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Purification and Characterization of the Lipid A 1-Phosphatase LpxE of *Rhizobium leguminosarum*[*] [S]

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LpxE, a membrane-bound phosphatase found in *Rhizobium leguminosarum* and some other Gram-negative bacteria, selectively dephosphorylates the 1-position of lipid A on the outer surface of the inner membrane. LpxE belongs to the family of lipid phosphate phosphatases that contain a tripartite active site motif and six predicted transmembrane helices. Here we report the purification and characterization of *R. leguminosarum* LpxE. A modified *lpxE* gene, encoding a protein with an N-terminal His_6 tag, was expressed in *Escherichia coli*. The protein was solubilized with Triton X-100 and purified to near-homogeneity. Gel electrophoresis reveals a molecular weight consistent with the predicted 31 kDa. LpxE activity is dependent upon Triton X-100, optimal near pH 6.5, and Mg^{2+}-independent. The H197A and R133A substitutions inactivate LpxE, as does treatment with diethyl pyrocarbonate. In a mixed micelle assay system, the apparent K_m for the precursor lipid IV_A is 11 μM. Substrates containing the 3-deoxy-D-*manno*-oct-2-ulosonic acid disaccharide are dephosphorylated at similar rates to lipid IV_A, whereas glycerophospholipids like phosphatidic acid or phosphatidylglycerol phosphate are very poor substrates. However, an LpxE homologue present in *Agrobacterium tumefaciens* is selective for phosphatidylglycerol phosphate, demonstrating the importance of determining substrate specificity before assigning the functions of LpxE-related proteins. The availability of purified LpxE will facilitate the preparation of novel 1-dephosphorylated lipid A molecules that are not readily accessible by chemical methods.

Lipopolysaccharide (LPS)[5] is a prominent component of the outer monolayer of the outer membranes of Gram-negative bacteria (1, 2). LPS is classified as a saccharolipid glycan (3), and it maintains the permeability barrier and structural integrity of the bacterial outer membrane (4). The hydrophobic membrane anchor of LPS, termed lipid A, is required for growth in most Gram-negative bacteria (5, 6), whereas the outer O-antigen sugars of LPS provide resistance to complement and enhance bacterial survival in the environment (1, 7). Lipid A, which is also known as endotoxin, is a potent stimulator of the innate immune response in animals via the Toll-like receptor-4-MD-2 (TLR4-MD-2) complex (8–11), the x-ray crystal structure of which was recently reported (12, 13).

In many enteric Gram-negative bacteria, including *Escherichia coli*, lipid A is a hexa-acylated disaccharide of glucosamine, derivatized with phosphate substituents at positions 1 and 4′ (1, 14, 15). Pharmacological studies have shown that the phosphate groups, the glucosamine disaccharide, and the appropriate arrangement of fatty acyl chains are crucial for full activation of TLR4-MD-2 (16). Lipid A analogues lacking the 1-phosphate group retain some of the adjuvant properties of *E. coli* lipid A, while showing greatly reduced whole animal toxicity (17, 18). Such mono-phosphorylated lipid A derivatives are being evaluated in human clinical trials (19).

Lipid A molecules from *Rhizobium leguminosarum* (20), *Rhizobium etli* CE3 (21, 22), *Helicobacter pylori* (23, 24), and *Francisella tularensis* (25–27) lack one or both of the phosphate groups present in *E. coli* lipid A. These bacteria nevertheless contain the genes encoding the seven enzymes that generate the 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo)-containing *E. coli* lipid A precursor, Kdo_2-lipid IV_A (2, 28), which contains both the 1- and the 4′-phosphate groups. We have previously demonstrated that *R. etli*, *R. leguminosarum,* and *Francisella novicida* possess selective lipid A 4′- and 1-phosphatases (29–33) that are not present in *E. coli* and account for the absence of one or both lipid A phosphate groups in these organisms.

Recently, the gene encoding the lipid A 1-phosphatase (Fig. 1), termed *lpxE*, of *R. leguminosarum* was identified by an expression cloning strategy (31). LpxE belongs to a large family of lipid phosphate phosphatases found in prokaryotes and eukaryotes (34–36). The type 2 enzymes in this family are usually magnesium-independent and share a tripartite sequence motif (Fig. 1, *panel A*), which is involved in the binding and hydrolysis of the phosphate moiety of the substrate (34). Most of the type 2 phosphatases are integral membrane proteins with six predicted transmembrane segments (35). No high resolu-

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Experimental Procedures, Table I, and additional references.

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[5] The abbreviations used are: LPS, lipopolysaccharide; *AtLpxE, A. tumefaciens* homologue of *LpxE*; DEPC, diethyl pyrocarbonate; IPTG, isopropyl β-D-1-thiogalactopyranoside; Kdo, 3-deoxy-D-*manno*-oct-2-ulosonic acid; LB, lysogeny broth; MES, 2-(*N*-morpholino)ethanesulfonic acid; NTA, nitriloacetate; PA, phosphatidic acid; PGP, phosphatidylglycerol phosphate; TLR4, Toll-like receptor-4; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; BisTris, bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane.

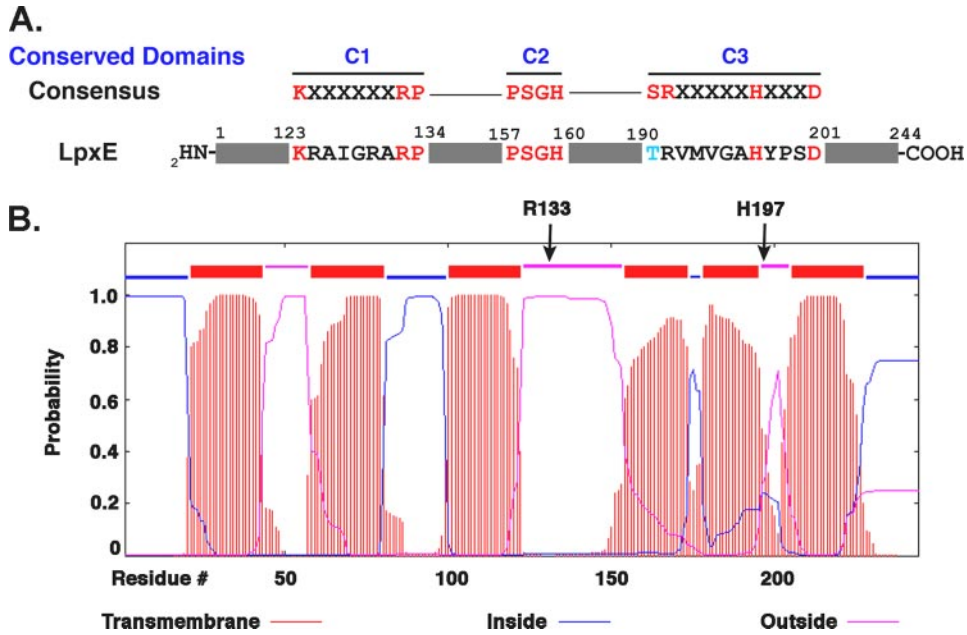

FIGURE 1. **LpxE active site motifs and predicted transmembrane topology.** The primary sequence of native *R. leguminosarum* LpxE shows homology to the conserved tripartite sequence motifs of the type 2 lipid phosphate phosphatase family (*panel A*). For *panel B,* the LpxE topology was predicted using the transmembrane hidden Markov model (TMHMM) (74). All three active site domains are predicted to face the periplasm in the inner membrane, based on this model. For LpxE, domain 1 begins near the extracellular end of helix 3, extending from residues 123–134; domain 2 is located within helix 4, residues 157–160; and domain 3, residues 190–201, encompasses part of helix 5 and the small downstream extracellular loop. The *arrows* denote the locations of key conserved residues (Arg-133 and His-197).

tion x-ray crystal structures or NMR structures of these membrane proteins have been reported. The type 2 phosphatases dephosphorylate diverse lipids, including phosphatidylglycerol phosphate (PGP), phosphatidic acid (PA), sphingosine phosphate, and lysophosphatidic acid (35, 36). *R. leguminosarum* LpxE was the first enzyme identified in this family that is selective for the 1-phosphate group of lipid A (31). Similar lipid A 1-phosphatases were later identified in *F. novicida* (32) and *H. pylori* (24). In cells, the dephosphorylation of lipid A by *F. novicida* and *H. pylori* LpxE is dependent upon the inner membrane flippase MsbA, indicating that the active sites of these LpxE orthologues face the periplasm (24, 32, 37), as predicted by hydropathy analysis (Fig. 1, *panel B*).

Given the importance of the phosphate groups of lipid A for its bioactivity and the need for structural studies of the lipid phosphate phosphatases, we now report the purification to near-homogeneity of a His_6-tagged derivative of *R. leguminosarum* LpxE. The purified enzyme is a highly selective lipid A 1-phosphatase (Fig. 2), with less than 1% activity against various glycerophospholipid substrates under matched assay conditions, and no detectable cleavage of the 4′-phosphate moiety of lipid A. Our chemical modification and site-directed mutagenesis data demonstrate that the catalytic mechanism of LpxE is similar to that of other members of the lipid phosphate phosphatase family (34, 35). However, LpxE homologues from other bacteria, such as *Agrobacterium tumefaciens* (38, 39), do not display the same substrate selectivity, demonstrating the need for developing biochemical assays in conjunction with the annotation of gene function.

