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An Alternative Route for UDP-Diacylglucosamine Hydrolysis in Bacterial Lipid A Biosynthesis[†]

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ABSTRACT: The outer leaflet of the outer membranes of Gram-negative bacteria is composed primarily of lipid A, the hydrophobic anchor of lipopolysaccharide. Like *Escherichia coli*, most Gram-negative bacteria encode one copy of each of the nine genes required for lipid A biosynthesis. An important exception exists in the case of the fourth enzyme, LpxH, a peripheral membrane protein that hydrolyzes UDP-2,3-diacylglucosamine to form 2,3-diacylglucosamine 1-phosphate and UMP by catalyzing the attack of water at the α-P atom. Many Gram-negative organisms, including all α-proteobacteria and diverse environmental isolates, lack LpxH. Here, we report a distinct UDP-2,3-diacylglucosamine pyrophosphatase, designated LpxI, which has no sequence similarity to LpxH but generates the same products by a different route. LpxI was identified because its structural gene is located between *lpxA* and *lpxB* in *Caulobacter crescentus*. The *lpxI* gene rescues the conditional lethality of *lpxH*-deficient *E. coli*. Lysates of *E. coli* in which *C. crescentus* LpxI (CcLpxI) is overexpressed display high levels of UDP-2,3-diacylglucosamine pyrophosphatase activity. CcLpxI was purified to >90% homogeneity. CcLpxI is stimulated by divalent cations and is inhibited by EDTA. Unlike *E. coli* LpxH, CcLpxI is not inhibited by an increase in the concentration of detergent, and its pH dependency is different. When the CcLpxI reaction is conducted in the presence of $H_2{}^{18}O$, the ^{18}O is incorporated exclusively into the 2,3-diacylglucosamine 1-phosphate product, as judged by mass spectrometry, demonstrating that CcLpxI catalyzes the attack of water on the β-P atom of UDP-2,3-diacylglucosamine.

Gram-negative bacteria possess inner and outer membranes. The outer membrane is asymmetric, having a periplasm-facing leaflet comprised of phospholipids, and an outer leaflet consisting primarily of lipid A, the hydrophobic anchor of lipopolysaccharide (LPS)[1] (*1*, *2*). The biosynthesis of lipid A is required for growth and viability in most Gram-negative bacteria (*1*, *3*). Therefore, the conserved, constitutive enzymes of lipid A biosynthesis (Scheme 1) are excellent targets against which to develop new antibiotics (*4*−*6*). This study focuses on the catalysis of UDP-2,3-diacylglucosamine hydrolysis (*7*, *8*), the fourth step of the pathway (Scheme 1).

The *lpxH* gene encodes the essential UDP-2,3-diacylglucosamine hydrolase of *Escherichia coli* (*7*, *8*). *E. coli* LpxH (EcLpxH) and its orthologues are distantly related to a large superfamily of metallo-phosphoesterases with diverse functions. EcLpxH is a peripheral membrane enzyme that displays apparent surface dilution kinetics (*8*), and it requires divalent cations, preferably Mn^{2+}, but not Mg^{2+}, for activity (*8*). ^{31}P NMR analysis of the EcLpxH products, UMP and 2,3-diacylglucosamine 1-phosphate (lipid X), generated from UDP-2,3-diacylglucosamine in the presence of $H_2{}^{18}O$, revealed that this hydrolase catalyzes the attack of water at the α-P atom of UDP-2,3-diacylglucosamine with incorporation of the ^{18}O isotope into the UMP product (*8*) (Scheme 2).

Most Gram-negative bacteria contain one gene encoding each enzyme of the lipid A biosynthetic pathway (Scheme 1) (*3*). These genes are easily identified by sequence comparisons (*9*). However, LpxH is a notable exception. Many bacterial species, including all α-proteobacteria, many δ-proteobacteria, all spirochetes, all cyanobacteria, and diverse environmental strains, such as the hyperthermophile *Aquifex aeolicus*, lack LpxH orthologues. They nevertheless produce lipid A using the same enzymes upstream and downstream of UDP-2,3-diacylglucosamine, as demonstrated for *Rhizobium leguminosarum* and *Rhizobium etli*, which lack LpxH (*10*, *11*). These findings suggest that alternative enzyme(s) catalyzing UDP-2,3-diacylglucosamine hydrolysis must exist in these bacteria to provide the appropriate substrates for LpxB (Scheme 1).

While a lack of sequence homology hampers the search for the genes encoding these alternative hydrolases, genomic context can sometimes assist in the identification of novel gene functions (*12*−*14*). In this work, we identify and characterize LpxI, a protein unrelated to LpxH that catalyzes UDP-2,3-diacylglucosamine hydrolysis by attack of water on the β-P atom instead of the α-P atom (Scheme 2). The gene encoding LpxI was identified because of its location between *lpxA* and *lpxB* in certain α-proteobacteria, such as *Caulobacter crescentus* and *Mesorhizobium loti*, which lack

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[1]Abbreviations: BCA, bicinchoninic acid; DTT, dithiothreitol; dUTPase, dUTP diphosphatase; ESI-MS, electrospray ionization mass spectrometry; FPLC, fast protein liquid chromatography; GlcN, glucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IPTG, isopropyl β-D-1-thiogalactopyranoside; LC, liquid chromatography; lipid X, 2,3-diacylglucosamine 1-phosphate; LPS, lipopolysaccharide; PAGE, polyacrylamide gel electrophoresis; PAP, purple acid phosphatase; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; TLC, thin layer chromatography.

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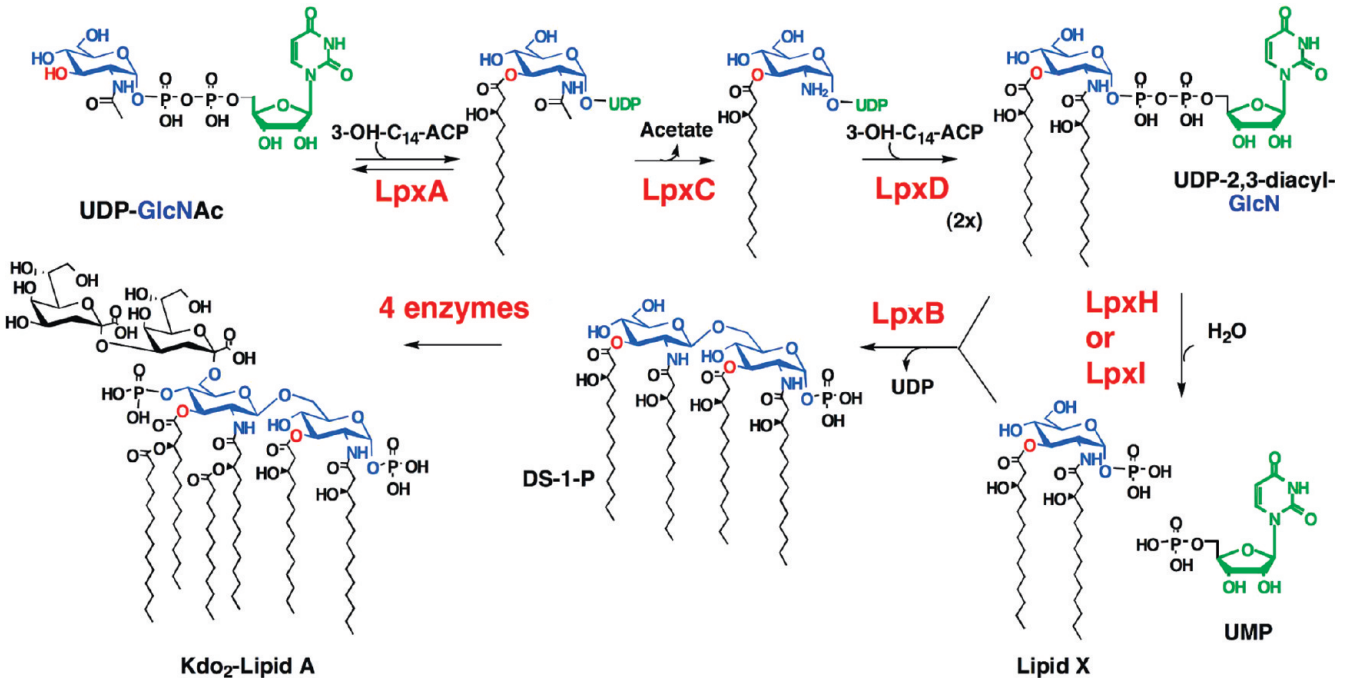
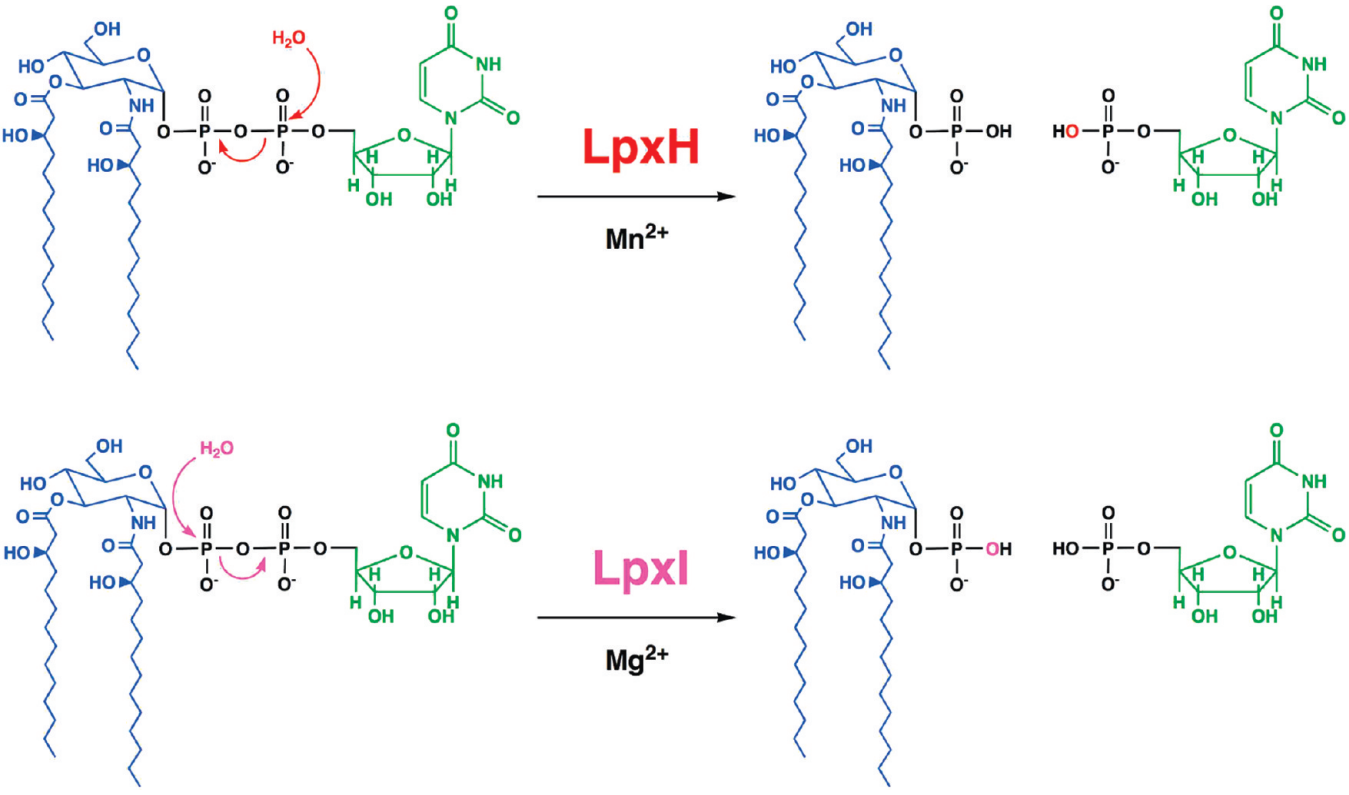
Scheme 1: Role of UDP-Diacylglucosamine Pyrophosphatases in Lipid A Biosynthesis[a]



