-binding site. The N-terminal primer was 5′-CCC AAA AAG CTT GGG AGA CCA CAA CGG TTT CCC-3′. The C-terminal primer was 5′-CCC GCC TCG AGG TCG ACG GAG CTC GAA TTC GGA TCC-3′. This fragment was then cloned into pCS411 using HindIII and XhoI digestion, which inserts the *lpxA* fragment behind the *gnnA* and *gnnB* genes. As noted above, these genes are themselves behind a ribosome-binding site from pET23c+ (subcloned from pCS355). The hybrid plasmid derived from this ligation was designated pCS439.

Cloning of lpxA from L. interrogans Serovar Icterohemeorrhagiae (Strain Verdun)—The whole genome sequence of the *L. interrogans* serovar Lai (21) revealed one gene (*LilpxA*) of 780 bp that displayed 41% identity and 59% similarity at the protein level with *E. coli* LpxA (9). The *LilpxA* gene of *L. interrogans* serovar Icterohemeorrhagiae (strain Verdun, virulent isolate) (22) was PCR-amplified with the *Pfu* polymerase using the oligonucleotide primers 5′-GGA ATT CCA TAT GAA AAT ACA TCC GAC TGC TAT TA-3′ (N-terminal primer) and 5′-GCG GAT CCT CAC CTG TGA TTT GTA ACT CCCC-3′ (C-terminal primer). The NdeI and BamHI sites, respectively, are underlined. The amplified gene was digested with NdeI and BamHI and then ligated with T4 DNA ligase into similarly digested pET30a+. The ligation mixture was transformed by electroporation into supercompetent XL1-Blue (Stratagene). Plasmid-containing transformants were selected at 37 °C on LB agar plates supplemented with kanamycin (30 μg/ml). Putative clones were repurified on LB kanamycin plates. Plasmid DNA was then isolated and screened for the presence of the desired insert by BamHI and NdeI digestion. One positive clone containing the *LilpxA* gene was designated pLP3 and confirmed by DNA sequencing. Only two silent changes, specific for the Verdun strain, were detected in comparison with the nucleotide sequence of *lpx*A from the Lai strain.

For preparation of cell extracts and assays, the *LilpxA* gene of pLP3 was overexpressed in *E. coli* BL21(DE3)/pLysS (Stratagene). The *LilpxA* gene was also subcloned from pLP3 into pBluescript II SK(+) using XbaI and XhoI to generate pCS611, as described for pCS421. The *LilpxA* gene was subcloned into the low copy vector pNGH100 to make pCS623, using SacI and BamHI as described for pCS449.

Preparation of Cell-free Extracts—To prepare *E. coli* extracts for assays, the constructs were grown at 30 °C in 50 ml of LB broth with 100 μg/ml ampicillin to A_{600} = 0.5, shifted to 18 °C for 10 min, induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside, and then grown overnight. The extracts were prepared by passage through a French pressure cell, as described (4). The protein concentrations were quantified using the Pierce bicinchoninic acid assay kit (23) with bovine serum albumin as the standard.

Cell-free extracts and membranes of *L. interrogans* strain Verdun (avirulent derivative) were prepared from a 4.3-g frozen cell pellet derived from a 10-liter culture (2×10^9 bacteria/ml) grown at the Institut Pasteur (Paris, France) (22). All of the steps were conducted at 0–4 °C. The pellet was resuspended in 20 ml of 50 mM HEPES, pH 7.5, containing 10% glycerol. A 5-ml portion of the cell suspension was diluted to 10 ml with the same buffer, and the remaining 15 ml was stored at −80 °C for later use. The cells were broken by three passages through a French pressure cell at 10,000 p.s.i. The lysate was centrifuged at 12,600 × *g* for 10 min to remove unbroken cells and large debris. The protein concentration of this extract was 6.9 mg/ml (23). A 7-ml portion of the extract was centrifuged at 100,000 × *g* for 1 h. The soluble fraction was stored at −80 °C, and the membranes were homogenized in ~1 ml of 50 mM HEPES, pH 7.5, to yield a final protein concentration of about 12 mg/ml. The membrane suspension was then divided into aliquots and stored at −80 °C.

Substrate Preparation—The [α-^{32}P]UDP-GlcNAc and all of the acyl-ACP substrates were prepared as previously described (24). To make [α-^{32}P]UDP-GlcNAc3N from [α-^{32}P]UDP-GlcNAc, 250 μCi of [α-^{32}P]UDP-GlcNAc was dissolved in 138 μl of deionized water. This radiolabeled substrate (final concentration, 1.5 μM) was incubated with an additional 48.5 μM UDP-GlcNAc carrier, 50 μM HEPES, pH 8.0, 100 mM L-glutamic acid, 1 mM NAD^+, and 0.5 mg/ml pCS355 cell-free extract (4) in a reaction volume of 200 μl for 2.5 h at 30 °C. About 50% of the [α-^{32}P]UDP-GlcNAc is converted to [α-^{32}P]UDP-GlcNAc3N under these conditions. This reaction was then chilled on ice and diluted 3-fold with cold 100% ethanol. After 10 min, the debris was removed by centrifugation. The supernatant was diluted 4-fold with cold water.

