Expression Cloning and Biochemical Characterization of a Rhizobium leguminosarum Lipid A 1-Phosphatase*

Received for publication, June 3, 2003, and in revised form, July 8, 2003
Published, JBC Papers in Press, July 16, 2003, DOI 10.1074/jbc.M305830200

Mark J. Karbarz‡§, Suzanne R. Kalb¶, Robert J. Cotter¶, and Christian R. H. Raetz‡¶
From the §Department of Biochemistry, Duke University Medical Center, Durham, North Carolina 27710 and the ¶Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Lipid A of Rhizobium leguminosarum, a nitrogen-fixing plant endosymbiont, displays several significant structural differences when compared with Escherichia coli. An especially striking feature of R. leguminosarum lipid A is that it lacks both the 1- and 4'-phosphate groups. Distinct lipid A phosphatases that attack either the 1 or the 4' positions have previously been identified in extracts of R. leguminosarum and Rhizobium etli but not Sinorhizobium meliloti or E. coli. Here we describe the identification of a hybrid cosmid (pMJK-1) containing a 23-kb R. leguminosarum 3841 DNA insert that directs the overexpression of the lipid A 1-phosphatase. Transfer of pMJK-1 into S. meliloti 1021 results in heterologous expression of 1-phosphatase activity, which is normally absent in extracts of strain 1021, and confers resistance to polymyxin. Sequencing of a 7-kb DNA fragment derived from the insert of pMJK-1 revealed the presence of a lipid phosphatase ortholog (designated LpxE). Expression of lpxE in E. coli behind the T7lac promoter results in the appearance of robust 1-phosphatase activity, which is normally absent in E. coli membranes. Matrix-assisted laser-desorption/time of flight and radiochemical analysis of the product generated in vitro from the model substrate lipid IVA confirms the selective removal of the 1-phosphate group. These findings show that lpxE is the structural gene for the 1-phosphatase. The availability of lpxE may facilitate the re-engineering of lipid A structures in diverse Gram-negative bacteria and allow assessment of the role of the 1-phosphatase in R. leguminosarum symbiosis with plants. Possible orthologs of LpxE are present in some intracellular human pathogens, including Francisella tularensis, Brucella melitensis, and Legionella pneumophila.

Lipopolysaccharide (LPS) is a macromolecular glycolipid found in the outer membranes of Gram-negative bacteria (1–4). The structure of LPS consists of three domains: the lipid A moiety that serves as the hydrophobic anchor, a nonrepeating core oligosaccharide, and a highly immunogenic, distal O-antigen polysaccharide (1–4). LPS acts as an efficient barrier to antibiotics (5, 6) and helps bacterial cells resist complement-mediated lysis (7). Lipid A (endotoxin) is essential for viability in almost all Gram-negative bacteria (3, 8, 9), and it is the active component of LPS that is responsible for some of the effects associated with severe Gram-negative infections and septic shock (4, 10–12).

The structure of lipid A in common Gram-negative animal pathogens, such as Escherichia coli, Salmonella typhimurium, or Pseudomonas aeruginosa, can vary slightly, but most of its distinguishing structural features are conserved (1, 3, 13). In contrast, the lipid A from the nitrogen-fixing Gram-negative endosymbionts, Rhizobium leguminosarum and Rhizobium etli CE3, is strikingly different (14–17). R. leguminosarum and R. etli lipid A species lack the usual 1- and 4'-phosphate groups (14–17), and a galacturonic acid residue is present in place of the 4'-phosphate moiety (14–17). The proximal glucosamine 1-phosphate unit may be replaced with 2-amino glucuronate (14–17). R. leguminosarum lipid A also lacks the secondary laurate and myristate residues present in E. coli lipid A (18–21) but instead is acylated with a secondary 27-hydroxyoctacosanoate chain (16, 17, 22) (see Fig. 1).

Recent pharmacological studies have shown that both phosphate groups and the correct number of fatty acyl chains are crucial for the cytokine-inducing activities of lipid A molecules in animal systems, reflecting the selectivity of the TLRs of the host (10, 23–27). These signaling proteins recognize conserved surface components present in pathogens, like the lipid A moiety of LPS, which specifically activates TLR-4 (12, 28–30). Engagement of the TLR receptors directly stimulates the mammalian innate immune system (12, 30–32). The TLR proteins are characterized by the presence of leucine-rich repeats in their extracellular domains (12, 30–32), which may be responsible for ligand binding in conjunction with other accessory proteins (33).

In the past year, a plant receptor-like kinase has been identified that is distantly related to the TLR family (34, 35) and is required for symbiotic nodule development in legumes. Considering the functions of the TLR receptors in animals (12, 30–32), a carbohydrate-based mechanism for recognizing microbes in plants, inducing either a symbiotic or a pathogenic response, might be the underlying basis for nodule accommodation and development (36). Given that R. leguminosarum lipid A (16, 17) lacks all of the structural features thought to be necessary for the stimulation of the innate immune system in animals (2, 10), it is conceivable that the unique structural features of R. leguminosarum lipid A might somehow play a role during symbiotic nodule formation.

In spite of the structural diversity of their lipid A molecules,
both E. coli and R. leguminosarum use the same seven enzymes to generate the conserved, phosphate containing precursor, Kdo₂-lipid IVₐ (37). Several additional enzymes exist in R. leguminosarum that catalyze the further conversion of Kdo₂-lipid IVₐ to R. leguminosarum lipid A. We have previously identified a 4'-phosphatase that catalyzes the further conversion of Kdo₂-lipid IVₐ in extracts of R. leguminosarum but not in E. coli. Recently, we have also discovered a novel oxidase that acts on 1-dephosphorylated lipid A species to generate the conserved, phosphate containing precursor, which includes lpxE (48).

### Experimental Procedures

**Chemicals and Materials**—[γ-³²P]ATP was obtained from Perkin-Elmer Life Sciences. Silica gel 60 thin layer plates (0.25 mm) were purchased from EM Separation Technology (Merrick), Trifton X-100 and bicinechonic acid were from Pierce. Yeast extract and tryptone were purchased from Difco. All other chemicals were reagent grade and were purchased from either Sigma or Mallinckrodt.