[a]LpxH is primarily found in γ- and β-proteobacteria, whereas LpxI is present in α-proteobacteria, many δ-proteobacteria, and some environmental isolates, as described in detail in Table 2 of the Supporting Information. Chlamydia and Cyanobacteria contain neither LpxH nor LpxI but do possess LpxD and LpxB. Protein sequence data can be found at http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi.

Scheme 2: LpxH and LpxI Catalyze UDP-Diacylglucosamine Hydrolysis by Different Mechanisms[a]



[a]Oxygen from the water attacking the α-phosphate in the LpxH-catalyzed reaction is colored red, whereas oxygen from the water attacking the β-phosphate in the LpxI-catalyzed reaction is colored magenta.

LpxH (Figure 1) (*15*, *16*). The different hydrolytic mechanism employed by LpxI explains why LpxI and LpxH share no overall sequence similarity or even any conserved active site residues (Figure 2). LpxI belongs to a unique protein superfamily (DUF1009) (*17*) for which no biochemical functions have been previously reported.

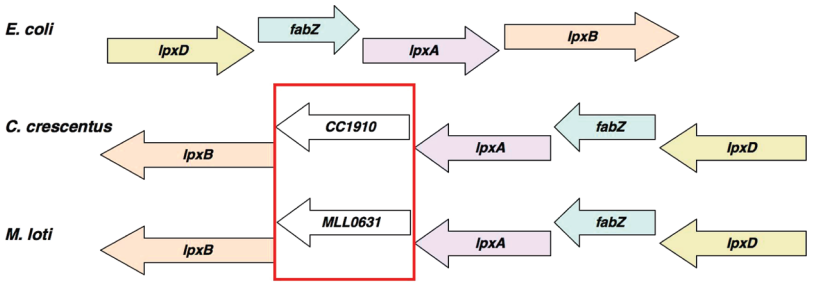


FIGURE 1: Comparison of the *lpxD-fabZ-lpxA-lpxB* gene clusters of *E. coli*, *C. crescentus*, and *M. loti*. Arrows represent open reading frames and their directions of transcription. A gene of unknown function encoding a protein designated as DUF2009 is situated between *lpxA* and *lpxB* in *C. cresentus* and *M. loti* and also is present in many other bacteria, like *A. aeolicus* (not shown), that lack LpxH (*15*, *16*, *32*). The DUF1009 orthologues, CC1910 and MLL0631, are highlighted with a red box. Gene lengths are not drawn exactly to scale.

MATERIALS AND METHODS

Chemicals and Reagents. Yeast extract, tryptone, and Bacto agar were purchased from Difco (Detroit, MI). Glass-backed silica gel 60 thin layer chromatography (TLC) plates were from EMD Chemicals (Darmstadt, Germany). Chloroform, pyridine, methanol, and acetic acid were from EMD Science (Gibbstown, NJ). γ-$^{32}P_i$ was purchased from PerkinElmer (Waltham, MA). The 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), phosphate-buffered saline components, HCl, and salts were purchased from Sigma-Aldrich (St. Louis, MO).

Cloning and Molecular Biology. Amplification of DNA segments, from either genomic or plasmid DNA, was accomplished using KOD Hot Start DNA polymerase, 8 mM dNTP stocks (dATP, dTTP, dGTP, and dCTP at 2 mM each), and KOD Hot Start reaction buffer, all obtained from EMD Chemicals (Gibbstown, NJ). Polymerase chain reaction (PCR) conditions were those recommended by the manufacturer, except for the inclusion of 1% (v/v) dimethyl sulfoxide and 1 M betaine in the reaction mixtures. The plasmids described in this study (see Table 1) were stored in and amplified from *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA). Qiagen Mini-Prep kits and QIAquick Spin kits (Qiagen, Valencia, CA) were used to purify plasmids and DNA fragments, respectively, by protocols described by the manufacturer. Restriction endonucleases, T4 DNA ligase, and calf intestinal alkaline phosphatase were obtained from New England Biolabs (Ipswich, MA). Unless otherwise stated, the following concentrations of antibiotics were used when appropriate: 100 μg/mL ampicillin, 50 μg/mL kanamycin, 20 μg/mL chloramphenicol, and 15 μg/mL tetracycline.

Construction of Plasmids. DNA oligomers (Integrated DNA Technologies, Coralville, IA) were used to amplify *E. coli* K-12 (NP_414724) *lpxH* and *C. crescentus* CB15 (NP_420716) LpxI from genomic DNA obtained from ATCC (Rockville, MD). Primers (Table 1 of the Supporting Information) were designed to confer 5′ *Nde*I and 3′ *Bam*HI restriction sites upon the ends each of the amplified oligonucleotides. A Mastercycler Gradient Thermocycler (Eppendorf, Hamburg, Germany) was used to amplify linear DNA. These PCR products were digested using *Nde*I and *Bam*HI, according to the manufacturers' protocols and subsequently ligated into a similarly digested recipient vector, pET21b (Novage/EMD Chemicals). These ligation reaction mixtures were then transformed into the XL1-Blue strain of chemically competent *E. coli* (Stratagene), according to the manufacturer's recommended procedure. Plasmids were isolated from transformants, and their sequences were confirmed by the Duke Cancer Center DNA Sequencing Facility. The resulting constructs were designated pEcH21b and pCcH21b (see Table 1). The restriction endonucelases *Xba*I and *Sal*I were used to digest these plasmids, liberating linear *E. coli lpxH* or *C. crescentus lpxI*, each flanked by 5′ *Xba* and 3′ *Sal*I restriction sites, and containing a pET21b-encoded ribosome binding site 7 bp upstream from the start codon. These segments were then ligated, as described above, into appropriately digested pBAD30 or pBAD33 plasmids (*18*). The resulting plasmids are listed in Table 1.

