# Purification and Characterization of the Lipid A 1-Phosphatase LpxE of *Rhizobium leguminosarum*

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## Supplementary Experimental Procedures

**Cloning of the *A. tumefaciens lpxE* homolog-** The *A. tumefaciens lpxE* homolog was cloned from genomic DNA with the primers shown in Supplementary Table 1. The *N*-terminal primer, AtLpxE forward, contains an *Eco*RI restriction site (in **bold**) upstream of the *A. tumefaciens lpxE* homolog initiation codon ATG. The *C*-terminal primer, AtLpxE reverse, also contains an *Eco*RI restriction site (in **bold**) that is downstream from the termination codon (TAA). The PCR reaction contained 150 ng of genomic DNA template, 0.1 μg of each primer, 200 μM of each deoxyribonucleotide triphosphate, 100 mM trishydroxymethylaminomethane (Tris)-HCl, pH 8.8, 35 mM $MgCl_2$, 250 mM KCl, and 2.5 units of Highfidelity PlatinumTaq polymerase (Invitrogen) in a reaction volume of 0.05 ml. The reaction mixture was subjected to a 1 min denaturation at 94 °C followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a final extension for 5 min at 72 °C, using the GeneAmp PCR system 2400 (PerkinElmer). The PCR product and the pET28a vector were digested with *Eco*RI, ligated together, and transformed into XL-1 Blue cells (Stratagene) for propagation of the plasmid, designated pAtLpxE. The orientation of the insert and its sequence were confirmed by DNA sequencing at the Duke University Sequence Analysis Facility.

**Generation of LpxE Point Mutants-** Mutated *R. leguminosarum lpxE* genes encoding LpxE with a single amino acid mutation in the catalytic arginine (R133) or histidine (H197) were generated by PCR-based mutagenesis. In this technique, two pieces of each variant gene with complementary 3' ends are generated by two separate PCR reactions. The desired mutation is introduced into these PCR products by a primer. In a subsequent PCR reaction, the two pieces are annealed together, and a third PCR reaction yields the full-length *lpxE* variant encoding the desired point mutant.

In the first set of PCR reactions, two products were generated for each point mutant. For example, in the case of R133A, the primers LpxE forward and R133A reverse were employed to generate one PCR product that spans the *N*-terminus of LpxE through the mutation. The second PCR product spans from the mutation to the *C*-terminus of LpxE and was generated with the primers R133A forward and LpxE reverse. The R133A forward and reverse primers are complementary to one another and contain the codon GCT (Ala) in place of the native codon CGT (Arg) as indicated in Supplementary Table 1. Both amplification reactions were carried out with pLpxE-4 (template) and *Pfu* DNA polymerase. Standard PCR conditions were used. The products were isolated from 1% agarose gels.

In the second set of PCR reactions, the two purified products from the first step were combined as the templates for a third PCR reaction employing LpxE forward and reverse primers to amplify the full-length *lpxE* variant encoding the desired point mutant R133A. The H179A mutation was generated in the same way, with the H179A forward and reverse primers containing the codon GCT (Ala) in place of the native codon CAT (His) as indicated in Supplementary Table 1. Each final PCR product containing the full-length *lpxE* variant was cloned into the pET28a cloning vector at the *Sac*I and *Xho*I sites as described in the Experimental Procedures. Each of the mutants was confirmed by DNA sequencing at the Duke University DNA Analysis Facility

**Preparation of the PGP substrate-** The [glycerol-$^{14}$C(U)]-PGP was prepared by enzymatic synthesis using cytidine 5′-diphosphate-diacylglycerol (CDP-DAG) and [$^{14}$C(U)]-glycerol-3-phosphate. Membranes of W3110 were used as the source of PGP synthase activity and were prepared as follows. *E. coli* strain W3110 was grown from a single colony in 400 ml of LB to an $A_{600}$ of 1.0. Cells were harvested and centrifuged at 9,000 x $g_{AV}$ for 20 min. All steps were carried out at 0-4 °C. The cells were resuspended in 20 mM potassium phosphate buffer, pH 7.0, at a protein concentration of 5-10 mg/ml. Washed membranes were obtained as previously described (1). The final membrane pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.0, at a protein concentration of 10 mg/ml. To remove as much PGP phosphatase activity as possible, membranes were solubilized, as follows (2). An appropriate volume of the following components were added to give a final concentration of 4% Triton X-100, 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, and a final washed membrane concentration of 5 mg/ml. The solubilization mixture was then incubated at 4 °C for 1 h with intermittent inversion on a rotating apparatus (2). Next, the mixture was centrifuged at 100,000 x $g_{AV}$ for 60 min at 4 °C to remove any remaining insoluble material. The above conditions solubilized PGP synthase, but leave most of the PGP phosphatase in the pellet (2).