**Bacterial Strains and Growth Conditions**—R. etli CE3, R. leguminosarum 3841, and S. meliloti 1021 (49) were grown as described previously (37, 40, and 49), and their properties are summarized in Table I.

| Strains | Genotype or description | Source or reference |
|---------|-------------------------|-------------------|
| R. etli CE3 | CFN42 Sm’ | Refs. 46 and 47 |
| R. leguminosarum 3841 | Wild type strain 300 biobar viciae Sm’ | Refs. 46 and 47 |
| S. meliloti 1021 | SU47 Sm’ | Sharon Long (49) |
| E. coli HB101 | hadS20 supE44 ara14 galK2 lacY1 proA2 rpsL20 | Invitrogen |
| XLI-Blue | recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lacF proAB lacZDMD5 Tn10 (Tet’) | Stratagene |
| MTK16 | pRK2013 Cm’ Km::Tn5 containing strain for tri-parental mating | Refs. 46 and 47 |
| Novablaue (DE3) | E. coli host strain used for expression | Novagen |
| Plasmids | | |
| pLAFR-1 | Broad host range P-group cloning vector, mobilizable RK2 cosmid Tet’ | Ref. 69 |
| pPK404a | Shuttle vector Tet’ | Ref. 65 |
| pET-28a | E. coli expression vector | Novagen |
| pMKJ-1 | pLAFR-1 derivative carrying a 25-kb fragment of R. leguminosarum 3841 = genomic DNA, which includes lpxE | This work |
| pLPXE-2 | pRK404a derivative carrying a 6.9-kb EcoRI fragment, which includes lpXE from pMKJ-1 | This work |
| pLPXE-3 | pRK404a derivative carrying a 4.9-kb HindIII fragment, which includes lpXE from pMKJ-1 | This work |
| pLPXE-4 | pET-28a derivative harboring lpxE behind the T1lac promoter | This work |

**Preparation of Cell-free Extracts and Washed Membranes**—All of the enzyme preparations were carried out at 0–4°C. Protein concentration was determined by the bicinchoninic acid method (51) using bovine serum albumin as a standard (Pierce). Cell-free extracts, cytosol, and washed membranes were prepared as described previously (40) and stored in aliquots at −80°C.

**Preparation of Radiolabeled Substrates**—The [4'-³²P]lipid IVₐ was prepared from [γ-³²P]ATP and the appropriate tetraacyl-disaccharide 1-phosphate acceptor, using the overexpressed 4'-kinase present in membranes of E. coli BLR(DE3)pLysS/pPJK2 (52, 53). The Kdo₂[4'-³²P]lipid IVₐ was then prepared from the [4'-³²P]lipid IVₐ by the action of the purified Kdo₂ lpxE gene (KdelA) (53, 54). The Kdo₂[4'-³²P]lipid IVₐ and [4'-³²P]lpxE were purified by preparative thin layer chromatography (63–55) and were stored as an aqueous dispersion at −20°C in 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Prior to use, all of the lipid substrates were dispersed by sonic irradiation for 1 min in a bath sonicator.

The substrate [³²P]labeled lipid X was prepared from [³²P]labeled E. coli strain MNT (56, 57), as previously described (58, 59). Tetraacetyl-disaccharide-1-phosphate was prepared from [³²P]labeled X and UDP-2,3-diacyl-glucoamine using a highly purified preparation of the E. coli LpxB disaccharide synthase (59). Nonradioactive tetraacetyl-disaccharide 1-phosphate carrier was prepared in the same way (59).

**Assay of the 1-Phosphatase**—Standard assay conditions for the 1-phosphatase activity are as follows. The reaction mixture (10 μl) contained 50 mM MES, pH 6.5, 1% Triton X-100, 10 mM NaCl, 2 mM diethiothreitol, and 5 μM Kdo₂[4'-³²P]lipid IVₐ (3000–6000 cpm/nmol). Protein concentration was adjusted as described below. Dephosphorylation reactions were performed at 30°C for 20 min or as indicated. The reactions were terminated by spotting 4-μl samples onto silica gel 60 TLC plates and dried under a cool air stream for 30 min. The TLC plates were developed in the solvent chloroform, pyridine, 88% formic acid, water (30:70:16:10 v/v/v/v). After drying and overnight exposure of the plate to a PhosphorImager screen, product formation was detected and quantified with a Molecular Dynamics Storm PhosphorImager equipped with ImageQuant software.

**Transfer of a R. leguminosarum 3841 Library into R. etli CE3**—A library of R. leguminosarum 3841 genomic DNA (~20–25 kb) (45), inserted into the cosmid pLAFR-1 (40) and harbored in E. coli 803 (61),
was provided by Dr. J. Downie of the John Innes Institute (Norwich, UK). Because R. leguminosarum promoters are not usually transcribed by E. coli RNA polymerase, colony lysates of the E. coli host cannot be assayed directly for production of 1-phosphatase. Accordingly, the entire library was transferred by tri-parental mating into R. etli CE3 (62), as described previously (44). E. coli strain 803 harboring the cosmid library served as the cosmid donor, and the helper strain E. coli MT616 (63) provided the transfer functions (Table 1).

**Screening of the R. leguminosarum 3841 Library for 1-Phosphatase Activity**—For screening, the glycerol stock of the mating mixture was thawed, appropriately diluted to obtain 50–100 colonies/plate, and spread onto TY agar containing 10 mM CaCl2, naldixic acid (20 μg/ml), streptomycin (200 μg/ml), and tetracycline (12.5 μg/ml) to ensure selection of R. etli CE3 cells harboring a hybrid cosmid. The plates were incubated for 40 h at 30°C. Individual colonies were picked with a sterile toothpick and inoculated into separate wells of a 96-well microtiter plate containing 150 μl of TY broth supplemented with 10 mM CaCl2, naldixic acid (20 μg/ml), streptomycin (200 μg/ml), and tetracycline (12.5 μg/ml). Each microtiter plate was incubated at 30°C with constant shaking for 40 h. To ensure consistency, growth was monitored until the A\text{600} was greater than 0.5. Next, 50 μl from each well was transferred to another microtiter plate, adjusted to 20% glycerol, and stored as a stock at −80°C for later use. The remaining 100 μl of cells were harvested by centrifugation at 3660 × g for 20 min at 4°C. The supernatant was decanted, and the cell pellet was re-suspended and washed with 50 μl of 50 mM Hepes, pH 7.5, and the washed cells were harvested by centrifugation at 3660 × g for 20 min at 4°C. The cells were resuspended and incubated with lysosome (1 mg/ml) and EDTA (2 mM) at 30°C for 20 min, followed by a single freeze-thaw cycle.