Construction of E. coli lpxH Deletion Strains. A deletion mutant of *E. coli* LpxH was generated with an in-frame kanamycin (Kan) insertion/substitution. Primers KanFlank_FW and KanFlank_RV (see Table 1 of the Supporting Information) were used to amplify a *Kan* cassette from plasmid pET28b (Novagen/EMD Chemicals). These primers were designed with 40 bp overhangs complementary to the regions of the *E. coli* chromosome immediately flanking *lpxH*. KanFlank_FW also added a ribosome binding site in the optimal position 7 bp 5′ to the *kan* cassette's start codon. The resulting linear oligonucleotide was electroporated into temperature-shifted *E. coli* strain DY330 (*19*), harboring either pBAD30, pBAD30Ec, or pBAD30Cc [strain DY330VC, DY330Ec, or DY330Cc, respectively (see Table 1)], as previously described (*20*). The strains were plated on LB-agar supplemented with kanamycin and ampicillin and grown for 18 h at 30 °C. Transformants were repurified, and colony PCR was performed using primers Kan_FW and Kan_RV (Table 1 of the Supporting Information) to amplify the *lpxH* locus and its 100 bp flanking regions. The resulting oligonucleotides were sequenced to confirm their identities. DY330 strains with *lpxH::Kan* replacements, covered by pBAD30Ec and pBAD30Cc, were designated DY330Δ*H*Ec and DY330Δ*H*Cc, respectively (Table 1).

A P1*vir* lysate was prepared (*21*) from DY330ΔHEc and used to infect *E. coli* W3110A cells harboring either pBAD33, pBAD33Ec, or pBAD33Cc (strain W3110AVC, W3110AEc, or W3110ACc, respectively) (Table 1). Following infection and outgrowth as previously described (*21*), cells were spread onto LB-agar plates supplemented with chloramphenicol, kanamycin, and 5 mM sodium citrate and allowed to grow for 20 h at 30 °C. Colonies were selected and repurified twice to remove traces of contaminating phage. Colony PCR was performed to amplify the chromosomal region ±100 bp from the *lpxH* locus, and the resulting oligonucleotides were confirmed by sequencing. The strains thereby created from the transduction of *lpxH::Kan* into W3110ACc and W3110AEc were designated W3110AΔHEc and W3110AΔHCc, respectively (see Table 1).

UDP-2,3-diacylglucosamine Hydrolase Expression and in Vitro TLC Assay. Plasmids pET21b and pCcI21b were transformed into *E. coli* strain C41(DE3) by electroporation and grown for 18 h at 30 °C on LB-agar plates supplemented with

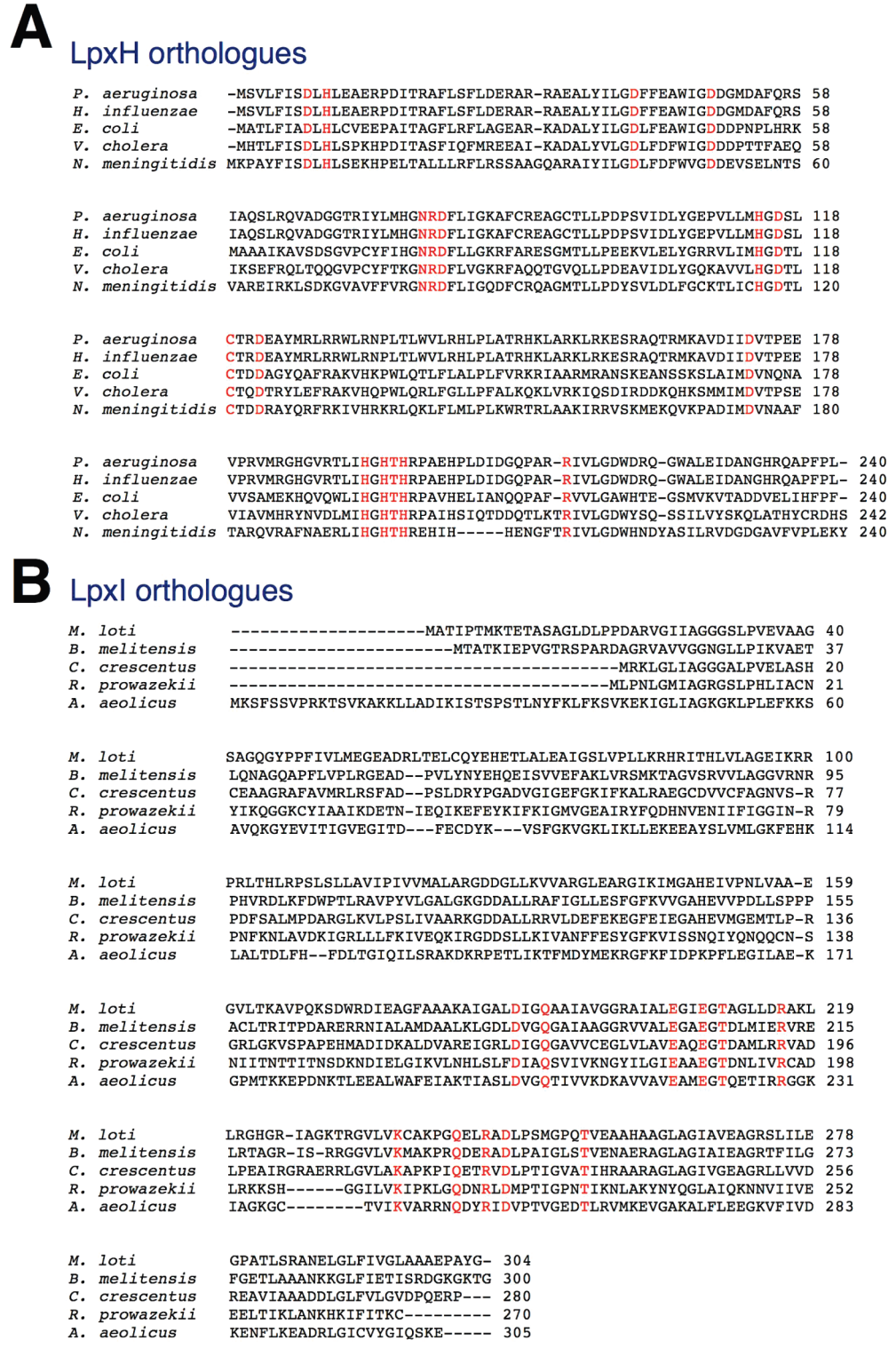


FIGURE 2: Sequence alignments of LpxH and LpxI orthologues. (A) Sequence alignments of five representative LpxH orthologues. (B) Alignments of five typical LpxI orthologues. In each panel, the absolutely conserved residues are colored red. There is no conservation of domains or of possible active site motifs between LpxI and LpxH, which are members of distinctly different protein families. The sequences were obtained from http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi.

ampicillin. Single colonies of resulting strains VC_21b and CcI_21b (see Table 1) were used to inoculate 5 mL overnight cultures. These, in turn, were used to inoculate 50 mL cultures of appropriately supplemented LB medium, at an initial OD_{600} of ~0.02. Strains VC_21b and CcI_21b were then grown at 30 °C with aeration at 220 rpm, until the A_{600} reached ~0.5 (~5 h). Expression was induced by addition isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 250 μM. The cells were grown for an additional 5 h, until the final A_{600} of the induced cells was ~3. Cells were collected by centrifugation at 3000*g*, washed with phosphate-buffered saline (PBS) (*22*), and resuspended in 3 mL of ice-cold PBS. The washed, resuspended cells were passed twice through a French pressure cell at 18000 psi, and cell debris was removed by centrifugation at 10000*g* for 30 min. Lysates were analyzed by SDS−PAGE and were assayed for UDP-2,3-diacylglucosamine hydrolase activity (*8*).