To separate the [α-^{32}P]UDP-GlcNAc and [α-^{32}P]UDP-GlcNAc3N, anion exchange chromatography was performed using a 2-ml DEAE-cellulose column (Whatman DE52) equilibrated with 10 mM BisTris, pH 6.0. The diluted reaction mixture was loaded onto the column, which

was washed with 8 ml of deionized water and then with 8 ml of 10 mM BisTris, pH 6.0. The column was eluted with increasing concentrations of BisTris, pH 6.0, according to the following scheme: 8 ml at 20 mM, 8 ml at 30 mM, 8 ml at 40 mM, 4 ml at 50 mM, and 12 ml at 80 mM. The fractions (2 ml) were analyzed by TLC on PEI-cellulose plates, developed with 0.2 M aqueous guanidine HCl. Most of the [α-^{32}P]UDP-GlcNAc3N elutes with the 20 and 30 mM BisTris. The relevant fractions were pooled, diluted 4-fold with water, and loaded onto a second DEAE column to remove NAD^+ (4).

In Vitro lpxA Assay Conditions—The LpxA-catalyzed conversion of [α-^{32}P]UDP-GlcNAc or [α-^{32}P]UDP-GlcNAc3N to [α-^{32}P]UDP-(3-*O*-acyl)-GlcNAc and [α-^{32}P]UDP-(3-*N*-acyl)-GlcNAc3N, respectively, is monitored by TLC on a silica plate because the acylated product migrates more rapidly than the substrate (8, 24, 25). Each 10-μl reaction tube contained 40 mM HEPES, pH 8.0, 1 mg/ml bovine serum albumin, 0.2 mg/ml LpxC inhibitor L-573–655 (14), and 10 μM acyl donor (as indicated). Either 10 μM [α-^{32}P]UDP-GlcNAc or 10 μM [α-^{32}P]UDP-GlcNAc3N was used as the acyl acceptor at 2×10^5 dpm/tube. The reaction was started at 30 °C by the addition of an appropriate amount of cell-free extract. Conversion to product was analyzed by spotting 1 μl of each reaction mixture on a silica TLC plate. The plate was developed with chloroform/methanol/water/acetic acid (25:15:4:2, v/v) and analyzed with a PhosphorImager.

Site-directed Mutagenesis of LilpxA—Using pLP3 as the template, the QuikChange site-directed mutagenesis kit (Stratagene) was used to generate the H120A substitution, which corresponds to the H125A mutation in *E. coli* (11). The structure of the mutated plasmid, designated pLP3-1, was confirmed by DNA sequencing. The pLP3-1 plasmid was then transformed into competent cells of BL21(DE3)/pLysS.

Structural Analysis of Lipid A Species Isolated from Various E. coli Constructs—Lipid A was isolated from the *E. coli lpxA2* mutant RO138 complemented with different sets of genes. The first construct expressed *gnnA*, *gnnB*, and *AflpxA* off of a single hybrid plasmid (RO138/pCS439) (4), whereas the second construct expressed *gnnA*, *gnnB*, and *LilpxA* on separate hybrid plasmids (RO138/pCS411/pCS623) (Table I).

For preparation of the lipid A, RO138/pCS439 was grown without shaking in 1 liter of LB medium for 48 h at 42 °C in the presence of 50 μg/ml ampicillin and 12 μg/ml tetracycline. A 100-ml culture of RO138/pCS411/pCS623 was grown with shaking at 250 rpm in LB medium at 30 °C. The latter construct was not viable at 42 °C. A 100-ml culture of RO138/pCS61 also was grown at 42 °C in LB medium with shaking, given that it grows normally and produces normal amounts of wild-type lipid A.

Extraction, hydrolysis at pH 4.5, and purification of lipid A 1,4′-bisphosphate species by DEAE-cellulose chromatography was carried out as previously described (17, 26). Prior to mass spectrometry, the purified lipid A was subjected to base hydrolysis by incubation in freshly made chloroform, methanol, 1.7 M NaOH (2:1:0.4, v/v/v) at room temperature (≈25 °C) for 2 h, with occasional mixing of the two phases (27). Following hydrolysis, the organic phase was dried under N_2, and the lipid was redissolved in chloroform, methanol (4:1, v/v). A portion was spotted onto a silica gel 60 TLC plate, developed in chloroform, pyridine, formic acid, water (50:50:16:5, v/v/v/v). After spraying with 10% sulfuric acid in ethanol, the lipids were visualized by charring.

Mass Spectrometry of Base-treated Lipid A Samples—MALDI-TOF mass spectra were acquired in the negative-ion linear modes using a Kratos Analytical (Manchester, UK) MALDI-TOF mass spectrometer, operated with a 337-nm nitrogen laser, a 20-kV extraction voltage, and time-delayed extraction (24).