**Assay of the Pooled R. leguminosarum 3841 Library Lysates for 1-Phosphatase Activity**—Four μl from each well of two microtiter plates were pooled into a single new microtiter dish (8 μl/well). The pooled cell lysates were assayed for their ability to metabolize Kdo₂-[4-32P]lipid IV A, as follows. A 96-well microtiter plate was prepared with each well containing 2 μl of 250 mM MES buffer, pH 6.5, 0.5% Triton X-100, 10 mM EDTA, and 1.0 μM Kdo₂-[4-32P]lipid IV A (−1000 cpm/well), to which 5 μl of pooled cell lysate was added. Each plate was then incubated at 30°C, and the reactions were terminated at the 10- and 20-min time points by spotting 5 μl portions onto a silica gel TLC plate with a multi-channel pipette. On each TLC plate, parallel reactions without enzyme or with R. leguminosarum CE3 crude cell lysate were also spotted as controls. The spots were dried and visualized under a UV box and then quantified with a scanner. To visualize phosphatase activity, the agarose in the ligase reaction mixtures was melted at 70°C, was further subjected to shrimp alkaline phosphatase treatment for 1 h at 37°C, and the reaction was heat-inactivated at 65°C for 20 min.

Next, the samples were resolved on a 0.7% Seaplaque low melting agarose gel at 20–25 volts at 4°C overnight. The bands were visualized with a “Dark Reader,” ensuring that no UV light (260 nm) was introduced. One EcoRI (7 kb) and one HindIII (4.9 kb) fragment derived from pMKJ-1 were selected for ligation into the multiple cloning cassette of pRK404a (65). The desired bands were excised from the gel using a razor blade and carefully placed into conical plastic microcentrifuge tubes.

For ligation of selected DNA fragments derived from pMKJ-1 with digested pRK404a, agarose plugs were melted at 70°C for 10 min without denaturing the DNA. Melted gel slices were then combined as indicated and held at 37°C, which keeps the agarose from solidifying. Typically, the melted agarose preparations (containing the vector or insert) were mixed in a 3:2 ratio, and water was added to bring the volume to 9 μl. Next, 1 μl of 2× concentrated ligation buffer (consisting of 30 μl of 10× ligation buffer (500 mM Tris-HCl, pH 7.5, 100 mM MgCl2, 100 mM dithiothreitol, 10 mM ATP, and 250 μg/ml bovine serum albumin), 20 μl of 400 units/μl T4 DNA ligase, and 10 μl of H2O) was added into each μl DNA mixture. The reactions were incubated at 16°C overnight.

The agarase in the ligase reaction mixtures were melted at 70°C for 10 min before transformation into appropriate host strains. Typically, a 5–7 μl portion of each ligation mixture was added to a 50-μl aliquot of HB101 competent cells (Invitrogen), transformed, and plated on LB agar containing tetracycline (12.5 μg/ml). Resistant colonies were screened for the desired insert by digestion with either EcoRI or HindIII. Subclones were designated pLPx-2 (containing the 7-kb insert) or pLPx-3 (containing the 4.9-kb insert), respectively.

Next, pLPx-2 and pLPx-3 were transferred into R. etli CE3 and S. meliloti 1021 by tri-parental mating. R. etli CE3 cells harboring pLPx-2 and pLPx-3 were plated on TY agar containing 10 mM CaCl2, naldixic acid (20 μg/ml), streptomycin (200 μg/ml), and tetracycline (12.5 μg/ml), whereas S. meliloti 1021 cells harboring pLPx-2 and pLPx-3 were selected on TY plates containing 10 mM CaCl2, naldixic acid (20 μg/ml), streptomycin (200 μg/ml), and tetracycline (12.5 μg/ml). The cell lysates were prepared as described above and analyzed for 1-phosphatase activity.

The DNA inserts of pLPx-2 and pLPx-3 were purified with the Qiagen Plasmid Maxi-prep kit. DNA sequencing of both strands was done at Duke University using the Terminator Cycle sequencing system with AmpliTaq DNA polymerase and an ABI 377 PRISM DNA sequencing instrument.

**Expression of the 1-Phosphatase Gene behind the T1lac Promoter—**PCR-amplified lpxE DNA was cloned into the pET-28a vector behind the T1lac promoter. The forward PCR primer (5'-ACCTTAGAGCTCAT-GGCGCGATTATTGG-3') was designed with a clamp region, a Sec I restriction site (underlined), and a match of the coding strand starting at the translation initiation site. The reverse primer (5'-ACTAGTCAGT- TATGCAGTGCGGAAC-3') was designed with a clamp region, a Xho I restriction site (underlined), and a match to the anti-coding strand that included the stop codon. The PCR was performed using Ffu polymerase. The hybrid cosmid pMKJ-1 was used as the template. Amplification was carried out in a 100-μl reaction mixture containing 100 ng of template, 20 μl Tris-HCl, pH 8.8, 10 mM KCl, 100 μM dNTPs, 0.1% Triton X-100, 0.1% bovine serum albumin, 100 ng of pET-28a, and 1000 units of Ffu polymerase. The reaction was subjected to a hot start (1 min at 94°C) followed by 25 cycles of 25°C for 10 min of cycles, annealing for 1 min at 94°C, annealing (1 min at 55°C), and extension (2 min at 72°C). After the 25th cycle, a 10-min extension time was used. The reaction product was analyzed on a 1% agarose gel. The desired band was excised and gel-purified using a Qiagen gel extraction kit. The purified PCR product was digested using ScaI and Xhol and ligated into the expression vector, pET-28a, that had been similarly digested. Ligase reactions were transformed into XL1-Blue cells (Stratagene) and screened for positive inserts. The desired construct was named pLPx-4. The lpxE insert was confirmed by DNA sequencing. The plasmid pLPx-4 was transformed into the E. coli expression strain N101 (43).