Table 1: Relevant Strains and Plasmids

strain	description	source
C41(DE3)	F^- *ompT hsd*S_B(r_B^- m_B^-) *gal dcm* (DE3) D(srl-recA)306::Tn*10*	*51*
W3110A	F^- *aroA::Tn10 msbA*$^+$, Tet^R	*28*
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [*F′proAB lacIqZDM15 Tn10* (Tet^R)]	Strategene
MN7	K-12-derived *pgsA444 lpxB1*; accumulates lipid X	*52*
DY330	W3110 Δ*lacU169 gal490* λcI857 Δ(*cro-bioA*)	*19*
Ec21XB	XL1-Blue harboring pEcH21b, Amp^R	this work
Cc21XB	XL1-Blue harboring pCcI21b, Amp^R	this work
EcH33XB	XL1-Blue harboring pBAD33Ec, Cam^R	this work
CcI33XB	XL1-Blue harboring pBAD33Cc, Cam^R	this work
EcH30XB	XL1-Blue harboring pBAD30Ec, Amp^R	this work
CcI30XB	XL1-Blue harboring pBAD30Cc, Amp^R	this work
DY330VC	DY330 harboring pBAD30, Amp^R	this work
DY330Ec	DY330 harboring pBAD30Ec, Amp^R	this work
DY330Cc	DY330 harboring pBAD30Cc, Amp^R	this work
DY330ΔHEc	DY330 *lpxH::Kan* harboring pBAD30Ec, Kan^R, Amp^R	this work
DY330ΔHCc	DY330 *lpxH::Kan* harboring pBAD30Cc, Kan^R, Amp^R	this work
W3110AVC	W3110A harboring pBAD33, Cam^R	this work
W3110AEc	W3110A harboring pBAD33Ec, Cam^R	this work
W3110ACc	W3110A harboring pBAD33Cc, Cam^R	this work
W3110AΔHEc	W3110A *lpxH::Kan* harboring pBAD33Ec, Cam^R, Amp^R	this work
W3110AΔHCc	W3110A *lpxH::Kan* harboring pBAD33Cc, Cam^R, Amp^R	this work
VC_21b	C41(DE3) harboring pET21b, Amp^R	this work
EcH_21b	C41(DE3) harboring pEcH21b, Amp^R	this work
CcI_21b	C41(DE3) harboring pCcI21b, Amp^R	this work

plasmid	description	source
pET21b	high-copy number expression vector containing a T7 promoter, Amp^R	Novagen
pET28b	high-copy number expression vector containing a T7 promoter, Kan^R	Novagen
pBAD30	arabinose inducible vector, Amp^R	*18*
pBAD33	arabinose inducible vector, Cam^R	*18*
pEcH21b	pET21b containing *E. coli lpxH*, Amp^R	this work
pCcI21b	pET21b containing *C. crescentus lpxI*, Amp^R	this work
pBAD33Ec	pBAD33 containing *E. coli lpxH*, Cam^R	this work
pBAD33Cc	pBAD33 containing *C. crescentus lpxI*, Cam^R	this work
pBAD30Ec	pBAD33 containing *E. coli lpxH*, Amp^R	this work
pBAD30Cc	pBAD33 containing *C. crescentus lpxI*, Amp^R	this work

For our initial analysis, the addition of 5 μL of diluted lysate from strain VC_21b or CcI_21b was used to start the UDP-2,3-diacylglucosamine hydrolase reactions in a total volume of 25 μL. Nonradiolabeled UDP-2,3-diacylglucosamine was prepared as previously described (*8*). Briefly, the final reaction mixtures contained 1 mM UDP-2,3-diacylglucosamine, 20 mM HEPES (pH 8.0), and 2 mM $MnCl_2$, and they were equilibrated at 30 °C for 15 min in 0.5 mL polypropylene Eppendorf tubes. Reactions were started by addition of crude lysates from VC_21b or CcI_21b, diluted as appropriate with PBS. At various time points, the reactions were quenched by spotting 5 μL portions onto 10 cm × 10 cm high-performance silica TLC plates (Merck, Darmstadt, Germany). These were developed and dried as described previously (*8*). Reaction products were visualized by spraying the dried TLC plate with 10% H_2SO_4 in ethanol and subsequent charring on a hot plate.

Preparation of [β-^{32}P]UDP-2,3-diacylglucosamine. ^{32}P-labeled lipid X was prepared as previously reported (*23*). Following purification, lipid X was redissolved in 2 mL of a $CHCl_3$/MeOH mixture (2:1, v/v), transferred to a clean screw-capped glass tube, and thoroughly dried under a nitrogen stream. Next, 10 mg of UMP-morpholidate, 50 μL of 0.2 M 1-*H*-tetrazole in acetonitrile, and 1 mL of dry pyridine were added. The tube was immediately sealed with a Teflon-coated screw cap and subjected to sonic irradiation for 5 min in a bath sonicator (Avanti Polar Lipids, Alabaster, AL). The mixture was incubated in a rotary water-bath shaker for at least 20 h at 37 °C, with shaking at 100 rpm. The reaction mixture was dried under nitrogen until no traces of pyridine remained (~2 h). The residue at the bottom of the tube was redissolved in 100−300 μL of 20 mM HEPES (pH 8.0) containing 0.02% (w/v) Triton X-100 and subjected to bath sonic irradiation for 2 min. This material was split into aliquots and stored at −80 °C. With this method, we could consistently convert more than 95% of [^{32}P]lipid X to [β-^{32}P]UDP-2,3-diacylglucosamine.

Expression and Purification of C. crescentus LpxI. A colony of *E. coli* strain CcI_21b (that overexpresses CcLpxI upon induction) was used to inoculate a 25 mL overnight LB medium culture supplemented with ampicillin. Following growth for 16 h at 30 °C, this culture was used to inoculate 1 L of LB broth containing ampicillin to an initial A_{600} of ~0.05. Growth proceeded at 30 °C with aeration at 220 rpm, until A_{600} reached ~0.5. IPTG was then added to a final concentration of 250 μM, and growth was continued for 5 h at 30 °C. Cells, which typically grew to an A_{600} of ~3.5, were harvested by centrifugation at 3000*g* for 30 min and subsequently washed with 80 mL of 20 mM HEPES (pH 8.0) containing 50 mM NaCl. The washed pellets were stored at −80 °C. In a typical purification, ~4 g of wet cell pellet was resuspended in ~300 mL of lysis buffer [20 mM HEPES (pH 8.0) containing 50 mM NaCl], and cells were lysed by three passages through an ice-cold Cell-Cracker pressure disruption chamber (Microfluidics International Corp., Newton,

MA). Cell debris and membranes were removed by ultracentrifugation at 150000*g* for 1 h. A Rabbit-Plus peristaltic pump (Rainin Instruments, LLC, Oakland, CA) was used to load the membrane-free lysate at ~2 mL/min onto a 5 mL High-Trap Q Sepharose Fast-Flow anion exchange cartridge (GE Health Sciences, Pittsburgh, PA), previously equilibrated in lysis buffer. The column was washed with 20 volumes (100 mL) of lysis buffer at 3 mL/min and was then attached to an AKTA 600 FPLC system (GE Health Sciences). A 30 column volume (150 mL) continuous gradient from 100% buffer A [20 mM HEPES (pH 8.0) containing 50 mM NaCl] to 100% buffer B [20 mM HEPES (pH 8.0) containing 200 mM NaCl] was applied at a flow rate of 2 mL/min. The protein, which was collected in 5 mL fractions, began to elute at ~100 mM NaCl. SDS−PAGE was used to determine which fractions to pool; typically, six fractions (30 mL) were combined. The partially purified CcLpxI was concentrated to ~10 mL using two 15 mL Amicon Ultra 10000 molecular weight cutoff centrifuge concentrators (Millipore, Billerica, MA), which had been washed in buffer B. The AKTA 600 FPLC system was used to load the concentrated, pooled Q Sepharose fractions at 1.5 mL/min onto a 330 mL size-exclusion column [Superdex 200 XK26/70 (GE Healthcare, Waukesha, WI)], equilibrated with buffer B. The peak of LpxI eluted at ~210 mL. Fractions of 5 mL were collected, and their purity determined by SDS−PAGE. Typically, four fractions (20 mL) were pooled and subsequently concentrated to ~25 mg/mL as described above. Protein aliquots were stored at −80 °C and subjected to fast freeze−thaw cycles upon storage and retrieval.

Autoradiographic in Vitro Assay of CcLpxI. Unless otherwise noted, 25 μL reaction mixtures containing 20 mM HEPES (pH 8.0), 0.5% (w/v) fatty acid-free bovine serum albumin (BSA), 0.05% (w/v) Triton X-100, 2 mM $MgCl_2$, 100 μM UDP-2,3-diacylglucosamine, 1000 cpm/μL [β-^{32}P]UDP-2,3-diacylglucosamine, and enzyme were prepared in 0.5 mL polypropylene tubes. Prior to addition of 5 μL of enzyme as the last step, the other reaction components were equilibrated at 30 °C for 15 min. Unless otherwise noted, enzyme samples were diluted in a buffer identical to the assay mixture, but lacking UDP-2,3-diacylglucosamine. At various time points, 3 μL reaction portions were removed and spotted onto 20 cm × 20 cm silica gel TLC plates (EMD Chemicals). These were then developed, dried, scanned, and quantified as previously described (*8*).

Kinetic Parameters, pH Optimum, and Detergent Dependence of CcLpxI. To determine the effect of pH on the apparent specific activity of CcLxpI, partially purified enzyme was assayed as described above, but with the usual 20 mM HEPES (pH 8.0) replaced with a triple-buffer system consisting of 100 mM sodium acetate, 50 mM bis(2-hydroxyethyl)iminotris-(hydroxymethyl)hexane, and 50 mM Tris (*24*). CcLpxI activity was measured from pH 4.0 to 9.0. A single-limb pK_a curve was fit to the data using KaleidaGraph. Typically, the enzyme was present at a concentration of 5−50 nM.

To determine the K_M and V_{max} of CcLpxI with respect to UDP-2,3-diacylglucosamine, the purified enzyme was assayed as described above, but with the concentration of UDP-2,3-diacylglucosamine varied from 5 to 1000 μM. KaleidaGraph was used to fit velocities to the Michaelis−Menten equation (*25*). The concentration of CcLpxI in the assay was varied from 2 and 200 nM to maintain linear conversion to product with time at different UDP-2,3-diacylglucosamine concentrations.