Solubilized PGP synthase was used to prepare [glycerol-$^{14}$C(U)]-PGP. In a 0.4-ml reaction tube, the following components were added: 40 µl of 2.5 M Tris-HCl, pH 8.0, 40 µl of 10% Triton X-100, 40 µl of 1 M $MgCl_2$, 40 µl of 2 mM CDP-DAG, 40 µl of 143 mCi/mmole [$^{14}$C(U)]-glycerol-3-phosphate, 150 µl of solubilized W3110 membranes, 20 µl of 10 mM $HgCl_2$, and 30 µl of deionized $H_2O$. After several hours at room temperature, the reaction mixture was converted to a 2-phase Bligh-Dyer system with the addition of 444 µl of chloroform and 444 µl of methanol, and it was centrifuged for 10 min to facilitate removal of the lower phase. The lower phase was washed once with fresh pre-equilibrated upper phase, and centrifuged for 10 min. To recover the lipid, the lower phase was collected and dried under a stream of $N_2$ and stored at -20 °C. By TLC, approximately 95% of the [$^{14}$C(U)]-glycerol-3-phosphate was converted to [$^{14}$C(U)-glycerol]-PGP.

**PGP Phosphatase and PA Phosphatase Assays-** The *in vitro* assay for PGP phosphatase activity was essentially the same as that described previously (3). Briefly, the reaction mixture contained 50 mM Tris-HCl, pH 7.5, 0.1% Triton X-100, 2 mM $MgCl_2$, 5 mM b-mercaptoethanol, ~ 50 µM [glycerol-$^{14}$C(U)]-PGP (140 mCi/mmole), and 0.5 mg/ml of membrane protein. The reaction was allowed to proceed for up to 60 min at 30 °C, and then quenched by spotting 4 µl directly onto a TLC plate. After drying the spots under cool air, the plate was developed in the solvent chloroform, pyridine, 88% formic acid, water, (50:50:16:5, *v*/*v*/*v*/*v*). Following removal of the solvent with a stream of hot air, the plate was analyzed with a PhosphorImager.

The dephosphorylation of PA was measured under similar conditions described for the lipid A 1-phosphatase, except that 50 µM [glycerol-$^{14}$C(U)]-PA (150 mCi/mmole, PerkinElmer Life Sciences) was substituted for lipid $IV_A$. Reactions were followed by conversion of radioactive PA to diacylglycerol. After drying the spots, the TLC plate was developed in the solvent chloroform, pyridine, 88% formic acid, water (50:50:16:5, *v*/*v*/*v*/*v*) and analyzed with a PhosphorImager.

**Supporting References-**


1. Trent, M. S., Pabich, W., Raetz, C. R. H., and Miller, S. I. (2001) *J. Biol. Chem.* **276**, 9083-9092
2. Dowhan, W., and Hirabayashi, T. (1981) *Methods Enzymol.* **71 Pt C**, 555-561
3. Chang, Y. Y., and Kennedy, E. P. (1967) *J. Lipid Res.* **8**(5), 456-462


**Supplementary Table I**

*Primers for the cloning of AtlpxE and for the generation of lpxE site-directed mutants*

| Name | Sequence[a] |
|---|---|
| AtLpxE forward: | 5'-GGGCCCG**GAATTC**ATGCAAGAAAAAGACAAGAGG-3' |
| AtLpxE reverse: | 5'-AGG**GAATTC**TTATCTTGTTTCAGCCGCCGG-3' |
| LpxE forward: | 5'-ACTTA**GAGCTC**ATGCGGGCATTTTGG-3' |
| LpxE reverse: | 5'-ACTA**CTCGAG**TTATGCAGTGCGGAAC-3' |
| R133A forward:[b] | 5'-GCGATCGGCAGGGCG*GCT*CCCGATCATTTCCAC-3' |
| R133A reverse: | 5'-GTGGAAATGATCGGG*AGC*CGCCCTGCCGATCGC-3' |
| H197A forward: | 5'-CGCGTGTCATGGTCGGCGCC*GCT*TATCCGAGCGACGTCAT-3' |
| H197A reverse: | 5'-ATGACGTCGCTCGGATA*AGC*GGCGCCGACCATGACACGCG-3' |

[a]The underlined 5' nucleotides were added for efficient cleavage by the restrictions enzymes. The bold nucleotides are the restriction sites.

[b]The underlined and italicized codon accomplished the point mutation indicated.