**Preparation of Membrane Fractions for SDS-PAGE Analysis**—In the handling of membrane fractions, it was found that incubation of the sample in SDS at temperatures above 50°C prior to electrophoresis led to LpxE aggregation in the stacking gel, a common phenomenon with membrane proteins (66). Consequently, the samples were prepared for SDS-PAGE analysis by incubation of a 4°C cold solution, consisting of 200 mM Tris, pH 6.8, 50% glycerol, 12% SDS, 200 mM dithiothreitol, and 0.02% bromphenol blue (66). Incubation for 30 min at 40°C was optimal for LpxE membrane protein solubilization, and the samples were analyzed using 12% SDS-polyacrylamide gels.

**Purification of the 1-Dephosphorylated Reaction Product Generated In Vitro from Lipid IVA**—To increase the efficiency of subcloning, a low temperature ligation protocol was used. The LpxE Lipid A 1-Phosphatase of R. leguminosarum

### References

1. MT 616 (63) provided the transfer functions.
2. R. etli CE3 provided the transfer functions.
3. E. coli strains 803 harboring the cosmid library served as the cosmid donor, and the helper strain E. coli MT616 (63) provided the transfer functions.
4. The library was transferred by tri-parental mating into R. etli CE3 (62), as described previously (44).
5. E. coli strains 803 harboring the cosmid library served as the cosmid donor, and the helper strain E. coli MT616 (63) provided the transfer functions.
6. R. etli CE3 or S. meliloti 1021 were used as host cells in the tri-parental mating.
7. The library served as the cosmid donor, and the helper strain E. coli MT616 (63) provided the transfer functions.
8. The library was transferred by tri-parental mating into R. etli CE3 (62), as described previously (44).
9. E. coli strains 803 harboring the cosmid library served as the cosmid donor, and the helper strain E. coli MT616 (63) provided the transfer functions.
10. The library was transferred by tri-parental mating into R. etli CE3 (62), as described previously (44).
protein concentration was 1 mg/ml. The mixtures were incubated at 30°C for 18 h. Product formation was monitored by TLC, as described above for the assays, but the 1-phosphatase reaction product was detected by charring the plates after spraying with 10% sulfuric acid in ethanol. For product isolation, the aqueous reaction mixture was converted to a single-phase Bligh/Dyer mixture (67), consisting of chloroform/methanol/water (1:2:0.8, v/v/v). The sample was thoroughly mixed and centrifuged at 3,000 x g for 20 min at room temperature to remove precipitated proteins. The supernatant was then converted to a two-phase Bligh/Dyer mixture, consisting of chloroform/methanol/water (2:2:1.8, v/v/v) by the addition of appropriate amounts of chloroform and water. After mixing, the phases were separated by centrifugation. The lower phase was recovered and dried by rotary evaporation. The sample was redissolved in ~5 ml of chloroform/methanol/H₂O (2:3:1, v/v/v) and loaded onto a 0.5-ml DEAE-cellulose column (Whatman DE-52), equilibrated as the acetate form in the same solvent mixture (68, 69). The column was washed with the same solvent mixture and then eluted with 5-ml portions of chloroform/methanol/aqueous ammonium acetate (2:3:1, v/v/v), in which the ammonium acetate concentration in the aqueous component was increased stepwise from 60, 120, 240, to 480 mM. The elution of the substrate and product was monitored by spotting 5 μl of each fraction onto a silica gel TLC plate, which was developed in the chloroform, pyridine, 88% formic acid, H₂O (50:50:16:10, v/v/v/v). After drying, the lipids were detected by charring as described above. Fractions containing 1-dephosphorylated lipid IVₐ eluted with the 60 mM ammonium acetate component, whereas the remaining substrate eluted with 240 mM. To recover the product, the fractions containing the 1-dephosphorylated lipid IVₐ were pooled and were converted to a
two-phase Bligh-Dyer mixture. The lower phases were dried under a stream of N₂ and stored at −20 °C until further analysis.

MALDI/TOF Analysis of 1-Dephosphorylated Lipid IVₐ—Spectra were acquired in the positive and negative-ion linear modes using a Kratos Analytical (Manchester, UK) MALDI/TOF mass spectrometer, as described previously (43, 44).

Assay of Polymyxin Sensitivity—The outer membrane integrity of R. etli or S. meliloti cells harboring either control or pLpxE expressing plasmids was evaluated by examining their sensitivity to the cationic antibacterial peptide polymyxin B sulfate (Sigma). The assay was performed by placing 6-mm filter discs containing varying amounts of polymyxin (2, 10, or 20 µg) onto a lawn of cells that had been freshly plated onto TY agar with a saturated cotton swab from a culture at A₅₆₀ of ~0.2. After 40 h of growth at 30 °C, the diameters of the zones of clearing around each disc (itself 6 mm in diameter) were measured, providing an assessment of the relative polymyxin sensitivity.

RESULTS

Screening of a R. leguminosarum Genomic DNA Library for Clones Overexpressing Lipid A Phosphatase Activity—Previous attempts to clone the R. leguminosarum 1- and/or 4'-phosphatases by assaying ~4000 clones of a R. leguminosarum 3841 cosmid library harbored in S. meliloti 1021 were unsuccessful (40). The lipid A of Sinorhizobium strains normally contain phosphate groups at the 1 and 4' positions (70, 71), and it may be that the R. leguminosarum lipid A phosphatases are toxic to S. meliloti under some conditions. Consequently, an alternate expression cloning strategy described under “Experimental Procedures” was developed. The entire R. leguminosarum 3841 genomic DNA library (20–25-kb insert size) in the cosmid pLAFR-1, harbored in E. coli S103, was transferred into R. etli CE3 by tri-parental mating. Membranes of R. etli CE3 normally contain 1- and 4'-phosphatase activities, and CE3 lipid A is completely devoid of phosphate residues (14, 16, 17). Expression cloning with R. leguminosarum DNA cannot be carried out directly in E. coli, because E. coli RNA polymerase does not recognize R. leguminosarum promoters.