To probe whether CcLpxI activity is affected by detergent, the typical assay conditions were employed, except that the concentration of Triton X-100 was varied from 0 to 1.0% (w/v). To measure LpxI activity in the absence of detergent, the [β-^{32}P]UDP-2,3-diacylglucosamine was suspended in buffer lacking Triton X-100. An enzyme concentration of 10 nM was used in these assays.

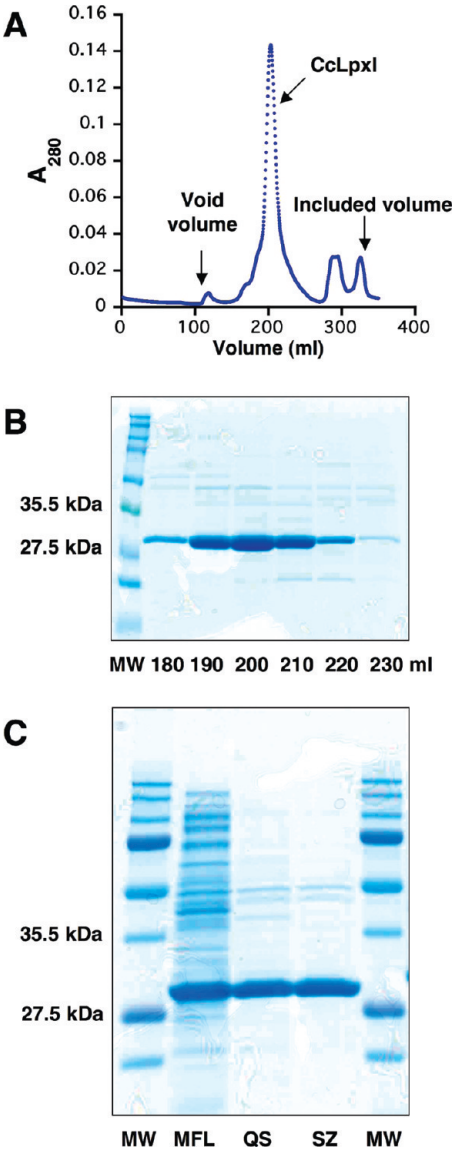


FIGURE 3: Purification of untagged recombinant CcLpxI. (A) Elution of CcLpxI monitored at A_{280} from a size-exclusion column. (B) A 12% SDS−PAGE analysis of selected fractions from the gel filtration column. The lanes correspond to the elution volume of the trace shown in panel A. Equal volumes were loaded. (C) SDS−PAGE analysis of protein from each step of the CcLpxI purification. MFL, QS, and SZ denote membrane-free lysate, pooled fractions from the Q-Sepharose anion exchange column, and pooled fractions from the Superdex S200 sizing column, respectively. Approximately 20 μg of protein was loaded in each lane.

Metal Dependence of CcLpxI. To determine whether divalent metal ions stimulate CcLpxI activity in vitro, the standard assay condition was employed, except that 2 mM $MgCl_2$ was replaced by no additive or each of the following chloride salts at 2.0, 0.2, or 0.02 mM: Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+}; a similar EDTA control was also included. The concentration of CcLpxI in the assay was varied from 2 and 200 nM to follow linear conversion to product with time in the presence of the different additives. A separate assay, in which $MgCl_2$ was titrated from 0 to 20 mM, was performed in the presence of 20 nM CcLpxI.

Table 2: Purification of CcLpxI from *E. coli* Strain CcI_21b

step	protein (mg)	volume (mL)	units (mmol/min)	specific activity (μmol min^{-1} mg^{-1})	yield (%)	*x*-fold purification
membane-free lysate	168	280	4.1	25	100	1.0
Q-Sepharose anion exchange	34	30	0.9	26	22	1.1
S200 size exclusion	27	20	0.8	30	20	1.2

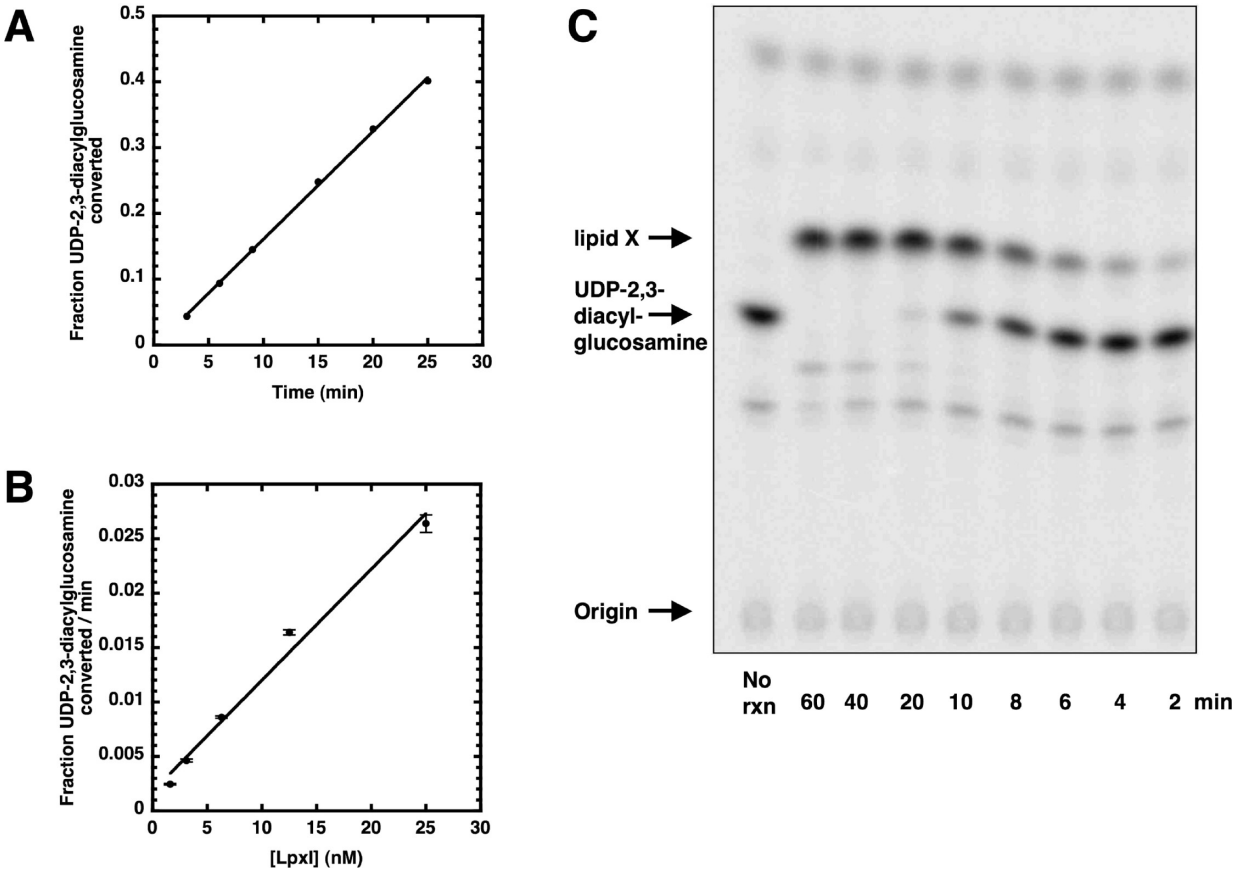


FIGURE 4: Linearity of CcLpxI activity with time and protein concentration. (A) Fraction of 100 μM [β-^{32}P]UDP-2,3-diacylglucosamine converted to the product lipid X at various times under standard assay conditions in the presence of 12.5 nM CcLpxI. (B) Fraction of 100 μM [β-^{32}P]UDP-2,3-diacylglucosamine converted to lipid X per minute as a function of enzyme concentration. (C) Image of a silica TLC plate of an in vitro CcLpxI reaction run at an enzyme concentration of 40 nM.