EXPERIMENTAL PROCEDURES

Chemicals and Materials—The [γ-^{32}P] ATP, [*glycerol*-U-^{14}C]PA, and [U-^{14}C]glycerol-3-phosphate were purchased from PerkinElmer Life Sciences. Kdo, NaF, NH_4VO_3, disodium EDTA, diethyl pyrocarbonate (DEPC), HEPES, and MES were obtained from Sigma. Bicinchoninic protein assay reagents (40) and Triton X-100 were purchased from Fisher. The *Non-Interfering*™ protein assay was from G-Biosciences. Yeast extract and bacto-tryptone were from BD Biosciences. Silica Gel 60 TLC plates were obtained from EMD Chemicals. DNA primers were purchased from MWG Biotech. T4 DNA ligase was from Invitrogen. PCR buffer was purchased from Sigma. Reagent grade pyridine, chloroform, and methanol were from Mallinckrodt Baker.

Bacterial Strains, Growth Conditions, and Molecular Biology Techniques—E. coli strain Novablue(DE3) (Table 1) was purchased from EMD Chemicals. All bacteria were grown in lysogeny broth (LB, 10 g of NaCl, 10 g of bacto-tryptone and 5 g of yeast extract per liter) (41). When necessary, the cultures were supplemented with tetracycline (12 μg/ml) or kanamycin (30 μg/ml). Plasmids were prepared using the Qiagen mini-prep kit (Qiagen). Restriction endonucleases (New England Biolabs), shrimp alkaline phosphatase, and T4 ligase (Invitrogen) were used according to the manufacturer's instructions. Competent cells were prepared for transformation using the calcium chloride method (42). LpxE was expressed with an N-terminal His_6 tag from the pET-28a-derived plasmid pLpxE-4 in Novablue(DE3) as described previously (31).

Preparation of A. tumefaciens Cell-free Extracts and Membranes—To prepare extracts for assays, *A. tumefaciens* strain C58 was grown at 28 °C in modified LB containing 10 g/liter tryptone, 5 g/liter yeast extract, 5 g/liter NaCl, and 10 μg/ml gentamycin to an A_{600} of 1.0. All cells and extract preparations were performed at 0–4 °C in the same manner as described below for *E. coli*.

PCR Amplification and Cloning of A. tumefaciens C58 lpxE—The *A. tumefaciens* (*AtLpxE*) homologue of *R. leguminosarum lpxE* was PCR-amplified and subcloned from *A. tumefaciens* C58 genomic DNA. Genomic DNA was prepared from 0.5 ml of cells using the Easy-DNA genomic preparation kit from Invitrogen. *AtLpxE* was amplified by PCR followed by ligation into the pET-28a expression vector to yield the plasmid, pAtLpxE. The primers and PCR conditions are described in the supplementary material. The plasmid was then transformed into Novablue(DE3) for overexpression of the protein. First, a

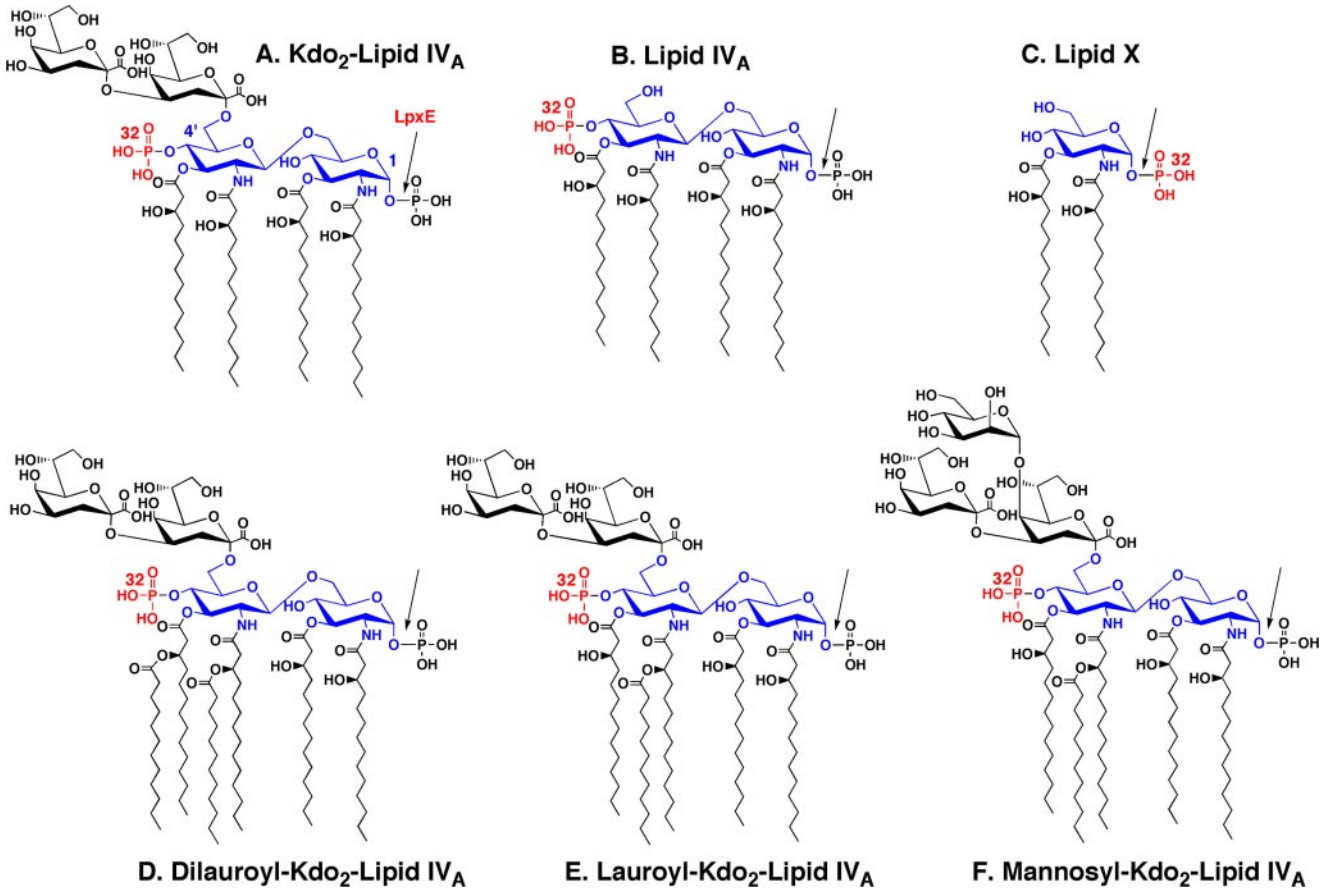

FIGURE 2. **Structures of Kdo_2-lipid IV_A and related substrates used to assay LpxE.** The seven enzymes that make Kdo_2-lipid IV_A in *E. coli* are also found in *R. leguminosarum* and *R. etli* (28), and with the possible exception of *lpxH* (50, 73), the genes encoding them are present in single copy in the *R. leguminosarum* genome. The glucosamine residues and their numbering are shown in *blue*, and the bond cleaved by LpxE is indicated with the *arrow*. The location of the radiolabeled phosphate moiety in each substrate is indicated in *red*. Although most active with substrates containing a Kdo disaccharide and one secondary acyl chain, the LpxE phosphatase does not require the Kdo moiety, allowing the use of the precursor lipid IV_A as a model substrate. The monosaccharide lipid A precursor lipid X is a poor substrate. Because the active site of LpxE is oriented toward the periplasmic surface of the inner membrane, however, it does not normally have access to LPS precursors lacking the Kdo moiety or the 2′ secondary acyl chain.

TABLE 1
Relevant bacterial strains and plasmids

Strain/plasmid	Description	Source or Ref.
***E. coli* strains**		
BLR(DE3)pLysS	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3) D(srl-recA)306::Tn*10* pLysS (Cam^R, Tet^R)	EMD Chemicals
NovaBlue(DE3)	*endA1 hsdR17*(r_{K12}^- m_{K12}^+) *supE44 thi-1 recA1 gyrA96 relA1 lac* (DE3) F′[*proA*$^+$*B*$^+$ *lacI*^{q} Δ*Z M15*::Tn*10*] (Tet^R)	EMD Chemicals
WBB06	W3110 (K12) *mtl* Δ(*WaaC-WaaF*)::*tet6*; LPS is Kdo_2-lipid A	52
MN7	K12-derived *pgsA444 lpxB1;* accumulates lipid X	45
Other strains		
A. tumefaciens C58	Wild-type pathogenic strain of *A. tumefaciens*	ATCC 33970
S. meliloti 1021	Gram-negative, nitrogen-fixing symbiont of alfalfa; Nal^R, Str^R	51
Plasmids		
pIJ1848	*R. leguminosarum* bv. phaseoli 8002 cosmid; *dctABD, lpcABC*	51
pJK2	pET3a containing *E. coli lpxK* behind T7 promoter; Amp^R	46
pET28a	Expression vector with T7 promoter; confers His_6 tag; Kan^R	EMD Chemicals
pAtLpxE	pET28a containing the *A. tumefaciens lpxE* homologue	This study
pLpxE-4	pET28a containing *R. leguminosarum lpxE*	31
pR133A	pLpxE-4 with the LpxE R133A mutation	This study
pLpxE-41 (H197A)	pLpxE-4 with the LpxE H197A mutation	This study

repurified single colony of *E. coli* containing pAtLpxE was inoculated into 20 ml of LB containing kanamycin (12 μg/ml) and grown in a rotary shaker at 37 °C to an A_{600} of 1.0. The culture was then used to inoculate 500 ml of fresh LB containing kanamycin (12 μg/ml), and when A_{600} reached 0.6, the culture was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h. Crude extracts, membrane-free cytosol, and washed membranes were prepared as described below for *E. coli*.