A clone that overexpresses the 1-phosphatase was identified by assaysing pools of two extracts for their ability to shift E. coli Kdo₂-[4',3₂P]lipid IVₐ (Fig. 1) to a more rapidly migrating species (data not shown). Extract a in pool 692 was the active lyase. Of the ~1200 lysozyme/EDTA lysates screened, only one clone (R. etli CE3/pMJK-1) was identified that overexpressed the putative 1-phosphatase activity. This plasmid was used for further studies. No clones overexpressing the 4'-phosphatase activity were identified.

Verification of 1-Phosphatase Overproduction in Membranes of R. etli CE3/pMJK-1—To confirm the initial screening results obtained with the above lysates, membranes of late log phase cultures of R. etli CE3/pLAFR-1 (the vector control) and R. etli CE3/pMJK-1 were prepared and assayed under conditions optimized for the 1-phosphatase (Fig. 2). In membranes of R. leguminosarum or R. etli CE3, the C28-dependent long chain acyltransferase (22) and the 1-phosphatase (22, 39) are the acyltransferase also requires the soluble factor C28-AcpXL (22, 25-kb insert size) and does not require cytosolic factors (data not shown). The optimal in vitro assay conditions for the activity overexpressed in R. etli CE3/pMJK-1, such as the Triton X-100 optimum of 1%, are very similar to those for the 1-phosphatase present in membranes of wild type R. leguminosarum or R. etli (39). Sucrose density gradient centrifugation revealed exclusive localization of the activity to the inner membranes (data not shown). Similar levels of 4'-phosphatase activity are seen in membranes of R. etli CE3 cells harboring the empty vector control or the hybrid cosmid (pMJK-1) (Fig. 2), consistent with previous biochemical evidence that distinct enzymes catalyze 1- and 4'-dephosphorylation (38, 39).

Subcloning of the pMJK-1 ~25-kb Insert for the Gene Encoding LpxE Activity—Restriction fragments of the ~25-kb R. leguminosarum 3841 genomic DNA insert in pMJK-1 were subcloned into the shuttle vector pRK404a (see “Experimental Procedures”). Plasmids obtained in this manner were individually transferred by tri-parental mating from E. coli host into both R. etli CE3 and S. meliloti 1021. The latter normally lacks 1-phosphatase activity. Only the membranes of S. meliloti 1021 harboring pLpxE-2 or pLpxE-3, containing DNA inserts of ~7 and 4.9 kb, respectively, exhibited high levels of 1-phosphatase activity (Fig. 3). Further analysis of the pLpxE-2-directed phosphatase demonstrated that it is Kdo-independent and membrane-associated (data not shown). As shown in Fig. 4, 1-dephosphorylation of 5 µM Kdo₂-[4',3₂P]lipid IVₐ by 0.5 mg/ml S. meliloti 1021/pLpxE-2 membranes is linear with time for about 1 h at 30 °C and nearly goes to completion after 7 h. The initial rate of dephosphorylation is proportional to membrane protein concentrations up to 1.5 mg/ml (data not shown). About 5–7 fold higher 1-phosphatase specific activity (Table II) is detected in washed membranes of S. meliloti 1021/pMJK-1,
S. meliloti 1021/pLpxE-2, or S. meliloti 1021/pLpxE-3 than wild type R. leguminosarum 3841. The 1-phosphatase activity present in membranes of R. etli CE3 or S. meliloti 1021 containing the indicated hybrid plasmids or vector controls (65) was assayed with 5 μM Kdo₂[4'-32P]lipid IV₄₆ as described under “Experimental Procedures.”

Identification of the Putative lpxE Gene—Sequencing of the DNA inserts in pLpxE-2 and pLpxE-3 revealed the presence of five putative open reading frames (Fig. 5A), which were identified using the programs DNA Strider and ORF Finder (72) (www.ncbi.nlm.nih.gov/gorf/gorf.html). Factors considered in determining the locations of lpxE and other genes on the insert include homology to sequences of known proteins, as well as the presence of possible promoter sequences and ribosomal binding sites. The complete nucleotide sequence relevant to the present paper has been submitted to GenBank™ under accession number AY371492.

Table III lists some of the closest homologs in the nonredundant data base of the polypeptides encoded by the five predicted open reading frames in the ~7-kb insert of pLpxE-2. Of particular interest is the distant, yet highly significant similarity of the predicted product of orf2 to enzymes that dephosphorylate various phospholipids. Orf2, which is predicted to be 244 amino acid residues long (Fig. 5 legend), contains the conserved, tri-partite lipid phosphatase catalytic motif (Fig. 5B) (73), and it is also related to various other acid phosphatases and glucose 6-phosphatases.

Orf2 is predicted to have six transmembrane helices (data not shown). The topography strongly suggests that the residues of the tri-partite active site motif face the periplasmic surface of the inner membrane. The closest Orf2 orthologs (Table IV) are found in Agrobacterium tumefaciens, S. meliloti, Mesorhizobium loti, and Franciscella tularensis. Although not extensively studied, the first three of these bacteria are thought to synthesize fully phosphorylated lipid A molecules (70, 71, 74). However, it may be that their 1-phosphatase orthologs are induced under special growth conditions. F. tularensis has recently been shown to contain lipid A molecules lacking the 1-phosphate moiety (75). We propose that orf2 be named lpxE (from the German eins for 1), given its function as the structural gene of the lipid A 1-phosphatase.