RESULTS

Selectivity of LpxA Acyltransferases in Extracts of M. loti and L. interrogans—Of the three organisms used for studying the origin of GlcN3N-containing lipid A molecules, *M. loti* is easiest to grow (6). As shown in Fig. 2*A* and Table II, 10 μM [α-^{32}P]UDP-GlcNAc is inactive as an acceptor substrate in extracts of *M. loti* when tested with either 10-, 12-, 14-, or 16-carbon 3-hydroxyacyl-ACP donors (each added at 10 μM). In contrast, [α-^{32}P]UDP-GlcNAc3N is efficiently acylated in the presence of 10-, 12-, or 14-carbon 3-hydroxyacyl-ACPs under matched conditions (Fig. 2*B* and Table II). The specific activity of *M. loti* LpxA was the highest with 3-hydroxymyristoyl-ACP (Table II), consistent with the reported fatty acid composition of *M. loti* lipid A (6). Prolonged incubation (Fig. 2*B*, *lanes 10* and *14*) resulted in nearly quantitative conversion of

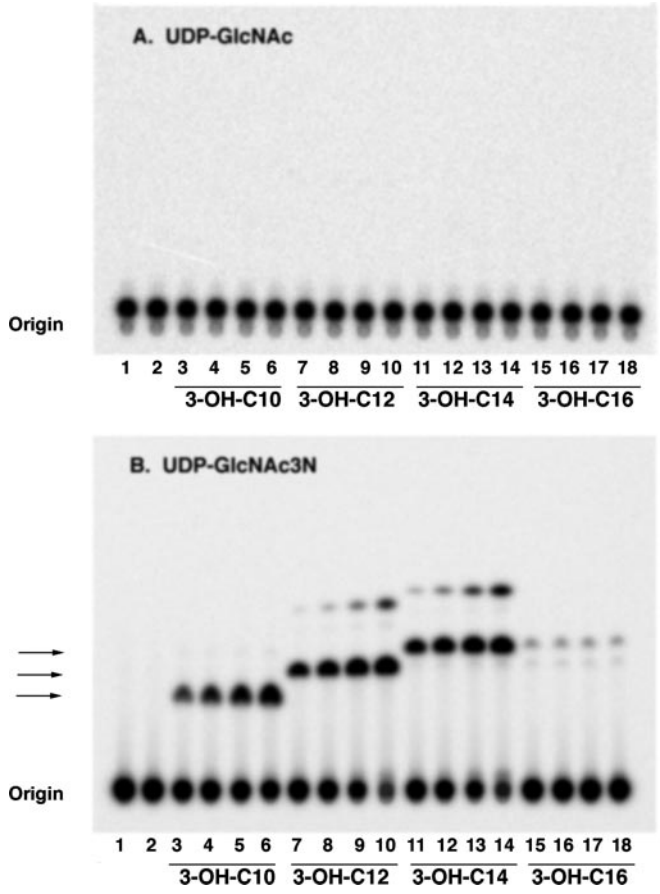

FIG. 2. **Acylation of [α-^{32}P]UDP-GlcNAc3N but not [α-^{32}P]UDP-GlcNAc in extracts of *M. loti*.** Cell-free extracts were assayed at 0.05 mg/ml protein with 10 μM each of donor and acceptor substrates, as indicated. The four time points for each indicated hydroxyacyl-ACP (*lanes 3–6*, *7–10*, *11–14*, and *15–18*) were 5, 10, 20, and 30 min. *Lanes 1* and *2* are no enzyme controls at 0 and 30 min. *A*, [α-^{32}P]UDP-GlcNAc as acceptor substrate. *B*, [α-^{32}P]UDP-GlcNAc3N as acceptor substrate. The *arrows* indicate R_F values of monoacylated products formed with the indicated acyl-ACP donors.

TABLE II
Sugar nucleotide and acyl-ACP selectivity of LpxA acyltransferases in extracts of E. coli, M. loti, and L. interrogans

Cell-free extracts were assayed in the linear range with 0.05–0.5 mg/ml protein, as appropriate. Conversion of [α -^{32}P]UDP-GlcNAc or [α-^{32}P]UDP-GlcNAc3N to the corresponding acylated derivative was monitored by TLC, followed by analysis with a PhosphorImager. The standard deviation of the specific activities was approximately ± 20% for triplicate or quadruplicate determinations.

Extract	Specific activity with indicated acyl donor			
	3-OH-C10-ACP	3-OH-C12-ACP	3-OH-C14-ACP	3-OH-C16-ACP
	pmol/min/mg % of maximal specific activity			
UDP-GlcNAc				
E. coli/pET23c+	<2	<2	51	<2
M. loti	<2	<2	<2	<2
L. interrogans	<2	<2	<2	<2
UDP-GlcNAc3N				
E. coli/pET23c+	<2	<2	84	<2
M. loti	520 (26)	800 (40)	2000 (100)	4 (0.2)
L. interrogans	<2	1400	<2	<2

[α-^{32}P]UDP-GlcNAc3N to acylated product, indicating that *N*-acylation by *M. loti* LpxA is thermodynamically favorable. This contrasts with the 3-*O*-acylation of [α-^{32}P]UDP-GlcNAc by *E. coli* LpxA, which is thermodynamically unfavorable (11, 28).