Preparation of Radiolabeled Substrates—As described previously (43, 44), [4′-^{32}P]lipid IV_A (Fig. 2) was prepared starting with 100 μCi of [γ-^{32}P]ATP, the tetra-acylated disaccharide 1-phosphate precursor of lipid A (the LpxB product) (45), and membranes of an *E. coli* construct that overexpresses the lipid

A 4′-kinase (46). Kdo_2-[4′-^{32}P]lipid IV_A (Fig. 1) was then prepared from [4′-^{32}P]lipid IV_A using purified *E. coli* Kdo transferase and Kdo cytidyltransferase (43, 47). Alternatively, Kdo_2-[4′-^{32}P]lipid IV_A was prepared directly from the disaccharide 1-phosphate precursor and [γ-^{32}P]ATP without purification of the [4′-^{32}P]lipid IV_A. After the Kdo transferase reaction was completed, 90–95% of the [γ-^{32}P]ATP was incorporated into the Kdo_2-[4′-^{32}P]lipid IV_A, as judged by analytical TLC and analysis with a STORM 840 PhosphorImager (GE Healthcare). The pure, dry Kdo_2-[4′-^{32}P]lipid IV_A was resuspended by sonic irradiation for 60 s in 200 μl of 10 mM Tris-HCl, pH 7.8, containing 1 mM EDTA and 1 mM EGTA, and the tube was stored at −20 °C. The typical yield of Kdo_2-[4′-^{32}P]lipid IV_A (specific activity ~ 8 μCi/nmol) following these extractions was 50–60% of the input [γ-^{32}P]ATP.

^{32}P-Lipid X (Fig. 2) was prepared from $^{32}P_i$-labeled cells of *E. coli* MN7, as described previously (49, 50). The intermediate disaccharide-1-^{32}P was synthesized from ^{32}P-lipid X and uridine diphosphate-2,3-diacylglucosamine, using a purified preparation of *E. coli* LpxB disaccharide synthase (45, 46).

Mannosyl-Kdo_2-[4′-^{32}P]lipid IV_A (Fig. 2) was prepared as a 5 μM stock (20,000 cpm/nmol) from Kdo_2-[4′-^{32}P]lipid IV_A, using membranes of *S. meliloti* 1021/pIJ1848, which overexpresses the LpcC mannosyltransferase of *R. leguminosarum* (51). The reaction mixture contained 0.5 mg/ml LpcC membrane protein and 10 mM guanosine-5′-diphosphate mannose. It was incubated at 30 °C for 60 min, and the membranes were then inactivated by heating to 65 °C for 10 min. To test the mannosyl-Kdo_2-[4′-^{32}P]lipid IV_A as a substrate for the lipid A 1-phosphatase, purified LpxE was added to the same reaction tube without purification of the mannosyl-Kdo_2-[4′-^{32}P]lipid IV_A.

Lauroyl-Kdo_2-[4′-^{32}P]lipid IV_A (Fig. 2) was synthesized from Kdo_2-[4′-^{32}P]lipid IV_A using the Kdo-dependent lauroyltransferase (LpxL) of *E. coli* in membranes of Novablue(DE3)/pLpxL (24). The reaction conditions for the lauroyltransferase were as follows: 0.2 mg/ml Novablue(DE3)/pLpxL membranes, 10 μM Kdo_2-[4′-^{32}P]lipid IV_A (20,000 cpm/nmol), lauroyl-acyl carrier protein (30 μM), 0.2% Triton X-100, 5 mM $MgCl_2$, and 50 mM NaCl in 50 mM HEPES, pH 7.5. The reaction mixture was incubated at 30 °C for 60 min, and then the LpxL membranes were inactivated by heating to 65 °C for 10 min. The heat-inactivated mixture was then tested directly as the substrate for the LpxE 1-phosphatase without purification. Dilauroyl-Kdo_2-[4′-^{32}P]lipid IV_A (Fig. 2) was prepared as described by Reynolds *et al.* (52). Nonradiolabeled Kdo_2-lipid A was purified from the WBB06 strain of *E. coli* and thus contains predominantly 2′-lauroyl and 3′-myristoyl secondary acyl chains (52). The preparation of the PGP substrate is described in the supplemental material.

LpxE 1-Phosphatase Assay—The lipid A 1-phosphatase LpxE was assayed unless otherwise indicated with the tetra-acylated lipid A precursor, [4′-^{32}P]lipid IV_A, which was stored frozen as an aqueous dispersion. Prior to use, the [4′-^{32}P]lipid IV_A substrate was subjected to ultrasonic irradiation in a bath apparatus for 1 min. The 1-phosphatase reaction mixture (10 μl final volume, unless indicated otherwise) contained 50 mM MES, pH 6.5, 1% Triton X-100, and 5 μM [4′-^{32}P]lipid IV_A (3000–6000 cpm/nmol). The reaction was started by addition of an appropriate amount of enzyme (0.01 mg/ml of pure enzyme or about 0.1 mg/ml of solubilized *E. coli* membranes obtained from cells overexpressing *lpxE* behind the T7*lac* promoter) and incubated for various times at 30 °C. The reactions were stopped by spotting 4-μl samples directly onto a silica gel TLC plate. After drying at room temperature, the plate was developed in the solvent chloroform, pyridine, 88% formic acid, H_2O (50:50:16:5, v/v). Following removal of the solvent with a stream of hot air, the plate was analyzed with a PhosphorImager, equipped with ImageQuant software (GE Healthcare).

PGP Phosphatase and PA Phosphatase Assays—The *in vitro* assays for PGP phosphatase and PA phosphatase activity are described in the supplemental material.

Growth of Cells, Preparation of Membranes, and Purification of LpxE—Novablue(DE3)/pLpxE-4 (31) was grown at 37 °C from a single colony in 1 liter of LB containing 30 μg/ml kanamycin. When the A_{600} reached 0.6, the culture was cooled to 26 °C, then induced with 1 mM IPTG, and incubated with shaking at 250 rpm for an additional 4 h at 26 °C. Cells were harvested at 4 °C by centrifugation at 9000 rpm for 20 min, resuspended in 30 ml of 50 mM HEPES, pH 7.5, and broken by passage through a French pressure cell at 18,000 p.s.i. These and all subsequent steps were carried out at 4 °C. Cell debris and unbroken cells were removed by centrifugation at 9000 rpm for 20 min. Membranes were prepared by ultracentrifugation at 100,000 × g_{AV} for 60 min. The membranes were resuspended in 20 ml of the same buffer, and the centrifugation at 100,000 × g_{AV} was repeated to remove remaining cytosol. Protein concentration was determined by the bicinchoninic acid method using bovine serum albumin as the standard (40). The membrane pellet was resuspended at 8–15 mg/ml protein and stored at −80 °C.

Solubilization of His-tagged LpxE—The membranes were solubilized by adding appropriate volumes of 10% aqueous Triton X-100 (to give a final concentration of 4% Triton X-100) and of 4 or 5 M aqueous NaCl (to yield 0.5 M NaCl). The mixture was incubated at 4 °C for 1 h with intermittent inversion on a rotating apparatus and was then centrifuged at 100,000 × g_{AV} for 60 min at 4 °C to remove any remaining insoluble material.

Metal Affinity Chromatography—Typically, 1 ml of Ni^{2+}-nitriloacetate (NTA)-agarose (50% slurry; Sigma) was poured into a disposable plastic gravity-flow column (15 ml) and allowed to settle. Next, 8 ml of the solubilized membrane protein preparation, described above, was diluted 4-fold with 50 mM sodium phosphate, pH 8, containing 500 mM NaCl, 10 mM imidazole, and 0.5% Triton X-100. This solution was applied to the column at 4 °C. The column was washed three times with 10 ml of 50 mM sodium phosphate, pH 8, containing 500 mM NaCl, 10 mM imidazole, and 0.5% Triton X-100. The column was eluted sequentially with 10-ml portions of a similar buffer (50 mM sodium phosphate, pH 8, 150 mM NaCl, and 0.5% Triton X-100), containing either 10, 20, 50, or 500 mM imidazole, and 1-ml fractions were collected. The first few 1-ml fractions of the last elution step contained most of the LpxE. The protein concentration and enzyme activity of each fraction were determined. For samples containing detergent and/or imidazole, the Non-Interfering™ protein assay from G-Biosciences was uti-

lized. Proteins were visualized by PAGE in the presence of 12% SDS, using the Mini Protean II electrophoresis system (Bio-Rad.). LpxE-containing protein samples were prepared for SDS-PAGE by treatment at 40 °C for 30 min as described (31).

LpxE Activity Optimization—The pH-rate profile of LpxE was determined with a uniform triple buffer system, consisting of 100 mM sodium acetate, 50 mM BisTris, and 50 mM Tris from pH 4 to 9, as described previously (6 and references therein). The presence of 10 mM EDTA in the assay had no significant effect on LpxE activity, confirming that purified LpxE has no divalent cation requirement (data not shown). LpxE was assayed with varying concentrations of $CaCl_2$, $MgCl_2$, or $MnCl_2$ (from 0.1 to 5 mM). The addition of divalent cations had no effect on the R_f values of lipid IV_A or its 1-dephosphorylated product (data not shown).