Preparation of the CcLpxI Reaction Products in the Presence of $H_2{}^{18}O$. Reaction mixtures (50 μL each) consisting of 100 μM UDP-2,3-diacylglucosamine, 2 mM $MgCl_2$, 20 mM HEPES (pH 8.0), and 66 nM purified CcLpxI were set up in the presence of either 100% $H_2{}^{16}O$ or an $H_2{}^{18}O/H_2{}^{16}O$ mixture (77:23, v/v) (Cambridge Isotopes, Andover, MA). The reactions were allowed to proceed at 30 °C for 2 h and were quenched by conversion to a 1.9 mL single-phase, acidic Bligh-Dyer system (*26*). A 20 μL portion of this material, containing 37 ng of lipid X, was analyzed by normal-phase liquid chromatography (LC) and mass spectrometry. Briefly, the sample was loaded onto a 25 cm × 2.1 mm Ascentis silica HPLC column (Sigma-Aldrich) using an Agilent (Santa Clara, CA) 1200 Quaternary LC system, coupled to a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA), operating in the ESI negative ion mode. Following injection onto the column, which was equilibrated in mobile phase A consisting of $CHCl_3$, methanol, and saturated aqueous NH_4OH (160:39:1, v/v/v), the sample was subjected to chromatography at a constant flow rate of 300 μL/min. First, an isocratic gradient of 100% mobile phase A was applied for 2 min, followed by a linear gradient from 100% mobile phase A to 100% mobile phase B consisting of $CHCl_3$, methanol, water, and saturated aqueous NH_4OH (120:68:10:1, v/v/v/v) over 14 min. Next, 100% mobile phase B was applied for 11 min, followed by a linear gradient from 100% mobile phase B to 100% mobile phase C consisting of $CHCl_3$, methanol, water, and saturated aqueous NH_4OH (90:90:19:1, v/v/v/v) over 3 min. Finally, mobile phase C was applied for 3 min, followed by a linear transition to mobile phase A over 30 s; the latter was passed over the column for an additional 5 min. The following parameters were used for negative ion electrospray mass spectrometry (ESI-MS) and MS/MS analysis: ionization source energy of −4500 V, curtain gas pressure of 20 psi, GSI of 20 psi, DP of −55 V, and FP of −150 V, with nitrogen used as the collision gas. The Analyst QS software suite (Applied Biosystems) was employed for data analysis.

RESULTS

Identification of a Novel UDP-2,3-Diacylglucosamine Hydrolase in Bacteria Lacking lpxH. The Clusters of Orthologous Genes database (*17*) was used to compare the genomes of

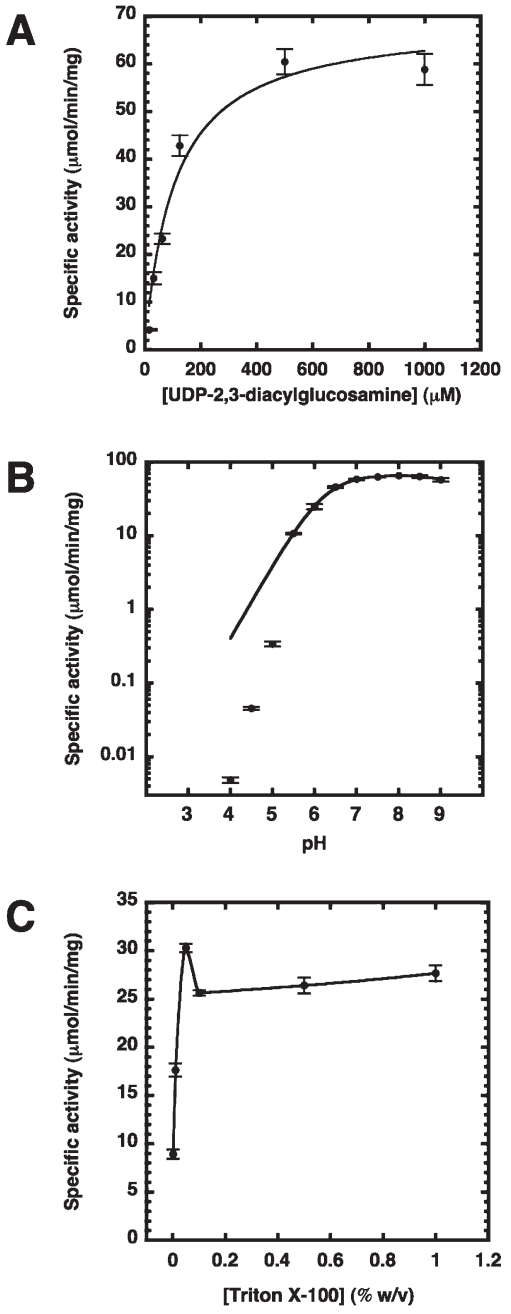


FIGURE 5: Substrate, pH, and detergent dependence of CcLpxI activity. (A) UDP-2,3-diacylglucosamine concentration dependence of CcLpxI activity under standard assay conditions. The apparent K_M, fit using Kaleidograph, was $105 \pm 25\ \mu$M; the apparent V_{max} was $69 \pm 5\ \mu$mol min^{-1} mg^{-1}. (B) CcLpxI activity as a function of pH. The activity is relatively constant between pH 6.5 and 9 but declines sharply below pH 6. (C) Effect of Triton X-100 concentration on the apparent specific activity of CcLpxI at 100 μM UDP-2,3-diacylglucosamine. Surface dilution kinetics are not apparent, and there is measurable activity in the absence of Triton.

Gram-negative bacteria lacking *lpxH* orthologues. Given that the gene(s) encoding a novel UDP-2,3-diacylglucosamine hydrolase analogue(s) might be located near other lipid A biosynthetic genes and that in many species *lpxD*, *fabZ*, *lpxA*, and *lpxB* cluster in an operon, we searched for species that lacked *lpxH* but possessed additional open reading frames of unknown function in the *lpxD-fabZ-lpxA-lpxB* locus. Several bacteria, including *C. crescentus* (*16*), *Sinorhizobium meliloti* (*27*), and *M. loti* (*15*), met this criterion in that an ~1000 bp open reading frame of unknown function was inserted between *lpxA* and *lpxB* (Figure 1). The corresponding gene product, annotated as DUF1009 in each of these three strains (*17*), is also present in many (though not all) of the other organisms that produce lipid A but lack *lpxH*. LpxH orthologues display no sequence similarity to the DUF1009 sequences (Figure 2), which have a distinctly different set of conserved amino acid residues.

The gene encoding the *C. crescentus* DUF1009 orthologue, CC1910 (*16*), was amplified from genomic DNA, cloned into the high-copy number pET21b plasmid, and expressed in *E. coli* C41(DE3), yielding strain CcI_21b. This construct was grown in parallel with its empty vector control (VC_21b) and induced to overexpress the CC1910 protein. Cells from each strain were harvested and lysed. Overexpression of the expected 29 kDa CC1910 protein was confirmed by SDS−PAGE (Figure 1A of the Supporting Information). Equal concentrations of lysates from VC_21b versus CcI_21b were used to detect UDP-2,3-diacylglucosamine hydrolase activity by TLC, similar to the method described for *E. coli* LpxH (*8*). This analysis revealed that the CcI_21b lysate contained >1000-fold higher UDP-2,3-diacylglucosamine hydrolase activity than its matched vector control (in Figure 1B of the Supporting Information, lane 2 vs lane 4), suggesting that CC1910, hereafter termed CcLpxI, is functionally similar to LpxH. Upon incubation of these lysates with CDP-diacylglycerol, under otherwise similar assay conditions, no difference in the rate of CDP-diacylglycerol hydrolysis was observed between extracts of VC_21b and CcI_21b, showing that LpxI, in contrast to CDP-diacylglucosamine hydrolase (*7*, *8*), is selective for UDP-2,3-diacylglucosamine (data not shown).

C. crescentus lpxI Can Replace lpxH in E. coli. To determine whether *C. crescentus lpxI* possesses UDP-2,3-diacylglucosamine hydrolase activity in vivo, the essential *lpxH* gene was replaced in *E. coli* DY330 (*19*) with a *kan*^{R} cassette, and covering plasmids expressing either *E. coli lpxH* or *C. crescentus lpxI* were used to test for complementation of *lpxH* deficiency. Whereas the empty vector could not complement the deletion of the chromosomal *lpxH* gene, plasmids expressing either *E. coli lpxH* or *C. crescentus lpxI* allowed this deletion mutant to form colonies (Figure 2 of the Supporting Information). The *lpxH::kan* deletion could also subsequently be transduced into wild-type strain *E. coli* W3110A (*28*) harboring either *E. coli lpxH* or *C. crescentus lpxI* on a pBAD plasmid. The replacement of *lpxH* with *kan* in W3110A was confirmed by PCR amplification of the *lpxH* locus and its 100 bp flanking regions. The identities of these PCR products were confirmed by sequencing.

Expression and Purification of CcLpxI. Induced cells of *E. coli* strain CcI_21b massively overproduced a protein of the molecular weight expected for LpxI and its associated UDP-2,3-diacylglucosamine hydrolase activity. To characterize CcLpxI, it was purified to greater than 90% homogeneity using ion-exchange and size-exclusion chromatography (Figure 3). The pellet from 500 mL of CcI_21b cells grown to an A_{600} of ~3.5 yielded ~30 mg of CcLpxI. The specific activity of the protein increased only 1.2-fold during the purification, consistent with the efficient overexpression, and the total activity yield was ~20% (Table 2). CcLpxI elutes from a gel filtration column in a symmetric peak (Figure 3A), consistent with either a dimeric or monomeric solution structure. Unlike EcLpxH, which is a peripheral membrane protein, recombinant CcLpxI did not sediment with membranes but remained in the supernatant following ultracentrifugation.