Crude extracts of *L. interrogans* serovar Icterohemeorrhagiae (strain Verdun) were prepared from frozen cells (22) and

FIG. 3. **Sequence comparison of AfLpxA, EcLpxA, and LiLpxA.** This ClustalW alignment (www.embl-heidelberg.de/) shows the predicted protein sequences and conserved residues of AfLpxA, LiLpxA, and EcLpxA. The relatively conserved *E. coli* lysine 76 and the absolutely conserved *E. coli* histidine 125 residues are highlighted with *arrows* (11). In many but not all bacteria that make lipid A containing GlcN3N, the lysine 76 equivalent is substituted with glycine. Lysine 76 is in the vicinity of the *E. coli* LpxA active site and may contribute to substrate binding (11). However, the G71K substitution in LiLpxA had no effect on sugar nucleotide selectivity (not shown).

assayed as described above for *M. loti*. Acyltransferase activity was observed only with 10 μM [α-^{32}P]UDP-GlcNAc3N and 10 μM 3-hydroxylauroyl-ACP (Table II). Prolonged incubation resulted in complete conversion of 10 μM [α-^{32}P]UDP-GlcNAc3N to product, as with the *M. loti* extracts.

The quantification of LpxA specific activities observed in various cell extracts with different combinations of substrates is summarized in Table II. Unexpectedly, *E. coli* LpxA was found to acylate UDP-GlcNAc3N at a slightly faster rate than its natural substrate UDP-GlcNAc (10 μM acceptor and donor substrates).

Mild Alkaline Hydrolysis of Acylated [α-^{32}P]UDP-GlcNAc3N—To confirm that LpxA acylates [α-^{32}P]UDP-GlcNAc3N on the nitrogen atom at the pyranose 3-position, a portion of the LpxA product generated with either *E. coli* or *L. interrogans* LpxA was subjected to mild alkaline hydrolysis (27). In contrast to [α-^{32}P]UDP-3-*O*-acyl-GlcNAc synthesized by *E. coli* LpxA, which is deacylated by a 30-min exposure to dilute NaOH at room temperature (27), the acylated [α-^{32}P]UDP-GlcNAc3N generated either by *E. coli* or *L. interrogans* LpxA was unaffected. These observations, together with the thermodynamically favorable acylation seen with UDP-GlcNAc3N *versus* UDP-GlcNAc, are consistent with the formation of an amide-linked acyl chain at the 3-position of the pryanose ring of UDP-GlcNAc3N (Scheme 1 in Ref. 4).

Cloning of LpxA Orthologs from L. interrogans and A. ferrooxidans—The *lpxA* genes of *L. interrogans* (21) and *A. ferrooxidans* were identified by probing their genomes (www.ncbi.nlm.nih.gov/BLAST/) with the *E. coli* LpxA sequence (9). One 780-bp gene, designated *LilpxA*, encodes a predicted protein of 259 amino acids that displays 41% amino acid identity and 59% similarity with *E. coli* LpxA (21), with an *E* value of about 4×10^{-51} in a pair-wise comparison (29). The nearly identical *lpxA* gene from *L. interrogans* strain Verdun (22) was cloned by PCR from its genomic DNA, inserted into pET30a+, and expressed in *E. coli* BL21(DE3)/pLysS. The *lpxA* gene of *A. ferrooxidans* encodes a protein of 260 amino acid residues with 49% amino acid identity, 69% similarity with an *E* value $\approx 3 \times 10^{-71}$ when compared with *E. coli* LpxA (29). This gene was first cloned into pET23c+ and also into pBluescript II SK(+), pNGH100, or pCS411, depending on the experiment (see below). A ClustalW alignment of EcLpxA, AfLpxA, and LiLpxA is shown in Fig. 3.

In Vitro Assays of the Cloned LpxA Orthologs—The pET vector constructs harboring either *EclpxA*, *AflpxA*, or *LilpxA* were expressed in *E. coli* BL21 (DE3)/pLysS, and the extracts were assayed for their ability to acylate either UDP-GlcNAc or UDP-GlcNAc3N (Table III). Extracts of all three constructs demonstrated significant overexpression of acyltransferase activity with at least one combination of acyl-ACP donor and sugar nucleotide acceptor, when compared with the vector controls (Table III). In the case of recombinant *L. interrogans* LpxA, acylation was not measurable for any combination except UDP-GlcNAc3N and 3-hydroxylauroyl-ACP, consistent with the assays shown in Table II. The recombinant *A. ferrooxidans* enzyme, although selective for UDP-GlcNAc3N, displayed low but measurable activity with UDP-GlcNAc (Table III). The recombinant AfLpxA displayed relaxed acyl donor chain length selectivity (Table III), when compared with *E. coli* or *L. interrogans* LpxA.

The specific activity of the recombinant *E. coli* enzyme with 3-hydroxymyristoyl-ACP and UDP-GlcNAc is in agreement with published data (10, 11). EcLpxA is ~100-fold selective for the 3-hydroxymyristoyl-ACP over 3-hydroxylauroyl-ACP or 3-hydroxypalmitoyl-ACP (Table III) (10). Cloned *E. coli* LpxA showed robust activity with UDP-GlcNAc3N (Table III) and retained the same pattern of 3-hydroxyacyl-ACP chain length selectivity as with UDP-GlcNAc (Table III). No LpxA protein tested showed measurable activity with decanoyl-ACP, lauroyl-ACP, myristoyl-ACP, or palmitoyl-ACP (data not shown).

TABLE III

Sugar nucleotide and acyl-ACP selectivity of LpxA in extracts of E. coli expressing lpxA of E. coli, A. ferrooxidans, or L. interrogans

Induced extracts of BL21 (DE3)/pLysS, containing vector, pTO1, pCS311, or pLP3 were assayed with the indicated acyl donor and sugar nucleotide combinations, each at 10 μM. All of the assays were in the linear range with respect to time and extract concentrations. The standard deviation of the specific activities was approximately ± 20% for triplicate or quadruplicate determinations.