In investigating the dependence of purified LpxE on detergent, the concentration of Triton X-100 was varied in the assay from 0.006 to 2% (*w*/*v*). At an average molecular weight of 625 g/mol, this corresponds to a range of 100 μM to 32 mM. The critical micelle concentration of Triton X-100 is ~200 μM. The concentration of lipid IV_A remained fixed at 5 μM. LpxE was also assayed at varying levels of lipid IV_A with the concentration of Triton X-100 fixed at 0.1% w/v (1.6 mM).

Inactivation of LpxE with Diethyl Pyrocarbonate—To remove interfering imidazole, LpxE from the Ni^{2+}-NTA-agarose column pool (5 ml) was first subjected to rapid chromatography on Sephadex G-25 M (GE Healthcare) to replace the elution buffer with 50 mM MES, pH 6.5, in 0.1% Triton X-100. For inhibition studies, 20 μM purified LpxE was incubated for 10 min at room temperature in 40 mM potassium phosphate, pH 7, with various concentrations of DEPC, stock solutions of which were prepared in ethanol just prior to each experiment. DEPC concentrations were determined by reacting it with excess imidazole (Sigma) and measuring the A_{240} ($\epsilon = 3200\ M^{-1}$) (60). DEPC reactions with LpxE were quenched by a 1:50 dilution of the enzyme into 80 μM imidazole on ice (representing at least a 50-fold molar excess over the DEPC). Ethanol and imidazole at the concentrations carried over into the assay mixture had no effect on LpxE activity (not shown).

For NH_2OH reactivation studies, 20 μM purified LpxE was incubated for 5 min at room temperature with or without 80 μM DEPC in 40 mM potassium phosphate, pH 7. The reactions were quenched by 1:50 dilution into 80 μM imidazole. These mixtures were then diluted 1:2 into 40 mM potassium phosphate, pH 7, or into a matched solution containing 10 mM NH_2OH (Sigma). A 100 mM stock of NH_2OH in water was prepared immediately before each experiment. Various times after dilution into NH_2OH, appropriate portions were assayed for LpxE activity.

Modification of LpxE with Lysine, Arginine, and Serine Reagents—In all experiments, 20 μM purified LpxE was incubated at room temperature for 30 min with increasing concentrations of pyridoxal 5′-phosphate, phenylglyoxal, or phenylmethanesulfonyl fluoride. Pyridoxal 5′-phosphate reactions were quenched by a 1:2 dilution into 20 mM sodium borohydride (corresponding to at least a 10-fold molar excess over pyridoxal 5′-phosphate). All reactions were then diluted appropriately and assayed for LpxE activity. Pyridoxal 5′-phosphate (Sigma) was prepared in 50 mM HEPES, pH 8, whereas phenylglyoxal and sodium borohydride were dissolved in water. The phenylmethanesulfonyl fluoride stock solution was prepared in isopropyl alcohol. Isopropyl alcohol and sodium borohydride have no effect on LpxE activity at the levels carried over into the assay (data not shown). The pyridoxal 5′-phosphate and phenylglyoxal studies were performed in 50 mM HEPES, pH 8 (53, 54). The phenylmethanesulfonyl fluoride studies were performed in 40 mM potassium phosphate, pH 7 (55).

Modification of LpxE with Cysteine-specific Reagents—In all experiments, 20 μM purified LpxE was incubated at room temperature for 30 min with increasing concentrations of *N*-ethylmaleimide (Sigma). Stock solutions were prepared in water. After 30 min, samples were diluted with 1 mg/ml bovine serum albumin and assayed for LpxE activity.

Site-directed Mutagenesis—Mutated *R. leguminosarum lpxE* genes, encoding LpxE with a single amino acid substitution in the catalytic histidine (His-197) or arginine (Arg-133), were generated by PCR mutagenesis by minor modification of a method described previously (56). The mutant LpxE constructs are numbered according to the native LpxE primary sequence. The N-terminal His_6 tag and linker (38 amino acids total) are present in the mutants. The primers are described in supplemental Table 1. The resulting pET28a-derived plasmids are designated pR133A and pH197A.

RESULTS

Overexpression of LpxE in E. coli and Solubilization with Triton X-100—Over 95% of the LpxE protein and 1-phosphatase activity were recovered with the membrane fraction of Novablue(DE3)/pLpxE-4 cells induced at 26 °C for 4 h. The 1-phosphatase activity was not released from the membrane by washing with 5 M NaCl, consistent with six predicted membrane-spanning segments in the protein (31). To solubilize the enzyme, the detergents Triton X-100, octyl-β-D-glucoside, lauroyl dimethylamine *N*-oxide, CHAPS, and dodecyl maltoside were tested at concentrations ranging from 0.5 to 4%. Following exposure of the membranes to 4% Triton X-100, the detergent-treated LpxE was nearly 10-fold more active than a control in which *E. coli* Novablue(DE3)pLpxE-4 membranes were added directly to the assay system. After high speed centrifugation, the solubilized LpxE-specific activity further increased to as high as 50-fold more active than the control membranes (Table 2). As shown in Fig. 3, more than 95% of the 5 μM Kdo_2-[4′-^{32}P]lipid IV_A that was used as the substrate in this experiment was converted to 1-dephosphorylated-Kdo_2-[4′-^{32}P]lipid IV_A in 20 min by 10 μg/ml solubilized membrane protein (Fig. 3, *4th lane*), compared with only 20% conversion by 10 μg/ml Novablue(DE3)/pLpxE-4 membranes (Fig. 3, *2nd lane*) added directly to the assay system. A significant portion of the LpxE protein band was rendered soluble in parallel with the LpxE activity, as judged by centrifugation at 100,000 × g_{AV} and gel electrophoresis (Fig. 4, *lanes 3–5*). Treatment of membranes with Triton X-100 also increased the total measurable membrane protein concentration 2-fold. The higher value was used to normalize the amount of protein loaded on gels. Extraction of proteins from Novablue(DE3)/pLpxE-4 membranes with the

TABLE 2
Purification of LpxE from *E. coli* Novablue(DE3)/pLpxE-4

Step	Protein concentration	Total protein	Specific activity	Total activity	Overall purification	Overall yield	Activation
	mg/ml	*mg*	*nmol/min/mg*	*nmol/min*	*-fold*	*%*	*-fold*
Membranes	8.42[a]	72[a]	0.0048	0.35	NA[b]	NA	1
Detergent-treated membranes	4.21	72	0.043	3.1	NA	NA	9
Solubilized membranes	2.69	48	0.23	11	1	100	48
Ni^{2+}-NTA-purified LpxE	1.58	7.4	1.1	8.1	5	74	NA

[a] Data corrected to reflect the effect of detergent on the protein assay.
[b] NA means not applicable.

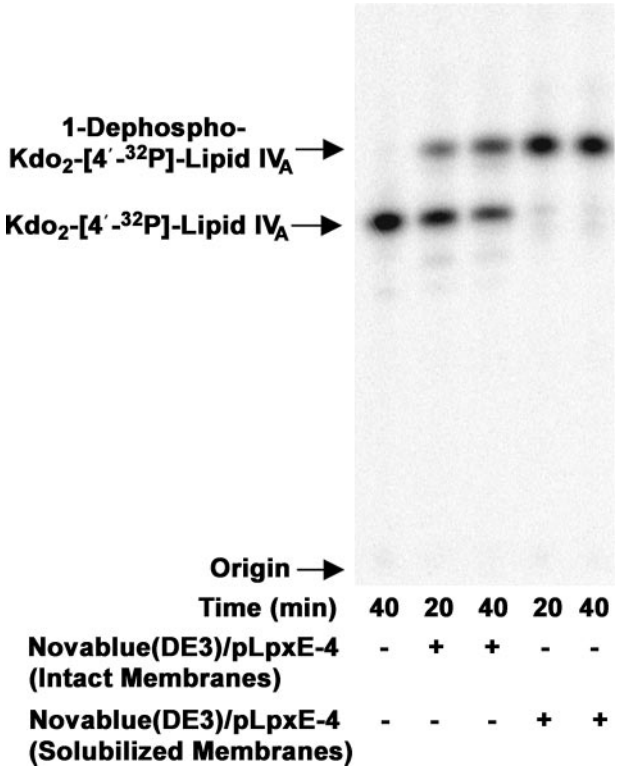

FIGURE 3. **Activation of LpxE by solubilization of Novablue(DE3)/pLpxE-4 membranes with 4% Triton X-100.** Membranes of Novablue(DE3)/pLpxE-4 were solubilized with 4% Triton X-100, ultracentrifuged to remove insoluble material, and assayed for 1-phosphatase activity with Kdo_2-[4′-^{32}P]lipid IV_A as substrate under otherwise standard conditions at 30 °C. The no enzyme control is shown in the *1st lane*. Membranes from Novablue (DE3)/pLpxE-4 that had not been solubilized with Triton X-100 were used at 0.5 mg/ml in the *2nd* and *3rd lanes*. Triton X-100-solubilized supernatant of Novablue(DE3)/pLpxE-4 membranes was used at 0.5 mg/ml in the *4th* and *5th lanes*. At each time point, a 4-μl portion was quenched, and the extent of dephosphorylation was assessed by TLC and PhosphorImager analysis.