An Autoradiographic Assay of CcLpxI Using [β-^{32}P]UDP-2,3-Diacylglucosamine. Using [β-^{32}P]UDP-2,3-diacylglucosamine as the substrate, we developed a quantitative TLC-based

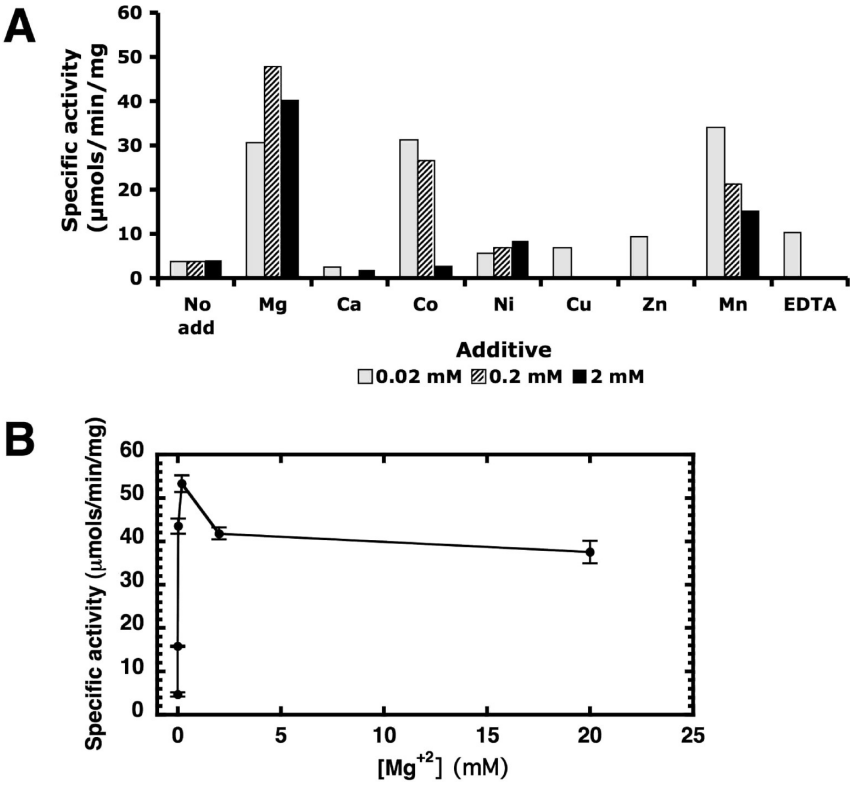


FIGURE 6: Metal dependence of CcLpxI. (A) Specific activity of CcLpxI in the presence of various divalent cations or EDTA. Three sets of assays were performed, each of which included a no additive (No add) control, as shown at the left. Each bar in the series of three bars represents a different concentration of each additive (e.g., 0.02, 0.2, or 2 mM). Standard assay conditions were used with an enzyme concentration of 10 nM. (B) The standard CcLpxI assay was used with 10 nM enzyme, but with the $MgCl_2$ concentration increasing from 0 to 20 mM.

assay for CcLpxI. Enzymatic activity is linear with time and with protein concentration (Figure 4A,B). Moreover, CcLpxI quantitatively converts [β-^{32}P]UDP-2,3-diacylglucosamine to [^{32}P]lipid X (Figure 4C).

Apparent Kinetic Parameters and Detergent Dependence of CcLpxI. The apparent K_M of purified CcLpxI with respect to UDP-2,3-diacylglucosamine was 105 ± 25 μM, while the apparent V_{max} was 69 ± 5 μmol min^{-1} mg^{-1} (Figure 5A). The pH−rate profile was determined for CcLpxI, and a single pK_a curve was fit to the data using Kaleidograph. The pK_a was estimated to be 6.15 ± 0.86 (Figure 5B), although the data deviate from a single-ionization model at low pH. The detergent dependence of CcLpxI was determined by varying the concentration of Triton X-100 in the assay from 0 to 1% (w/v). While stimulated ~3-fold in the presence of 0.05% (w/v) Triton X-100, the apparent activity did not decrease at high concentrations of Triton X-100 (Figure 5C), showing that CCLpxI activity is not apparently decreased by surface dilution of the substrate (*29*).

Metal Dependence of CcLpxI Activity in Vitro. The purified CcLpxI was assayed under the standard conditions, except that each of the following chloride salts was added at 0.02, 0.2, or 2 mM: Mg^{2+}, Ca^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} (explained in the legend of Figure 6). For each concentration, a control assay was included in which divalent cations were not added (Figure 6A, no add); in another set, 0.02, 0.2, or 2 mM EDTA was included in place of the divalent cations. The apparent specific activity of purified CcLpxI increased 10-fold in the presence of Mg^{2+} versus no added metal, or 6-fold in the presence of added Mn^{2+} or Co^{2+} (Figure 6A). The presence of EDTA at either 2.0 or 0.2 mM completely inhibited CcLpxI. This inhibition was reversed by dilution of the EDTA-treated enzyme into reaction mixtures containing 2 mM Mg^{2+} or Mn^{2+} (data not shown). Inhibition was also seen at the higher concentrations of Cu^{2+} and Zn^{2+}.

Next, LpxI activity was measured under standard assay conditions, but with the concentration of Mg^{2+} varied from 0 to 20 mM. CcLpxI activity was maximal at 0.2 mM Mg^{2+} and remained approximately the same up to 20 mM Mg^{2+} (Figure 6B). To determine whether divalent cations are associated with purified CcLpxI, inductively coupled plasma mass spectrometry was performed. No significant amounts of stoichiometric, tightly bound metal ions were detected in the purified protein in two separate determinations.

CcLpxI Catalyzes the Attack of H_2O on the β-Phosphorus Atom of UDP-2,3-Diacylglucosamine. To determine which phosphorus atom of UDP-2,3-diacylglucosamine is attacked during hydrolysis catalyzed by CcLpxI, excess purified enzyme was used to convert UDP-2,3-diacylglucosamine to products quantitatively in the presence of $H_2{}^{16}O$ or an $H_2{}^{18}O/H_2{}^{16}O$ mixture (77:23, v/v). Lipid X and UMP products were separated by normal-phase LC and analyzed by ESI-MS in the negative ion mode (Figure 7A,B). Lipid X (predicted $[M - H]^-$ at m/z 710.424) eluted between minutes 24.4 and 24.6; the UMP emerged during column regeneration between minutes 38.3 and 39.0. For the $H_2{}^{18}O$-labeled reaction, the proportional amount (~77%) of the lipid X product contained ^{18}O, as shown by the presence of a much more intense peak at m/z 712.441 versus m/z 710.440 (Figure 7B). This result is expected for the $[M - H]^-$ ion of a lipid X molecule containing a single ^{18}O atom (predicted $[M - H]^-$ at m/z 712.429) versus lipid X containing only ^{16}O (predicted $[M - H]^-$ at m/z 710.424). No ^{18}O was incorporated into the UMP product isolated from the CcLpxI ^{18}O

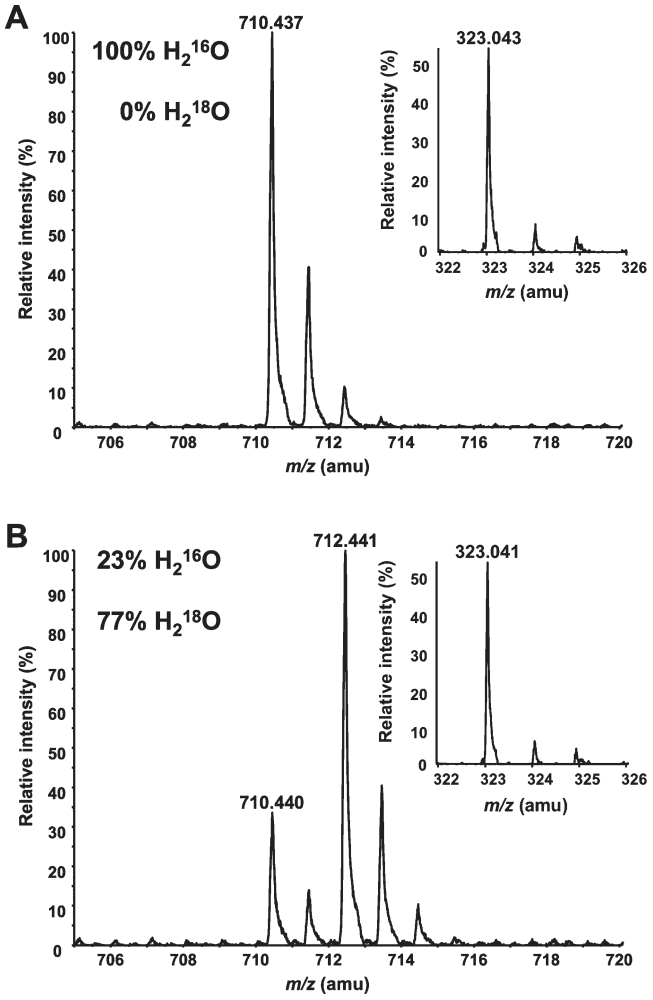


FIGURE 7: Incorporation of ^{18}O into lipid X during CcLpxI-catalyzed hydrolysis of UDP-2,3-diacylglucosamine in 77% $H_2{}^{18}O$. CcLpxI-catalyzed hydrolysis of UDP-2,3-diacylglucosamine was conducted in the absence or presence of 77% $H_2{}^{18}O$. (A) LC−ESI-MS analysis of lipid X generated by CCLpxI in the presence of 100% $H_2{}^{16}O$. (B) LC−ESI-MS analysis of lipid X generated by CCLpxI in the presence of an $H_2{}^{18}O/H_2{}^{16}O$ mixture (77:23, v/v). The insets show LC−ESI-MS analyses of the UMP generated in the reactions.

reaction mixture (Figure 7B, inset). We conclude that ccLpxI catalyzes the attack of water exclusively on the β-phosphorus atom of UDP-2,3-diacylglucosamine (Scheme 2).