Extract	Specific activity with indicated acyl donor			
	3-OH-C10-ACP	3-OH-C12-ACP	3-OH-C14-ACP	3-OH-C16-ACP
	pmol/min/mg % of maximal specific activity			
UDP-GlcNAc				
pET23c+ vector	<2	<2	51	<2
pTO1 (*EclpxA*)	54 (0.1)[a]	1600 (2.8)	58,000 (100)	300 (0.5)
pCS311 (*AflpxA*)	<2	10 (28)	36 (100)[b]	5.3 (15)
pLP3 (*LilpxA*)	<2	<2	< 50	<2
UDP-GlcNAc3N				
pET23c+ vector	<2	<2	84	<2
pTO1 (*EclpxA*)		4700 (5.2)	91,000 (100)	1700 (1.9)
pCS311 (*AflpxA*)	600 (18)	810 (24)	3400 (100)	500 (15)
pLP3 (*LilpxA*)	<2	60,000	<100	<2

[a] Data from Wyckoff *et al*. (10).

[b] See text regarding the reasons for specific activity below vector control.

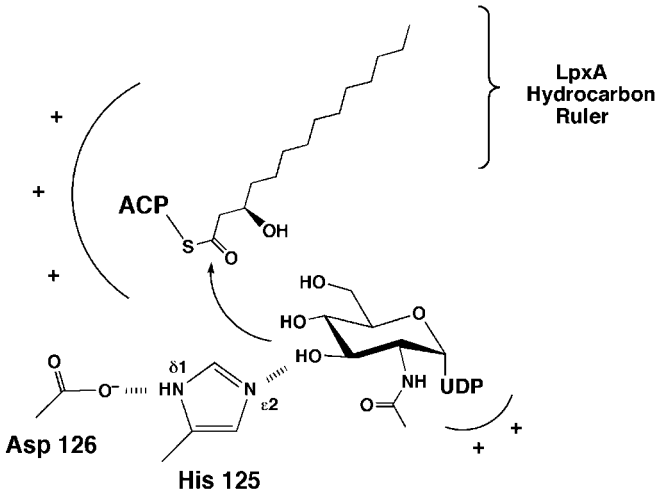

FIG. 4. **Possible role of His^{125} in the catalytic mechanism of *E. coli* LpxA.** In our previously published model (11), N$^{\delta 1}$ of histidine 125 (in the alternative tautomeric representation from the one shown above) was proposed to function as the general base. However, the His^{125} side chain of LpxA was flipped in the published x-ray structure (9) (S. Roderick, personal communication). With the revised conformation, N$^{\epsilon 2}$ of His^{125} is proposed to activate the glucosamine 3-OH group in the acceptor substrate, and the conserved Asp^{126} side chain would then be in a good position to orient and/or stabilize the His^{125} residue by hydrogen bonding the N$^{\delta 1}$ proton.

Site-directed Mutagenesis of L. interrogans LpxA—Previous studies demonstrated that His^{125} of *E. coli* is required for catalysis (11), perhaps as a general base to activate the glucosamine 3-OH group of UDP-GlcNAc during acyl chain transfer (Fig. 4). His^{125} is conserved and corresponds to His^{120} in *L. interrogans* (Fig. 3, *lower arrow*). Substitution of His^{120} with alanine inactivates the *L. interrogans* LpxA (Fig. 5), suggesting a similar function as in *E. coli*, despite the differences in acceptor and donor selectivity.

Partial Complementation of E. coli RO138 (lpxA2) by gnnA, gnnB, and/or AflpxA—The temperature-sensitive *E. coli lpxA2* mutant RO138 (12, 13) was transformed with hybrid plasmids harboring genes for the biosynthesis of UDP-GlcNAc3N and/or an LpxA ortholog specific for UDP-GlcNAc3N. The plasmids pCS411 (*gnnA gnnB*), pCS449 (low copy *AflpxA*), and pCS623 (*LilpxA*) did not complement RO138 at 42 °C, but pCS421 (high copy *AflpxA*) by itself was effective. The latter observation suggests that the AfLpxA acyltransferase activity seen with UDP-GlcNAc as the acceptor substrate (Table III) is sufficient to restore the growth of RO138 at 42 °C provided the AfLpxA protein is expressed at a high level. The transformation of RO138/pCS411 (*gnnA gnnB*) with pCS449 (low copy *AflpxA*) likewise did not rescue the temperature-sensitive phenotype of RO138, suggesting that there was not enough expression of AfLpxA from pCS449. To address this issue, RO138 was transformed with the pBluescript-derived high copy plasmid pCS439 (*gnnA gnnB AflpxA*). This strain grew slowly on LB plates or liquid medium at 42 °C, provided the culture was not shaken.