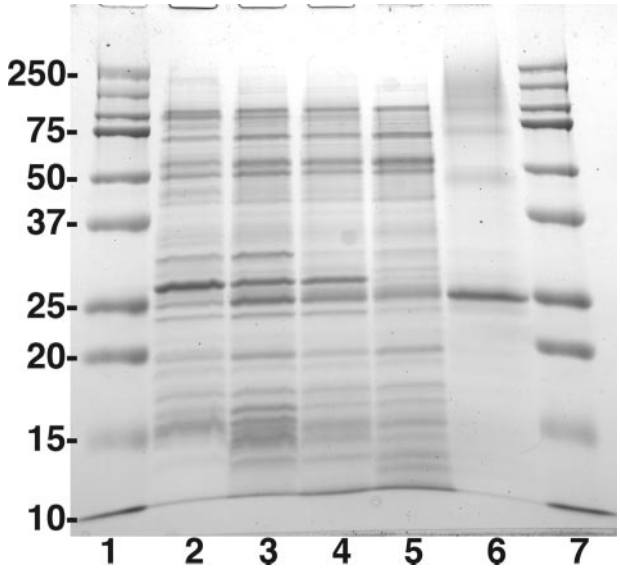

FIGURE 4. **Gel electrophoresis of LpxE at various stages of the purification.** The molecular weight standards, shown in *lanes 1* and *7*, are 10, 15, 20, 25, 37, 50, 75, 100, 150, and 250 kDa; *lane 2*, 10 μg of membrane protein from Novablue(DE3)/pET-28a induced with IPTG; *lane 3*, 10 μg of membrane protein from Novablue(DE3)/pLpxE-4 induced with IPTG; *lane 4*, 10 μg of 4% Triton X-100-treated LpxE membranes from Novablue(DE3)/pLpxE-4 induced with IPTG (pre-centrifugation); *lane 5*, 10 μg of 4% Triton X-100-solubilized LpxE membranes (post-centrifugation); *lane 6*, 10 μg of purified LpxE protein (after the Ni^{2+}-NTA column).

other detergents listed above was also quite efficient, but LpxE activity was stimulated only 2-fold (data not shown).

Purification of LpxE—The presence of the N-terminal His_6 tag in the recombinant *R. leguminosarum* LpxE protein permitted the use of immobilized metal affinity chromatography. The solubilized, activated protein was applied to a Ni^{2+}-NTA-agarose column prepared in 0.5% Triton X-100, as described under "Experimental Procedures." Most of the activity was retained and was eluted with 500 mM imidazole along with a protein of the molecular weight expected for LpxE (Fig. 4, *lane 6*). The specific activity of the partially purified enzyme was increased 5-fold relative to the solubilized, activated membrane fraction (Table 2). The purity of this material was greater than 95%, as judged by SDS-PAGE (Fig. 4, *lane 6*). The extent of purification required to reach homogeneity is consistent with the behavior of other membrane-associated enzymes involved in lipid metabolism (57).

Effects of pH, Divalent Cations, and Triton X-100 on LpxE Activity—The activity of the purified LpxE was measured in a uniform triple buffer system as described under "Experimental Procedures," allowing for a buffer-independent comparison of the pH dependence of LpxE activity from pH 4 and 9. LpxE activity is optimal around pH 6.5, although LpxE is similarly active between pH 5 and 8 (Fig. 5, *panel A*). The data form a bell-shaped curve. The pK_a and pK_b values for the acidic and basic limb, respectively, of the curve were determined as described previously (6 and references therein). The pK_a is 5.25 ± 0.07, ands the pK_b is 8.04 ± 0.07.

At the optimal pH of 6.5, the effects of the divalent cations Mg^{2+}, Mn^{2+}, or Ca^{2+} were tested at varying concentrations ranging from 0 to 5 mM (Fig. 5, *panel B*). LpxE was not strongly affected by Mg^{2+} or Ca^{2+} but was inhibited by Mn^{2+} with an IC_{50} value around 0.5 mM (Fig. 5, *panel B*). The steepness of the Mn^{2+} inhibition curve suggests the formation of inactive aggregates of LpxE and/or lipid IV_A.

As is observed with most enzymes of lipid metabolism (58), the detergent Triton X-100 was required for full activity (Table 2 and Fig. 5, *panel C*) (31). In addition to its direct activation

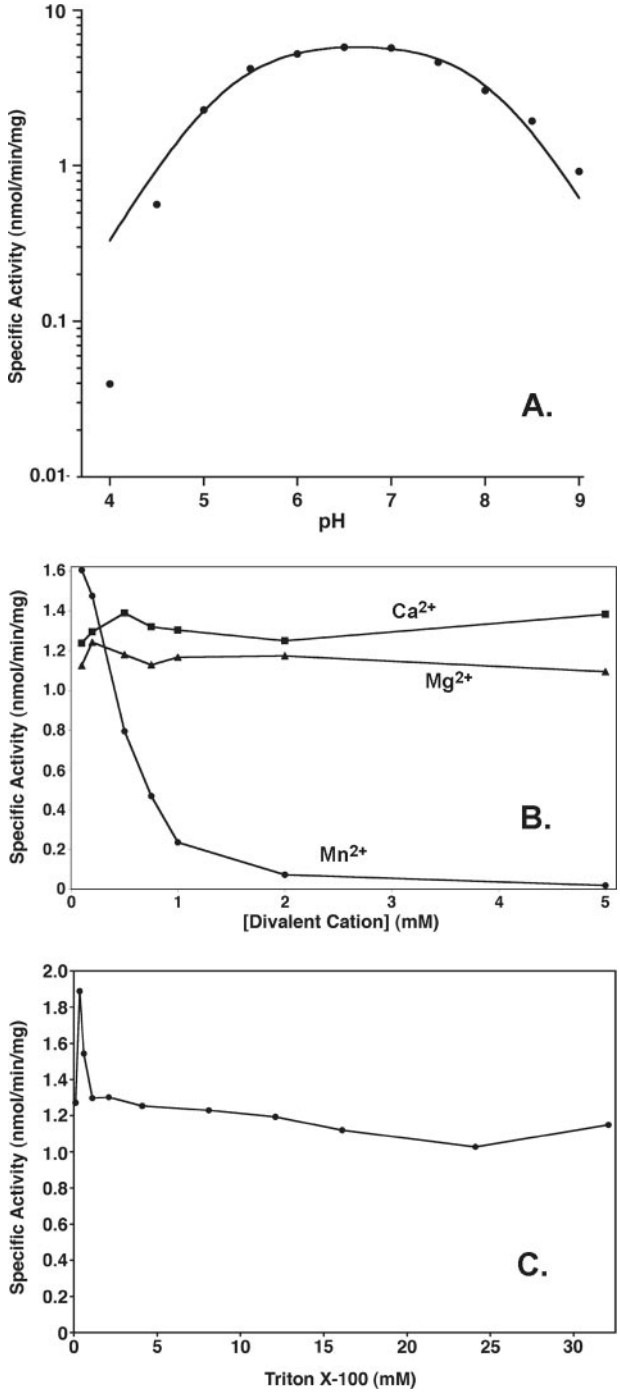

FIGURE 5. **Effect of pH, divalent cations, and Triton X-100 on LpxE activity.** The 1-phosphatase activity was measured at the indicated pH values in a uniform triple buffer system (*panel A*). The *curve* represents a fit of the pH-rate equation as described under "Experimental Procedures." The pK_a and pK_b values derived from the fit are 5.25 ± 0.07 and 8.04 ± 0.07. The 1-phosphatase activity was measured in the presence of $MgCl_2$, $CaCl_2$, or $MnCl_2$ (*panel B*) or at various concentrations of Triton X-100 (*panel C*) under otherwise standard conditions. The points are connected for ease of visualization. The data shown in each panel are from a single representative experiment.

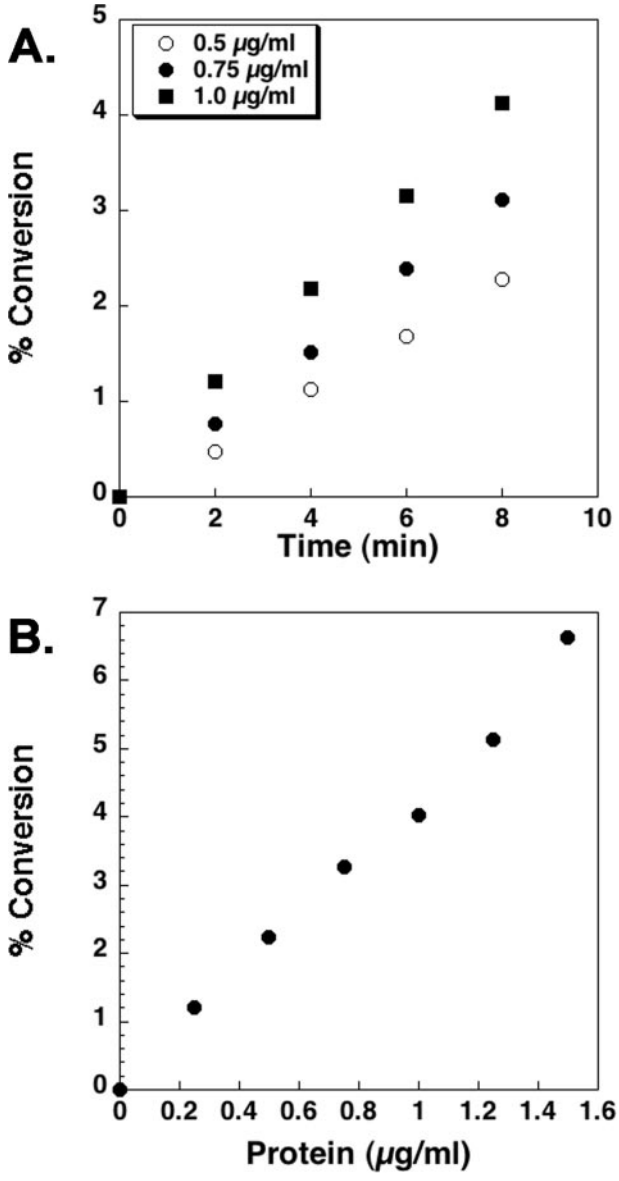

FIGURE 6. **Time and protein concentration dependence of *R. leguminosarum* LpxE.** Dephosphorylation of [4′-^{32}P]lipid IV_A is linear with time (*panel A*) and protein concentration when assayed for 10 min under standard conditions at pH 6.5 (*panel B*). After prolonged incubation, or in the presence of high enzyme concentrations, the reaction goes to completion (data not shown). The data shown in each panel are from a single representative experiment.

effects on LpxE, Triton X-100 could interact with lipid IV_A to generate mixed micelles, thereby giving the enzyme better access to its substrate. However, when the concentration of lipid IV_A was held constant at 5 μM, the effects of Triton X-100 were not consistent with surface dilution kinetics (58), as there was little to no decrease in the 1-phosphatase activity in the range of 1–30 mM Triton X-100 (Fig. 5, *panel C*). It was not possible to assay pure LpxE with less than 100 μM Triton, because of carryover from the enzyme and substrate preparations.