DISCUSSION

Although essential for growth in *E. coli* and present in most γ- and β-proteobacteria (*8*, *30*), LpxH is missing in α-proteobacteria and many other groups (Figure 8 and Table 1 of the Supporting Information). Here, we describe the identification of a different UDP-2,3-diacylglucosamine hydrolase present in many of the species that lack LpxH, including almost all α-proteobacteria, most strains of *Leptospira* (*31*), and many diverse organisms, like *A. aeolicus* (*32*). The relevant gene, which encodes a protein of unknown function annotated as DUF1009, was recognized because of its location between *lpxA* and *lpxB* in *C. crescentus* and *M. loti* (Figure 1) (*15*, *16*). Members of the DUF1009 family possess no sequence similarity to EcLpxH or any other type of protein (Figure 2A,B).

We cloned the gene encoding DUF1009 from *C. cresentus* (CC1910) into a high-copy number vector and expressed it in *E. coli*, resulting in massive overproduction of the expected 29 kDa protein (Figure 1A of the Supporting Information). *E. coli* lysates of strain CcI_21b displayed >1000-fold increased UDP-2,3-diacylglucosamine hydrolase activity relative to vector controls (Figure 1B of the Supporting Information). The presence of CC1910, renamed *lpxI*, permitted the deletion and replacement of the chromosomal *lpxH* gene with a kanamycin resistance cassette in *E. coli* (Figure 2 of the Supporting Information). These results demonstrate that LpxI functions as an alternative UDP-2,3-diacylglucosamine hydrolase in vivo.

Taken together with the dissimilarity of their conserved motifs (Figure 2), we propose that EcLpxH and CcLpxI evolved separately to generate the same products by different hydrolytic mechanisms. The presence of *lpxI*, but not *lpxH*, in more ancient species like *A. aeolicus*, suggests that *lpxI* may have evolved earlier than *lpxH*. Moreover, some groups of organisms that make lipid A, such as the Cyanobacteria (*33*) and the Chlamydiae (*34*, *35*), lack both *lpxH* and *lpxI*, suggesting that at least one additional class of UDP-2,3-diacylglucosamine hydrolases may exist.

To determine which P atom of UDP-2,3-diacylglucosamine is attacked by water during CcLpxI-catalyzed hydrolysis, reactions were run in the presence or absence of $H_2{}^{18}O$. Analysis of the products by ESI-MS (Figure 7) revealed that the ^{18}O was incorporated exclusively into lipid X. CcLpxI therefore catalyzes the attack of water on the β-P atom, whereas EcLpxH specifically attacks the α-P atom (Scheme 2) (*8*). This observation, together with the lack of homology between EcLpxH and CcLpxI, strongly suggests that these enzymes perform catalysis by different mechanisms.

The incorporation of $H_2{}^{18}O$ into the β-P atom of UDP-2,3-diacylglucosamine by LpxI is formally analogous to the chemistry performed by phosphatidylserine synthase (PssA) of *E. coli*, which catalyzes the transfer of the phosphatidyl moiety of CDP-diacylglycerol to the hydroxyl group of serine (*36*). PssA catalysis is thought to proceed via a phosphatidyl−enzyme intermediate (*37*), but PssA can also cleave CDP-diacylglycerol to generate phosphatidic acid and CMP in the absence of serine, with concomitant incorporation of $H_2{}^{18}O$ into the phosphatidic acid product by attack of water on the β-P atom of CDP-diacylglycerol (*36*). This consideration raises the intriguing converse possibility that LpxI (or other enzymes like it) might be able to transfer the 2,3-diacylglucosamine 1-phosphate moiety from UDP-2,3-diacylglucosamine to an acceptor molecule other than water, analogous to the process of phosphatidyl transfer in glycerophospholipid metabolism.

The significance of two mechanisms for cleaving UDP-2,3-diacylglucosamine remains unclear. Phosphohydrolases are ubiquitous in biology and have probably evolved many times on different protein scaffolds (*38*). Phosphohydrolases are often metal-dependent, and much is known about their mechanisms (*39*). They play diverse roles in the modification and processing of proteins (*40*, *41*), nucleic acids (*42*, *43*), lipids (*44*−*47*), and diverse small molecules (*39*, *48*−*50*). The mechanisms of these enzymes generally involve the positioning of a deprotonated water molecule for attack on a P atom of a coordinated phosphate moiety of the substrate (*39*). The roles played by the metal ions may vary. In some cases, the metal coordinates the oxygen atoms of the phosphate group, while in others, it positions the water for attack on the P atom (*39*, *48*−*50*). In still other instances, the metal appears to coordinate a network of ordered water molecules, which in turn position a "catalytic water" for attack on the substrate (*48*). Although purified CcLpxI does not contain any tightly bound divalent cations, it is dependent upon them for activity (Figure 6A). Structural and mechanistic studies will be required to elucidate the

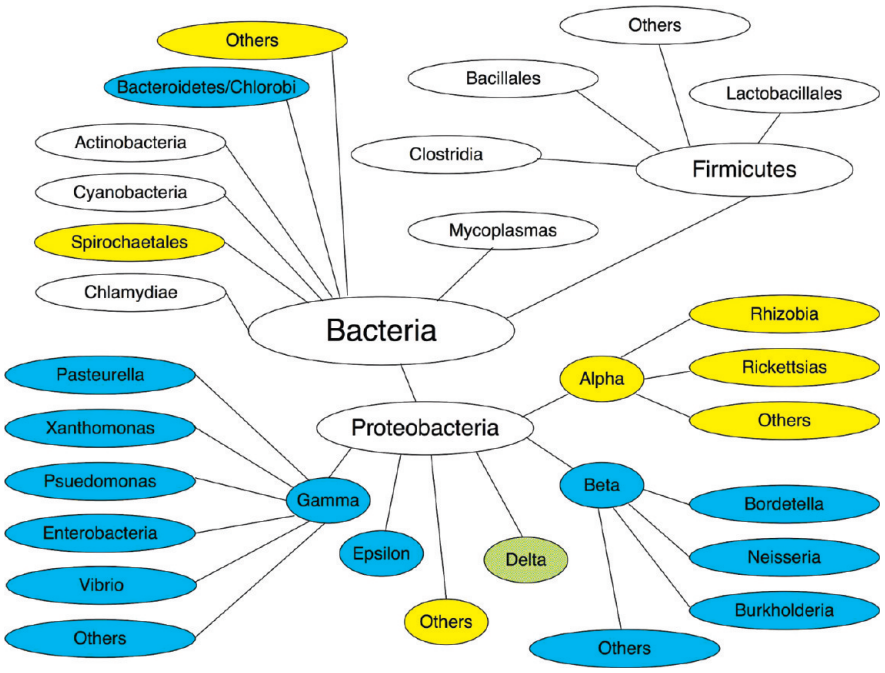


FIGURE 8: Distribution of LpxH and LpxI orthologues among diverse bacterial groups. This figure, modified from the NCBI bacterial genomes website (http://www.ncbi.nlm.nih.gov/sutils/genom_tree.cgi), shows the approximate distribution of putative LpxH (cyan) and LpxI orthologues (yellow) in diverse bacterial groups for which complete genome sequences are currently available. Light green shading indicates approximately equal proportions of organisms having putative LpxH and LpxI orthologues. Important Gram-negatives, such as the Chlamydiae and the Cyanobacteria, contain neither protein, even though both possess LpxC (Table 2 of the Supporting Information) and LpxB (Scheme 1). Many Spirochaetales, such as *Treponema pallidum* and *Borrelia burgdorferi*, do not synthesize lipid A. However, species of Leptospira do make lipid A and contain LpxI orthologues.

roles of metals in CcLpxI catalysis and the basis for the selectivity of CcLpxI for UDP-2,3-diacylglucosamine.

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SUPPORTING INFORMATION AVAILABLE

Primers used to construct various LpxI overexpressing strains (Table 1), additional information regarding the distributions of LpxI and LpxI among diverse bacteria (Table 2), additional information about the purification of LpxI (Figure 1), and additional information about the ability to *lpxI* to complement the *E. coli lpxH* mutation (Figure 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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