Biosynthesis of GlcN3N-containing Lipid A Molecules in

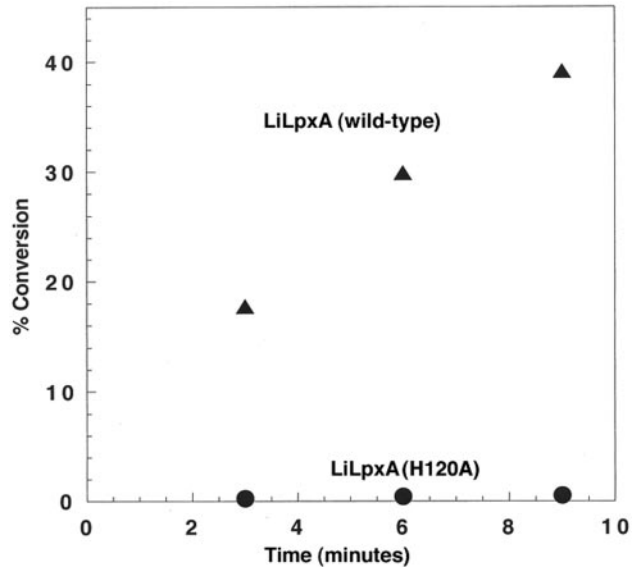

FIG. 5. **The H120A substitution inactivates *L. interrogans* LpxA.** His^{120} of *L. interrogans* LpxA is the equivalent of His^{125} in *E. coli* (see Fig. 3) (11).

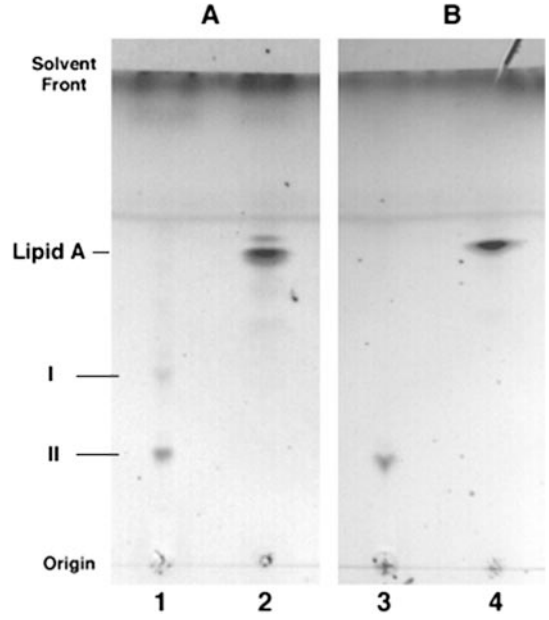

FIG. 6. **Mild alkaline hydrolysis of lipid A from RO138/pCS61 (*EclpxA*) (*A*) and RO138/pCS439 (*gnnA gnnB AflpxA*) (*B*).** The plate was developed in chloroform/pyridine/formic acid/water (50:50:16:5, v/v/v/v). The lipids were visualized by spraying with 10% sulfuric acid in ethanol, followed by charring on a hot plate. Nonhydrolyzed lipid A controls are shown for RO138/pCS439 (*lane 2*) and RO138/pCS61 (*lane 4*). Corresponding base-treated samples are shown in *lanes 1* and *3*, respectively.

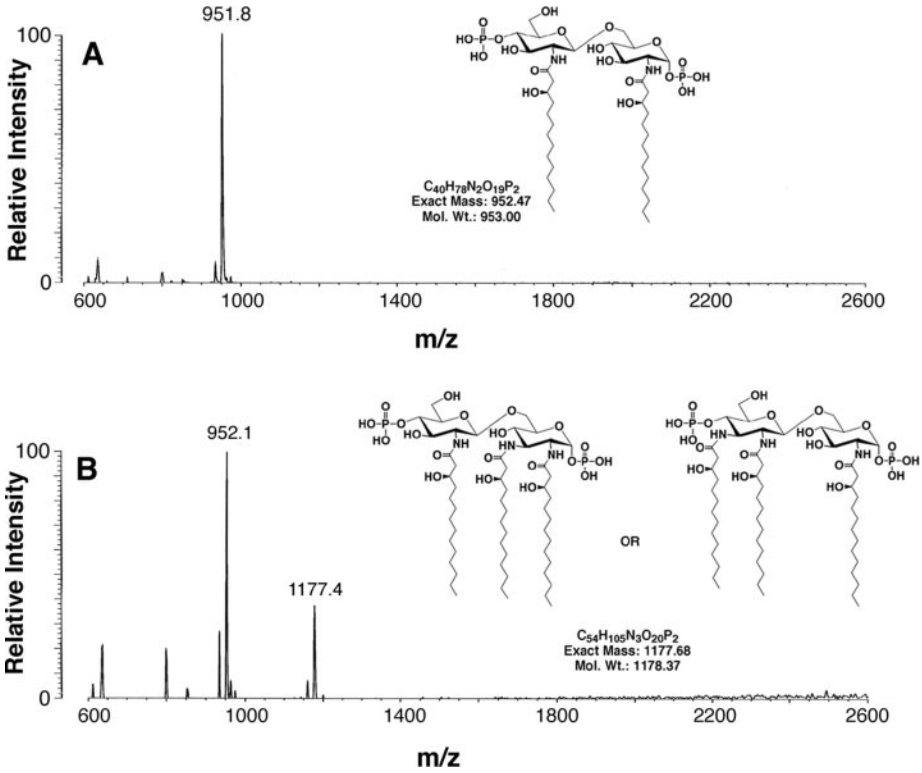

FIG. 7. **Negative ion mode MALDI-TOF mass spectrometry of base-hydrolyzed lipid A from RO138/pCS61 and RO138/pCS439.** A 10-fold excess of RO138/pCS61 (*EclpxA*) lipid A (*A*) was hydrolyzed, when compared with RO138/pCS439 (*gnnA gnnB AflpxA*) lipid A (*B*), to ensure that complete hydrolysis of all ester-linked acyl chains was taking place under all experimental conditions. The proposed structures of the mild alkaline hydrolysis products are indicated.