Kinetic Properties and Lipid Substrate Specificity of Purified LpxE—With lipid IV_A as the model substrate (Fig. 2), LpxE activity is linearly dependent upon both time and protein concentration (Fig. 6, *panels A* and *B*). The apparent K_m for lipid IV_A is 11 ± 2 μM, and the apparent V_{max} is 3.3 ± 0.2 nmol/min/mg (Fig. 7).

The substrate selectivity of the 1-phosphatase was investigated under standard assay conditions with 5 μM substrate. The addition of two Kdo units to lipid IV_A increases the specific

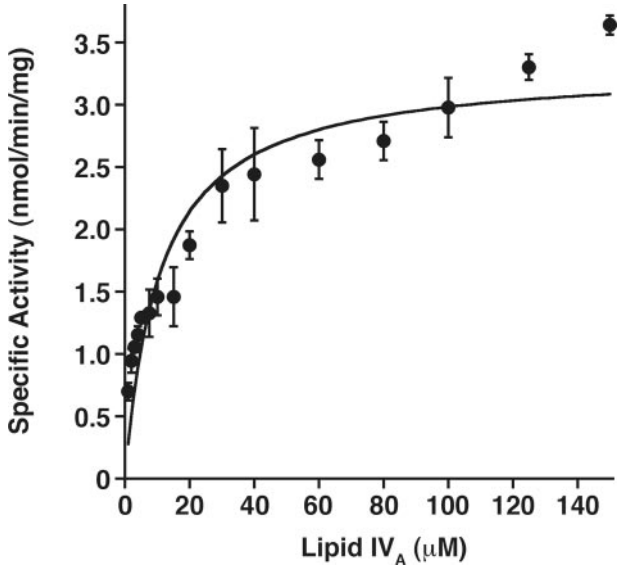

FIGURE 7. **Steady state kinetics of purified LpxE in a mixed micelle system.** Standard assay conditions were used, but the lipid IV_A substrate concentrations were varied. A fit of the Michaelis-Menten equation to the data gives an apparent K_m of 11 ± 2 μM for lipid IV_A and an apparent V_{max} of 3.3 ± 0.2 nmol/min/mg. The data shown are the average of several experiments with the standard deviation shown.

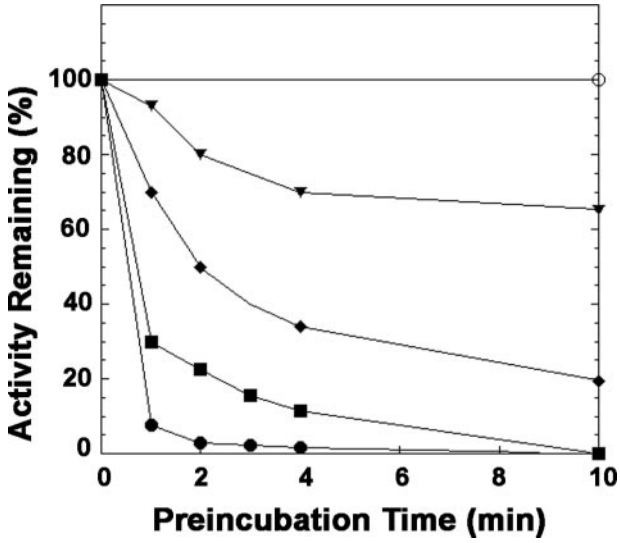

FIGURE 8. **Time course of inactivation of *R. leguminosarum* LpxE by DEPC.** Purified LpxE (20 μM) was preincubated at room temperature with 0 (○), 10 μM (▼), 20 μM (◆), 40 μM (■), or 80 μM (●) DEPC. At the indicated times, a portion of the preincubation mixture was quenched with imidazole, diluted, and assayed for remaining LpxE activity. The points are connected for ease of visualization. The data shown are from a single representative experiment.

TABLE 3

Relative specific activities of LpxE with different lipid substrates

The relative rates were determined under standard assay conditions with 5 μM substrate using purified LpxE. The structures of lipid A and its precursors are shown in Fig. 2.

Substrate	Relative specific activity
	%
Kdo_2-lipid A	30
Lauroyl-Kdo_2-lipid IV_A	100
Mannosyl-Kdo_2-lipid IV_A	50
Kdo_2-lipid IV_A	50
Lipid IV_A	11
Lipid X	0.5
Phosphatidic acid	0.033
Phosphatidylglycerol phosphate	0.043

activity of LpxE by about 5-fold (Table 3). Kdo_2-[4′-^{32}P]lipid A, mannosyl-Kdo_2-[4′-^{32}P]lipid IV_A, and lauroyl-Kdo_2-[4′-^{32}P]lipid IV_A (Fig. 2) were also tested; all three compounds were utilized efficiently, but lauroyl-Kdo_2-[4′-^{32}P]lipid IV_A was a better substrate than Kdo_2-[4′-^{32}P]lipid IV_A by a factor of two (Table 3). These findings are consistent with the fact that *R. leguminosarum* core-lipid A precursors are typically penta-acylated (21, 22). The presence of the additional core sugar mannose (51, 59) did not increase the rate of 1-dephosphorylation appreciably (Table 3).

R. leguminosarum LpxE is the first example of a purified enzyme that specifically dephosphorylates lipid A (or some of its precursors) at the 1-position. No release of inorganic phosphate from the 4′-position was detected, even after full 1-dephosphorylation (Fig. 3, *4th* and *5th lanes*). The rate of dephosphorylation of the monosaccharide precursor lipid X (Fig. 2) was 20-fold slower than that of lipid IV_A (Table 3), indicating that the distal diacylglucosamine unit of lipid IV_A somehow enhances catalytic efficiency.

Possible alternative glycerophospholipid substrates, such as PA and PGP (Table 3) are poor very substrates for LpxE. They are dephosphorylated at less than 1% the rate of lipid IV_A under otherwise matched conditions.

Inactivation of LpxE by DEPC—Incubation of purified LpxE with DEPC, a histidine-modifying reagent, causes a rapid loss of enzymatic activity (Fig. 8). LpxE is stable under the preincubation conditions in the absence of DEPC. The loss of activity depends upon the time of preincubation and the concentrations of DEPC. The inactivation is complete after 10 min with a 2–4-fold molar excess of DEPC over LpxE. The sensitivity of LpxE to DEPC is consistent with its proposed mechanism of action.

Reactivation of DEPC-inactivated LpxE by NH_2OH—In some cases, DEPC has been shown to react with side chains other than histidine (60). The small amount of DEPC needed for the inactivation of LpxE and the rapid rate of inactivation (Fig. 8) argue that side chains other than histidine are not being modified. In addition, the inactivation by DEPC is reversed (100% recovery after 30 min) by subsequent incubation with NH_2OH (data not shown). NH_2OH reverses DEPC modification of histidine and tyrosine residues but not of lysine or cysteine side chains (60). The reaction of DEPC with tyrosine residues on LpxE is unlikely, given that no change in the absorbance of LpxE occurs at 280 nm upon treatment with DEPC (data not shown). Thus, the inactivation of LpxE by DEPC can be attributed solely to the modification of key histidine residue(s).

Effects of Other Chemical Modification Reagents on LpxE Activity—We examined LpxE inactivation by pyridoxal 5′-phosphate/sodium borohydride, phenylglyoxal, and phenylmethanesulfonyl fluoride, which are lysine-, arginine-, and serine-specific reagents, respectively (61). Low millimolar concentrations of both pyridoxal 5′-phosphate/sodium borohydride and phenylgloxal inhibit LpxE completely (data not shown). Phenylmethanesulfonyl fluoride has little effect. No inactivation of LpxE is seen with the sulfhydryl reagent, *N*-ethylmaleimide. These findings suggest that lysine and/or arginine resi-

due(s) may play important roles in substrate binding and/or catalysis, whereas serine and cysteine residues do not, consistent with the fact that there are no conserved serine or cysteine residues in the lipid phosphate phosphatase motifs (34).