Heterologous Expression of the lpxE Gene in E. coli and Characterization of the Recombinant Enzyme—Unequivocal evidence

| Strain | Specific activity (nmol/min/mg) |
|--------|-------------------------------|
| R. etli CE3 | 0.002 |
| R. leguminosarum 3841 | 0.014 |
| R. etli CE3/pMKJ-1 | 0.098 |
| S. meliloti 1021 | ND |
| S. meliloti 1021/pLAFB-1 | ND |
| S. meliloti 1021/pMKJ-1 | 0.059 |

* ND, no detectable phosphatase activity is present in membranes. The specific activity of the 1-phosphatase was measured using washed membranes from the indicated strains of Rhizobium.
that LpxE encodes the lipid A 1-phosphatase is provided by heterologous expression in E. coli, extracts of which normally have no phosphatase activity against lipid IVA and other lipid A precursors (38, 39). A phosphatase that attacks the 1 position would release $^{32}$Pi from $[1-^{32}$P]lipid IVA.

E. coli membranes prepared from cells containing the vector control pET-28a did not catalyze any dephosphorylation of $[1-^{32}$P]lipid IVA (Fig. 6B). However, E. coli membranes from induced cells containing the plasmid pLpxE-4 rapidly dephosphorylated the 1 position of $[1-^{32}$P]lipid IVA, as judged by the appearance of radiolabeled inorganic phosphate with time (Fig. 6B). The 1-phosphatase in the membranes of E. coli Novablu (DE3)/pLpxE-4 has a pH optimum and Triton X-100 dependence similar to what is observed with the native R. leguminosarum 1-phosphatase (39).

Considering the high apparent level of protein expression (Fig. 6A), the estimated specific activity of the recombinant 1-phosphatase in E. coli Novablu (DE3)/pLpxE-4 membranes seems low at 0.1 nmol/min/mg. The low specific activity may be due in part to incorrect folding or inclusion body formation. Extraction of the 1-phosphatase with Triton X-100 increases its specific activity to about 1.6 nmol/min/mg, suggesting inhibitory substances in the crude membrane fraction.

**Mass Spectrometry of the Product Generated by the Recombinant 1-Phosphatase** — The reaction product generated from

---

**TABLE III**

**Possible functions of genes in the vicinity of LpxE based on similarities to selected gene products**

| Predicted protein (length) | Related protein with characterized function if any (length) | Accession number | Homology* | E value |
|---------------------------|-------------------------------------------------------------|-----------------|-----------|---------|
| Orf1 (628)                | Integral inner membrane metabolite transport protein, MtbA, *Bradyrhizobium japonicum* (555) | AAF78796         | 254/301/343 | $10^{-132}$ |
| LpxE (244)                | Putative phosphatic acid phosphatase, *Lactococcus lactis* (218) | AAC45388         | 43/56/125 | 0.001   |
| Orf3 (370)                | β-Isopropylmalate dehydrogenase, LeuB, *A. tumefaciens* (370) | AAA22089         | 326/344/370 | $10^{-175}$ |
| Orf4 (313)                | l-Serine dehydratase, SdhL, *Rattus norvegicus* (327) | CAA68721         | 144/196/313 | $10^{-57}$ |
| Orf5 (201)                | 3-Isopropylmalate dehydratase small subunit, LeuD, *Azotobacter vinelandii* (215) | CAA722150        | 107/135/201 | $2 \times 10^{-53}$ |

* Homology is given as the number of identities/number of positives/number of residues (including gaps) in the related segment. The gapped Blastp algorithm (86) was used to identify possible orthologs using the predicted *R. leguminosarum* proteins as the probe.
are dephosphorylated at the 1 and/or 4 positions. Porphyromonas gingivalis (79), contain lipid A moieties that are unique to this organism and provide definitive evidence that lpxE is especially effective (Fig. 6 and Table II).

Expression of lpxE in S. meliloti 1021 Renders Cells Polymyxin-resistant—The lipid A of S. meliloti 1021 differs from that of R. etli and R. leguminosarum in that it is fully phosphorylated at the 1 and 4' positions when grown under laboratory conditions (16, 17). Interestingly, S. meliloti 1021 is somewhat more sensitive to polymyxin at 10 or 20 μg (Table V) than is R. etli CE3 (Table V) or R. leguminosarum (not shown). To determine whether or not dephosphorylation at the 1 position might be playing a role, we tested the polymyxin sensitivity of S. meliloti 1021 harboring recombinant lpxE or the appropriate vector control (Table V). There was a significant decrease in the sensitivity to polymyxin when lpxE was expressed in S. meliloti, but not in R. etli, as judged by disc diffusion assays (Table V). Presumably, the reduced negative charge of the resulting 1-dephosphorylated lipid A molecules in S. meliloti harboring lpxE lowers the affinity of the outer membrane for polymyxin, preventing the drug from reaching the inner membrane and killing the cells (76).

**TABLE IV**

| Organism           | Homology (Gaps) | Approximate E values |
|--------------------|-----------------|----------------------|
| A. tumefaciens     | 84/125/214 (4)  | 5 × 10⁻³⁰            |
| S. meliloti        | 87/123/222 (2)  | 6 × 10⁻³⁰            |
| M. morganii        | 83/108/223      | 8 × 10⁻²⁶            |
| F. tularensis      | 51/65/112 (1)   | 1 × 10⁻¹¹            |
| Legionella pneumophila | 55/72/149       | 3 × 10⁻¹⁰            |
| Magnetospirillum magnetotacticum | 69/101/209 (18) | 4 × 10⁻¹⁴           |
| Brucella melitensis | 40/61/119       | 4 × 10⁻¹¹            |

Homology is given as the number of identities/number of positives/number of residues (including gaps) in the related segment when compared with R. leguminosarum LpxE, a hypothetical protein of 244 amino acid residues.

There are two separately sequenced A. tumefaciens strains. This is the strain sequenced by the University of Washington (88). The strain sequenced by the Cereon group contains the same ortholog (87).

This organism contains an LpxE ortholog and a cluster of genes similar to that present in R. leguminosarum 3841.