RO138 Complemented with gnnA, gnnB, and AflpxA—Lipid A samples from RO138/pCS61 (*EclpxA*) and RO138/pCS439 (*gnnA gnnB AflpxA*) grown without shaking at 42 °C were isolated and purified by ion exchange chromatography on DEAE cellulose. Each lipid A preparation was subjected to mild alkaline hydrolysis to remove all of the ester-linked acyl chains. TLC analysis of the hydrolyzed samples suggested that a portion (~30%) of the RO138/pCS439 (*gnnA gnnB AflpxA*) lipid A contained three base-resistant acyl chains (Fig. 6*A*, *Band I*) under conditions that degrade all of the RO138/pCS61 (*EclpxA*) lipid A to a single, diacylated derivative (Fig. 6*B*, *Band II*).

MALDI-TOF Mass Spectrometry of the Base-hydrolyzed Lipid A Samples—To confirm the identities of the base-treated lipid preparations, MALDI-TOF mass spectrometry was performed in the negative mode. Hydrolyzed lipid A from RO138/pCS61 (*EclpxA*) gave rise to a single peak at *m/z* 951.8 (Fig. 7*A*), interpreted as $[M - H]^-$ of a diacylated lipid A 1,4′-bisphosphate (Fig. 7*A*), formed during base hydrolysis by the loss of the four ester-linked acyl chains that are normally present in native *E. coli* lipid A (Fig. 1).

The base-treated lipid A from RO138/pCS439 (*gnnA gnnB AflpxA*) grown at 42 °C likewise shows a major peak at *m/z* 952.1 (Fig. 7*B*), interpreted as $[M - H]^-$ of the same diacylated lipid A species seen with the RO138/pCS61 (*EclpxA*) lipid A (Fig. 7*A*). However, a second peak is present at *m/z* 1177.4 (Fig. 7*B*), which would be expected for $[M - H]^-$ of a lipid A 1,4′ bisphosphate species with three *N*-linked hydroxymyristoyl chains (Fig. 7*B*). The latter would be formed by mild base hydrolysis of lipid A molecules consisting of one glucosamine residue and one GlcN3N unit. The negative mode data (Fig. 7*B*) do not reveal the extent to which the GlcN3N unit is incorporated into the proximal or distal positions.

DISCUSSION

LpxA is an essential cytoplasmic enzyme in *E. coli* that catalyzes the first step of lipid A biosynthesis according to the reaction: UDP-GlcNAc + *R*-3-hydroxymyristoyl-ACP → UDP-3-*O*-(*R*-3-hydroxymyristoyl)-GlcNAc + ACP (3, 8, 11, 30). The crystal structure of *E. coli* LpxA at 2.6-Å resolution suggests that the enzyme is homotrimer in which the active sites are situated between adjacent subunits (9–11). Each LpxA monomer is constructed around an unusual left-handed parallel β-helix, which is conserved in all LpxA orthologs and in many other bacterial acetyl- and acyltransferases (9, 31–33). The crystal structure of *E. coli* LpxA has not been determined in the presence of bound substrates or substrate analogs, but site-directed mutagenesis has demonstrated that histidine 125 is crucial for activity and that adjacent basic residues may contribute to substrate binding (Fig. 4) (11). The recent structure of *Helicobacter pylori* LpxA with a detergent molecule bound at the proposed active site supports the above conclusions (34).

The acyl-ACP donor selectivity of LpxA has previously been studied in several systems (8, 17, 24, 35–37). In general, LpxA orthologs show strong preferences for acyl chain length and the presence of the *R*-3-hydroxyl group (8, 17, 24, 35–37). The corresponding coenzyme A thioesters are not substrates (8, 25). *E. coli* LpxA is highly selective for *R*-3-hydroxymyristoyl-ACP (8, 25), whereas the *Pseudomonas aeruginosa* enzyme strongly prefers *R*-3-hydroxydecanoyl-ACP (35, 36). The G173M substitution converts the *E. coli* enzyme from a 14- to a 10-carbon-specific acyltransferase, whereas the converse M169G substitution in *P. aeruginosa* LpxA does the opposite (10). These findings show that precise hydrocarbon rulers are present in the active sites of these LpxA orthologs (Fig. 4). Cocrystals of LpxA variants with various acyl-ACPs will be required to gain further insights.