Site-directed Mutagenesis of R. leguminosarum LpxE—Alignment of the predicted LpxE proteins from *R. leguminosarum*, *Mesorhizobium loti*, *A. tumefaciens*, *S. meliloti*, and *F. tularensis* shows about 35% identity and 55% similarity (data not shown). Residues found in the tripartite active site motif (Fig. 1, *panel A*) are conserved among all of the LpxE orthologues. To test the biological significance of these sequence motifs, we expressed and assayed the LpxE point mutants R133A and H197A. These residues were chosen because of their critical roles in enzymatic catalysis for diacylglycerol pyrophosphate phosphatase from *Saccharomyces cerevisiae* (62), their conservation among enzymes that contain the lipid phosphate phosphatase motifs (34–36), and the clustering of the related active site side chains in the x-ray crystal structure of the soluble *Escherichia blattae* nonspecific acid phosphatase (63). Each mutant protein was expressed at similar levels, based on SDS-PAGE analysis and Western blotting (data not shown). As expected, LpxE H197A (Fig. 8) and LpxE R133A (data not shown), when overexpressed in *E. coli* and solubilized with 4% Triton X-100, showed negligible (less than 1% of wild type) lipid A 1-phosphatase activity in our *in vitro* assay system, clearly demonstrating that His-197 and Arg-133 are essential for catalysis. Purified preparations of these mutant enzymes were also completely inactive (data not shown).

A. tumefaciens LpxE Is a PGP Phosphatase—Membranes of *A. tumefaciens* do not catalyze the 1-dephosphorylation of lipid A, yet the genome of this organism contains an LpxE homologue that displays 39% identity and 58% similarity to *R. leguminosarum* LpxE. To determine the function of the *A. tumefaciens* LpxE homologue, we used PCR to amplify the gene from genomic DNA, subcloned it into a pET-28 expression vector, and assayed membranes from induced *E. coli* harboring the plasmid, pAtLpxE, for lipid A phosphatase activity. The induced membranes displayed no 1-phosphatase activity with lipid A or related substrates (Fig. 2), including Kdo_2-[4′-^{32}P]lipid A, lauroyl-Kdo_2-[4′-^{32}P]lipid IV_A, Kdo_2-[4′-^{32}P]lipid IV_A, [4′-^{32}P]lipid IV_A, and ^{32}P-lipid X (data not shown).

We next assayed membranes from *E. coli* cells that overexpress the *A. tumefaciens* LpxE homologue with two possible glycerophospholipid substrates, [*glycerol*-U-^{14}C]PA and [*glycerol*-U-^{14}C]PGP for phosphatase activity. As shown in Fig. 10, expression of the *A. tumefaciens lpxE* gene behind the T7*lac* promoter on plasmid pAtLpxE in *E. coli* resulted in the appearance of robust PGP phosphatase activity, as judged by the nearly complete conversion of [*glycerol*-U-^{14}C]PGP to [*glycerol*-U-^{14}C]phosphatidylglycerol within 10 min at 0.5 mg/ml membrane protein (Fig. 10, *lanes 5-8*). When assayed with [*glycerol*-U-^{14}C]PA, no activity was seen (data not shown). These findings demonstrate that the *R. leguminosarum* LpxE homologue present in *A. tumefaciens* is very likely a PGP phosphatase and not a lipid A 1-phosphatase.

DISCUSSION

The *lpxE* gene, which encodes the lipid A 1-phosphatase of *R. leguminosarum*, was previously identified by an expression cloning strategy (31). LpxE catalyzes the dephosphorylation of the proximal glucosamine moiety of lipid A, and it belongs to a large family of membrane-bound lipid phosphate phosphatases, characterized by a tripartite active site sequence motif highlighted in Fig. 1, *panel A* (34–36). Although not present in *E. coli* or *Salmonella*, LpxE orthologues are found in several important pathogens, including strains of *Francisella* and *Helicobacter* (24, 32). Expression of *Francisella* LpxE in *E. coli* or *Salmonella* does not inhibit cell growth but causes nearly quantitative dephosphorylation of the proximal glucosamine unit of lipid A (24, 32). *In vivo*, dephosphorylation of lipid A by LpxE is strictly dependent upon the proper functioning of the essential ABC transporter MsbA in such constructs (24, 32), indicating that the LpxE active site faces the periplasmic side of the inner membrane. The possibility of dephosphorylating lipid A at its 1-position in living bacterial cells could prove useful for studies of pathogenesis and for vaccine development.

Using a strain of *E. coli* that harbors the *lpxE* gene behind the T7*lac* promoter, we have now overexpressed, solubilized, and purified an N-terminally His_6-tagged version of the *R. leguminosarum* 1-phosphatase. Chromatography on a Ni^{2+}-NTA-agarose column yielded a nearly homogeneous polypeptide of 31 kDa, as judged by SDS-PAGE, in agreement with the predicted size of LpxE (31). The specific activity of the pure enzyme is 1.1 nmol/min/mg with lipid IV_A as the substrate under standard assay conditions. Western blotting confirms that the 1-phosphatase activity, the major protein band at 31 kDa, and the His_6 epitope co-elute (data not shown). Western blotting also confirmed the presence of higher molecular weight aggregates in the purified LpxE preparation that persist under denaturing SDS-PAGE conditions (data not shown). These aggregates suggest the presence of homo-oligomers of LpxE, consistent with the partial laddering of LpxE seen by Coomassie staining (Fig. 4, *lane 6*).

LpxE from membranes exhibits an unusual activation in the presence of Triton X-100. Why the recombinant LpxE is activated by exposure to 4% Triton X-100 prior to the assay is unclear. Inhibitory substances present in the crude membrane fraction may be removed by the solubilization process. Alternatively, the LpxE protein may be folded incorrectly until it is treated with 4% Triton X-100. It is also possible that the detergent facilitates formation of active oligomers of LpxE. The fact that high concentrations of Triton X-100 in the assay do not reduce the activity of LpxE by surface dilution (Fig. 5, *panel C*) suggests that it is the solubilization of LpxE and not the presentation of substrate at the mixed micelle interface that is critical.

The predicted transmembrane topology of LpxE (Fig. 1, *panel B*) suggests that all three of its phosphatase motifs face the periplasm. To validate the importance of these motifs, two of them were subjected to site-directed mutagenesis. The R133A and H197A substitutions (Fig. 1, *panel B*) completely inactivated the enzyme, as shown by assays of the H197A enzyme (Fig. 9). The decreased activities of the mutant enzymes

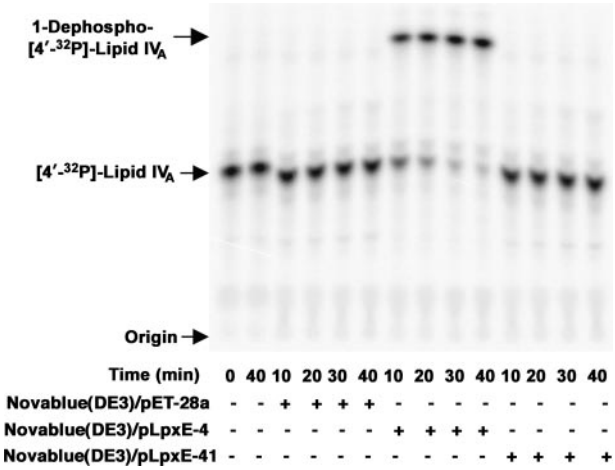

FIGURE 9. **Absence of LpxE activity of the H197A LpxE mutant.** Membranes isolated from Novablue(DE3)/pET-28a (empty vector), Novablue(DE3)/pLpxE-4 (wild-type LpxE), and Novablue(DE3)/pLpxE-41 (H197A) were solubilized with 4% Triton X-100 and assayed in the presence of 0.1 mg/ml protein for 1-phosphatase activity at 30 °C.

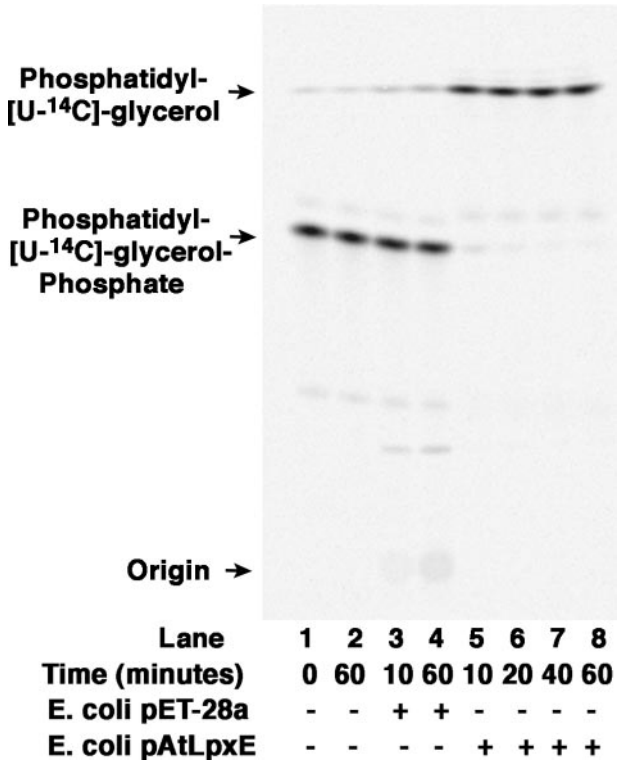

FIGURE 10. **The LpxE homologue of *A. tumefaciens* is a PGP phosphatase.** The assay was carried out under standard conditions at the indicated times with no protein added (*lanes 1* and *2*), 0.5 mg/ml membrane protein from cells harboring either the vector control (*lanes 3* and *4*), or pAtLpxE (*lanes 5-8*).

were not because of reduced enzyme expression, and the purified mutant enzymes (not shown) also displayed no detectable LpxE activity.