The lipopolysaccharides of some Gram-negative bacteria, including R. etli (14–17), R. leguminosarum (14–17), F. tularensis (75), Aquifex pyrophilus (77), Helicobacter pylori (78), and Porphyromonas gingivalis (79), contain lipid A moieties that are dephosphorylated at the 1 and/or 4' positions. In R. etli and R. leguminosarum, the structures of such phosphate-deficient lipid A molecules are well characterized (Fig. 1) (16, 17). Enzymatic studies with R. etli and R. leguminosarum membranes have also demonstrated the presence of distinct 1- and 4'-phosphatases in these organisms (38, 39), supporting the proposed pathway shown in Fig. 1. R. etli and R. leguminosarum first synthesize a lipid A structure resembling that of E. coli, but then modify the resulting substance further with additional enzymes not present in E. coli. Many of the enzymes that are unique to R. etli and R. leguminosarum may be located on the outer surface of the inner membrane, as suggested by the sequence of LpxE (Fig. 5 legend) or in the outer membrane as previously shown for LpxQ (Fig. 1) (43, 44).

We now report the expression cloning and characterization of a gene, designated lpxE, that encodes the lipid A 1-phosphatase of R. leguminosarum. This enzyme normally is not present in extracts of S. meliloti or E. coli (39). However, both S. meliloti 2011 and E. coli can express the R. leguminosarum 1-phosphatase when lpxE is introduced on an appropriate vector (Figs. 3, 6, and 7). Heterologous expression of lpxE behind the Tetlac promoter in E. coli is especially effective (Fig. 6 and Table II) and provides definitive evidence that lpxE is the structural gene for the 1-phosphatase.

An examination of the COG data base (72) shows that three distant orthologs of LpxE are present in E. coli, which include the phosphatidylglycerol-phosphate phosphatase PgpB (80, 81), a putative additional phosphatidylglycerol-phosphate phosphatase that can be assayed in mutants lacking pgpB (82), and the as yet uncharacterized undecaprenyl-diphosphate transferases (83).
phosphatase involved in peptidoglycan assembly (83, 84). Like PgpB and many of the eucaryotic phosphatases that act on glycerophospholipids (73, 81, 85), the *R. leguminosarum* lipid A 1-phosphatase is membrane-bound and requires detergent for activity. Compared with *E. coli* PgpB or the periplasmic alkaline phosphatase, the lipid A 1-phosphatase is distinct and highly selective for the 1 position of lipid A (Figs. 6 and 7).

Sequence comparisons using PSI-BLAST (86) indicate that LpxE shares key conserved active site residues with PgpB and related lipid phosphatases (Fig. 5B), but this limited similarity likely reflects a shared catalytic mechanism and not substrate specificity. The putative catalytic residues of LpxE (Fig. 5B) are predicted to face the periplasmic surface of the inner membrane (see www.cbs.dtu.dk/services/TMHMM-2.0/). LpxE must now be purified to homogeneity, characterized with regard to lipid substrate specificity and topography, and subjected to site-directed mutagenesis. However, the remarkable selectivity of LpxE for the 1 position of Kdo₂-lipid IV₅ or lipid IV₅A (Figs. 3, 6, and 7) argues against the idea that this enzyme is a nonspecific lipid phosphatase. Conversely, when commercially available, soluble phosphatases from calf intestine or shrimp were tested under the conditions optimized for the *R. leguminosarum* 1-phosphatase, no dephosphorylation of Kdo₂-[⁴⁻³²P]lipid IV₅A was observed (data not shown).

The presence of closely related LpxE orthologs in *A. tumefaciens* (87, 88), *M. loti* (89), and *S. meliloti* (49) (Table IV) is

---

**Table V**

| Strain                  | Zone of clearing diameter | 2 μg of polymyxin | 10 μg of polymyxin | 20 μg of polymyxin |
|-------------------------|----------------------------|-------------------|-------------------|-------------------|
| *R. etli* CE3/pRK404a   | 8                          | 12.5              | 14                |
| *R. etli* CE3/pLpxE-2   | 10                         | 13                | 15                |
| *R. etli* CE3/pLpxE-3   | 10                         | 13                | 15                |
| *S. meliloti* 1021/pRK404a | 9                         | 17                | 18                |
| *S. meliloti* 1021/pLpxE-2 | <6                        | <6                | 7                 |
| *S. meliloti* 1021/pLpxE-3 | <6                        | <6                | 7                 |
unexpected and especially intriguing. Limited available structural information suggests the presence of phosphate-containing lipid A molecules in these organisms (70, 71, 74). Under our standard assay conditions there is no endogenous 1-phosphatase activity in membranes of S. meliloti (44). This variant is fully functional as a lipid A oxidase when expressed in E. coli (44). Considering the confirmed function of A. tumefaciens LpxQ as a lipid A oxidase, it seems probable that the A. tumefaciens LpxE ortholog (Table IV) functions as the lipid A 1-phosphatase required for generating the LpxQ substrate (Fig. 1), although this remains to be demonstrated experimentally. The structural analysis of A. tumefaciens lipid A and a systematic search for an induced lipid A 1-phosphatase under special growth conditions might reveal how the LpxQ enzyme and LpxQ orthologs of these organisms are regulated. Enteric Gram-negative bacteria detect changes in environmental conditions using two-component regulatory systems (90). Although no two-component systems in R. leguminosarum or S. meliloti are known to be involved in the modification of lipid A, this possibility cannot be excluded. There is in fact evidence that the LPS of R. leguminosarum 3841 is altered under the conditions that would be encountered during symbiosis (91, 92). When comparing LPS of R. leguminosarum 3841 isolated from pea bacteroids to LPS from strains grown in the laboratory, distinct epitopes are observed by immunochromatographic methods (91–94). These epitopes can be induced ex planta by growth at low pH and/or at low oxygen concentrations (95, 96), indicating that LPS may be modified in a regulated manner. However, lipid A structure was not examined in these studies (95, 96). Various covalent modifications of lipid A, induced by growth on low magnesium ion concentrations or low pH via the PhoQ/P or the PmrA/B two-component regulatory systems, respectively, are known to occur in E. coli (96) (Table IV; Fig. 1), 980–982).