Although *E. coli* and related LpxA proteins have been characterized as UDP-*N*-acetylglucosamine 3-*O*-acyltransferases (8, 28, 35), their sugar nucleotide specificity has not been examined in depth (8). As proposed in Scheme 1 of the preceding manuscript (4), lipid A biosynthesis in bacteria that make GlcN3N-substituted lipid A molecules might start with the conversion of UDP-GlcNAc to the analog UDP-GlcNAc3N, followed by selective *N*-acylation catalyzed by special LpxA orthologs present in those organisms. The results shown in Fig. 2

and Tables II and III demonstrate that LpxA orthologs from *M. loti*, *L. interrogans*, and *A. ferrooxidans* do indeed utilize UDP-GlcNAc3N in strong or absolute preference to UDP-GlcNAc.

L. interrogans and *M. loti* make lipid A molecules based exclusively on a β-1′,6-linked GlcN3N disaccharide (6, 7), whereas *A. ferrooxidans* lipid A contains both GlcN3N and GlcN units (5). The LpxA with the most stringent substrate specificity is that of *L. interrogans* (Tables II and III). In agreement with the absence of glucosamine in the lipid A backbone of this organism (7), LiLpxA does not utilize UDP-GlcNAc at a measurable rate with any acyl donor substrate (limit of detection <2 pmol/min/mg of extract). Furthermore, LiLpxA does not acylate UDP-GlcNAc3N with any donor other than 3-hydroxylauroyl-ACP. Although most other LpxA proteins display a high degree of specificity for a particular acyl chain length, they do in fact function with alternative acyl donors at slow rates (Tables II and III) (8, 17, 24, 35, 37).

A. ferrooxidans LpxA displays some activity with all of the 3-hydroxyacyl-ACP donors tested and with both sugar nucleotides (Table III). *M. loti* cell extracts (Fig. 2) and the *Bordetella pertussis* and *B. bronchiseptica* LpxA orthologs (24) are likewise very relaxed with regard to acyl chain length selectivity. In the bordetellae, structural studies indicate that the acyl chains at the 3- and 3′-positions of lipid A can be different under different conditions, explaining the need for a relaxed LpxA ortholog (38–40). Structural characterization of *A. ferrooxidans* lipid A shows no such heterogeneity (5); however, this work was done with *A. ferrooxidans* IFO 14262 and may not reflect the exact lipid A structure of *A. ferrooxidans* ATCC 23270 from which our *AflpxA* gene was cloned. In RO138/pCS439, grown at 42 °C (Fig. 7*B*), mainly 3-hydroxymyristate was incorporated into the lipid A 3- and 3′-positions by AfLpxA. AfLpxA may be more selective *in vivo*, or more *R*-3-hydroxymyristoyl-ACP may be available inside the cells.

The composition of the lipid A synthesized by AfLpxA in living cells may be affected by the relative sizes of the UDP-GlcNAc and the UDP-GlcNAc3N pools. Despite reports of a mixed lipid A backbone in *A. ferrooxidans* (5) and our demonstration of a mixed lipid A composition in RO138/pCS439 (Figs. 6*A* and 7*B*), AfLpxA is ≈100-fold selective for UDP-GlcNAc3N over UDP-GlcNAc when assayed *in vitro* at 10 μM of each donor and acceptor substrate (Table III). Consequently, one could argue that a 100:1 concentration ratio of UDP-GlcNAc to UDP-GlcNAc3N might be needed *in vivo* to yield a mixed lipid A backbone containing equal amounts of GlcN3N and GlcN, as suggested in the literature (5). Because GlcN3N is roughly one-sixth of the total lipid A sugar in RO138/pCS439 (Fig. 7*B*), the *in vivo* ratio of UDP-GlcNAc to UDP-GlcNAc3N present in this *E. coli* construct might be ≈300:1.

The specific activity of AfLxpA-expressing *E. coli* extracts (36 pmol/min/mg) was significantly lower than that of the empty vector control (51 pmol/min/mg), when assayed with UDP-GlcNAc and 3-hydroxymyristoyl-ACP (Table III). Apparent suppression of the background chromosomal activity by heterologous overexpression of foreign LpxA orthologs has been noted previously (10, 17). This phenomenon may be due to the formation of inactive heterotrimers between EcLpxA and the foreign LpxA proteins. Because there was no measurable chromosomal activity with any acyl donor other than 3-hydroxymyristoyl-ACP in the vector control extracts under these assay conditions (Table III), the low but significant activity seen with UDP-GlcNAc when AfLpxA-expressing extracts were assayed with C-12 and C-16 hydroxyacyl-ACPs (Table III) likely represents true AfLpxA catalytic function.

E. coli LpxA has robust activity with UDP-GlcNAc3N and can tolerate the presence of some GlcN3N in its lipid A, as in RO138/pCS439 grown on plates or in nonshaking liquid culture medium at 42 °C (Fig. 7*B*). However, the complementation of the *lpxA2* phenotype was not complete. Very small colonies formed on plates, and the maximal A_{600} reached in nonshaken liquid medium was only ≈0.2. It may be that lipid A substituted with GlcN3N is toxic to *E. coli*. Whatever the explanation for the slow growth of RO138/pCS439 at 42 °C, it might yet be possible to substitute *E. coli* lipid A completely with GlcN3N, either by using alternative GnnA, GnnB, and/or LpxA orthologs or by introducing second site suppressor mutations into constructs like RO138/pCS439. Engineered strains containing GlcN3N-based lipid A molecules might be useful for the preparation of new endotoxin antagonists (41, 42) or novel vaccines.

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