The characterization of LpxE indicated that there is a broad pH optimum for activity (Fig. 5, *panel A*). The typical bell-shaped curve for the pH-rate profile indicated that at least one acidic and basic group contribute to the specific activity of LpxE. Although mutagenesis and chemical modification have identified His-197 and Arg-133 as key ionizable amino acid side chains, it is likely that the charge state of the lipid IV_A, in particular that of the 1-phosphate group, might also affect LpxE activity.

The x-ray crystal structure of the *E. blattae* nonspecific acid phosphatase, a soluble protein that is a distant member of the same phosphatase family, in that it possesses the tripartite active site motifs, reveals a phosphate-binding pocket in which a conserved histidine residue might form a phosphoenzyme intermediate (63). His-197 of LpxE (Fig. 1, *panel A*) could have a similar function. Because it is difficult to prepare lipid A substrates labeled with ^{32}P of high specific radioactivity at the 1-position, we were unable to demonstrate a phosphoenzyme intermediate for LpxE (data not shown). A x-ray crystal structure or NMR structure of LpxE will be required to elucidate its mechanism and the manner in which it achieves its remarkable selectivity for the 1-position of lipid A. *Helicobacter* LpxE (24) might be more amenable to structural studies, given its relatively small size, but a solubilization and purification scheme have not been reported.

Lipid A and related molecules are clearly the preferred substrates for *R. leguminosarum* LpxE, based on a comparison of specific activities under standard assay conditions (Table 3). Maximal 1-phosphatase activity is seen with lauroyl-Kdo_2-lipid IV_A, consistent with the fact that *R. leguminosarum* lipid A is penta-acylated (21, 22). No 4′-phosphatase activity is detected with the recombinant, purified enzyme under any condition. *R. leguminosarum* LpxE activity is enhanced about 10-fold by the presence of the Kdo disaccharide in its substrate (Table 3). This Kdo effect is further accentuated in the case of *Francisella* LpxE (32), possibly accounting for the absence of the 1-phosphate group in a portion of the *Francisella* LPS (25), but not in "free" *Francisella* lipid A (26), which is a prominent and unusual feature of the lipids found in that organism.

Purified LpxE dephosphorylates PA and PGP at a very slow rate (Table 3). This activity is not likely to be physiologically relevant. Interestingly, however, *A. tumefaciens* LpxE, which is the closest non-*Rhizobium* LpxE homologue in the current nonredundant data base, has no activity against lipid A but is very active with PGP. This unexpected reversal of selectivity demonstrates the importance of developing enzymatic assays in conjunction with the functional annotation of genomes. Although *Francisella* LpxE is less similar overall to *R. leguminosarum* LpxE than is *A. tumefaciens* LpxE, the C-terminal half of *Francisella* LpxE actually shows much greater similarity to *R. leguminosarum* LpxE. It may be that the C-terminal half of the protein is critical for determining substrate selectivity, a possibility that could be tested by constructing genes encoding hybrid LpxE proteins.

The lipid A of *A. tumefaciens* grown on nutrient broth possesses both the 1- and 4′-phosphate groups,[6] and we have shown that there is no detectible 1-phosphatase activity in *A. tumefaciens* membranes. Consistent with this observation, recombinant AtLpxE shows no lipid A 1-phosphatase activity but instead is a PGP-phosphatase (Fig. 10). Interestingly, the *A. tumefaciens* genome encodes a functional *lpxQ* orthologue (65). In order for LpxQ to function, the 1-phosphate group of

[6] N. Que-Gewirth and C. Raetz, unpublished observations.

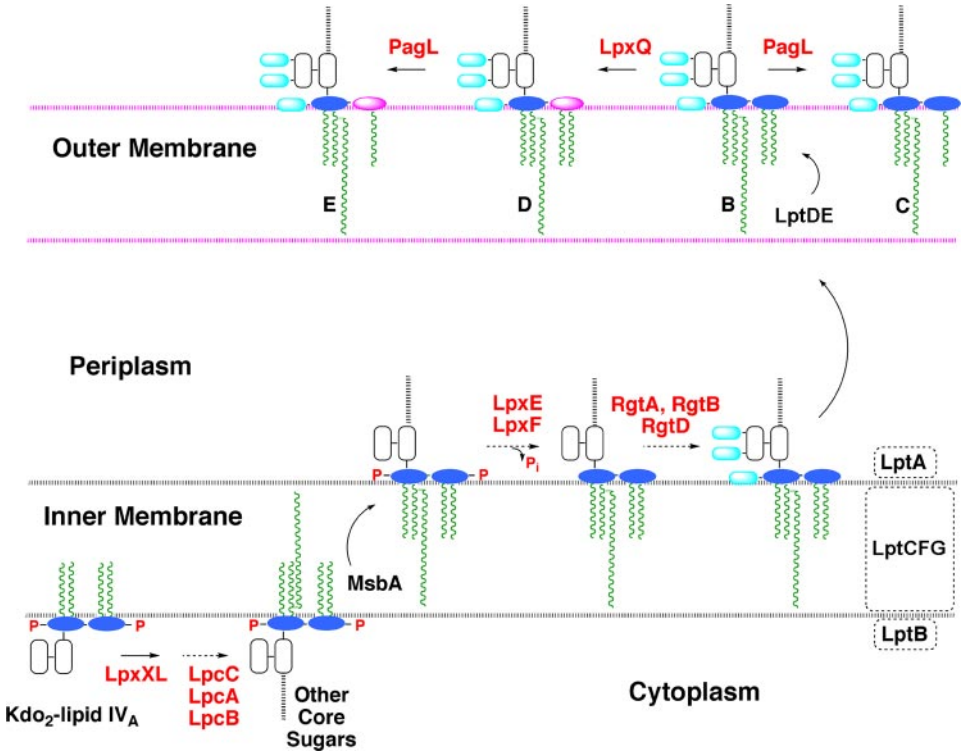

FIGURE 11. **Topography of the active sites of LpxE and other lipid A modification enzymes in *R. etli and R. leguminosarum*.** The structure of the conserved intermediate Kdo_2-lipid IV_A is shown in Fig. 2. The evidence for the existence and orientation of these enzymes and transporters is reviewed elsewhere (2). The color scheme is as follows: glucosamine, *blue*; Kdo, *white*; galacturonic acid, *cyan*; aminogluconate, *magenta*; phosphate groups, *red*; fatty acyl chains, *green*; enzymes, *red letters*; proposed transport proteins, *black letters*. The active sites of both LpxE (the 1-phosphatase) and LpxF (the 4′-phosphatase) face the periplasm, preventing premature dephosphorylation of key precursors such as lipid IV_A.

lipid A must first be removed (Fig. 11). These observations suggest that there might be a cryptic *A. tumefaciens* 1-phosphatase that is not expressed during growth on nutrient broth or is not active under our assay conditions.

The biosynthesis of lipid A in *R. leguminosarum* and *R. etli* diverges from that of other Gram-negative bacteria after the formation of the conserved intermediate Kdo_2-lipid IV_A (28–30, 64–67) (Figs. 2 and 11). An important generalization is that all lipid A modification enzymes unique to *Rhizobium* are located outside of the cytoplasmic compartment (2). The active sites of LpxE and LpxF (the 4′-phosphatase) face the periplasmic surface of the inner membrane (Fig. 11), and both are therefore MsbA-dependent (32, 33). Following the removal of the 4′-phosphate moiety, galacturonic acid is incorporated at position 4′ by RgtD, which is thought to use undecaprenyl-phosphate galacturonic acid as its donor substrate (Fig. 11) (66, 67). In addition, galacturonic acid residues are added to the outer Kdo unit of nascent *R. leguminosarum* LPS by the enzymes RgtA and -B (66, 67), which likewise use undecaprenyl-phosphate galacturonic acid as their sugar donor (Fig. 11). Once transported to the outer membrane, the oxidase LpxQ (64, 65) can convert the 1-dephosphorylated proximal glucosamine residue generated by LpxE to 2-aminogluconate in an oxygen-dependent reaction (Fig. 11, *magenta oval*). The outer membrane deacylase PagL can remove the ester-linked hydroxyacyl chain at position 3 (68). When the *lpxE* gene is inactivated in *R. etli*, the cells remain viable, but no 2-aminogluconate is synthesized, and the 1-position of lipid A retains its phosphate group.[7] These findings validate the physiological relevance of LpxE as the sole lipid A 1-phosphatase in *Rhizobium* and strongly support the scheme shown in Fig. 11.

What is the function of LpxE? Many studies have confirmed the importance of the phosphate groups of lipid A for its activity in the stimulation of mammalian immune cells (16, 19). Lipid A derivatives lacking the 1-phosphate group are potent, but nontoxic, partial agonists of the innate immunity receptor TLR4-MD-2 (16, 17, 19). Lipid A variants lacking the 1-phosphate group are currently in clinical trials as vaccine adjuvants (19, 69, 70). Some plants have also recently been shown to possess distinct systems of innate immunity (71, 72). The unusual lipid A of *R. leguminosarum* and *R. etli* might help bacteroids evade the innate immune response of plants during symbiosis in root cells, while allowing the plant to defend itself against Gram-negative pathogens that contain the more common bis-phosphorylated lipid A moiety. Failure to remove the 1-phosphate group could also enhance the sensitivity of *R. etli* and *R. leguminosarum* to endogenous cationic anti-microbial peptides. Characterization of the recently isolated *R. etli* mutants lacking *lpxE*[7] should shed light on these issues.

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