Many chemical studies have confirmed the importance of the phosphate groups of lipid A for stimulating mammalian immune cells (10). Although not yet well characterized in terms of their ability to respond to lipid A, plants have recently been shown to possess systems of innate immunity (105, 106) and to use lipid A as a ligand for pattern recognition. Recent studies with related preparations from Rhizobium sp. sin-1 suggest that some of these substances might even be novel LPS antagonists (107). Current methods to prepare 1-dephosphorylated lipid A species from bacterial sources or from enzymatically synthesized molecules require acid hydrolysis at 100 °C (43). This treatment is non-specific in that it also cleaves the labile Kdo linkages in compounds like Kdo2-lipid IVα (110–112). Consequently, the availability of LpxE will enable the preparation of novel molecules, such as 1-dephospho-Kdo2-lipid IVα, or 1-dephospho-Kdo2-lipid A, starting with Kdo2-lipid IVα (Fig. 1) or Kdo2-lipid A, which are readily accessible (54, 55, 113).

The expression of the lpxE gene behind the T7lac promoter in E. coli (Table II) will facilitate the purification of large amounts of the 1-phosphatase. With the pure enzyme, it will be possible to evaluate the functions of the conserved residues in the phosphatase motif (Fig. 5) and to examine the substrate selectivity of LpxE toward various lipids. It will also be possible to use LpxE to re-engineer lipid A structure in living Gram-negative bacteria and to investigate the effects of such structural modifications on outer membrane assembly, symbiosis, and pathogenesis. The induction of polymyxin resistance in S. meliloti by heterologous expression of R. leguminosarum lpxE (Table V) suggests that 1-dephosphorylation of lipid A can have important physiological consequences.

Acknowledgments—We thank Dr. J. A. Downie (John Innes Institute, University of East Anglia) for providing the R. leguminosarum genomic DNA cosmid library. We also thank Dr. D. Borthakur (University of Hawaii) for the pRK404a plasmid shuttle vector. We thank Drs. Shib Sankar Basu and M. Stephen Trent for help with the preparation of this manuscript.

REFERENCES

1. Raetz, C. R. H. (1990) Annu. Rev. Biochem. 59, 129–170
2. Raetz, C. R. H. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 1035–1063, American Society for Microbiology, Washington, D.C.
3. Raetz, C. R. H., and Whitfield, C. (2002) Annu. Rev. Biochem. 71, 635–700
4. Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C. (eds) (1999) Endotoxin in Health and Disease, Marcel Dekker, Inc., New York
5. Nikaido, H. (1996) in Escherichia coli and Salmonella: Cellular and Molecular Biology (Neidhardt, F. C., ed) Vol. 1, 2nd Ed., pp. 29–47, American Society for Microbiology, Washington, D.C.
6. Vaara, M. (1993) Antimicrob. Agents Chemother. 37, 2255–2260
7. Roantree, J. R. (1967) Annu. Rev. Microbiol. 21, 443–466
8. Garrow, S. M., and Raetz, C. R. H. (1990) J. Biol. Chem. 265, 6394–6402
9. Onishi, H. H., Pelak, B. A., Gerecke, L. S., Silver, L. L., Kahan, F. M., Chen, M. H., Patchett, A. A., Galloway, S. M., Hyland, S. A., Anderson, M. S., and Raetz, C. R. H. (1996) J. Biol. Chem. 271, 980–982
10. Rietschel, E. T., Kirikae, T., Schade, F. U., Mamat, U., Schmidt, G., Loppnow, H., Umler, A. J., Zahringer, U., Seydel, U., Di Padova, F., Schreier, M., and Brade, H. (1994) FASEB J. 8, 2257–2263
11. Papillo, J. E. (1993) N. Engl. J. Med. 328, 1471–1477
12. Aderem, A., and Ulevitch, R. J. (2000) Nature 406, 782–787
13. Zahringer, U., Landner, B., and Rietschel, E. T. (1999) in Endotoxin in Health and Disease (Brade, H., Opal, S. M., Vogel, S. N., and Morrison, D. C., ed) pp. 95–114, Marcel Dekker, Inc., New York
14. Bhat, U. R., Forsberg, L. S., and Carlson, R. W. (1994) J. Biol. Chem. 269, 14492–14499
15. Forsberg, L. S., and Carlson, R. W. (1998) J. Biol. Chem. 273, 2747–2757
16. Que, N. L. S., Lin, S., Cotter, R. J., and Raetz, C. R. H. (2000) J. Biol. Chem. 275, 28096–28016
17. Que, N. L. S., Ribeiro, A. A., and Raetz, C. R. H. (2000) J. Biol. Chem. 275, 28017–28027
18. Gobert, N., Takayama, K., Macagni, P., Honovich, J., Wong, R., and Cotter, R. J. (1988) J. Biol. Chem. 263, 11971–11976
19. Brozek, K. A., and Raetz, C. R. H. (1999) J. Biol. Chem. 274, 15410–15417
20. Clementz, T., Bednarski, J. J., and Raetz, C. R. H. (1997) J. Biol. Chem. 272, 12095–12102
21. Clementz, T., Zhou, Z., and Raetz, C. R. H. (1997) J. Biol. Chem. 272, 10353–10360
22. Breake, K. A., Carlson, R. W., and Raetz, C. R. H. (1996) J. Biol. Chem. 271, 32126–32136
23. Leppnow, H., Brade, H., Durrbaum, I., Dinarello, C. A., Kusumoto, S., Hormaeche, E. T., and Flatt, J. F. (1989) J. Immunol. 143, 3229–3234
24. Golenbock, D. T., Hampton, R. Y., Qureshi, N., Takayama, K., and Raetz, C. R. H. (1991) J. Biol. Chem. 266, 19490–19498
25. Lian, B., Meola, T. K., Heine, H., Yoshinuma, A., Kusumoto, S., Fukase, K., Mento, N. J., Oikawa, M., Qureshi, N., Monks, B., Finberg, R. W., Ingalls, R. R., and Golenbock, D. T. (2000) J. Clin. Invest. 105, 497–504
26. Pollaruk, A., Rizzardi-Castagnoli, P., Citerri, S., and Beutler, B. (2000) Proc. Natl. Acad. Sci. U.S.A. 97, 2163–2167
27. Persing, D. H., Coler, R. N., Lacy, M. J., Johnson, D. A., Baldridge, J. R., Hershberg, R. M., and Reed, S. G. (2002) Trends Microbiol. 10, 852–837

3 M. J. Karbarz and C. R. H. Raetz, unpublished observations.
