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The calcium-stimulated lipid A 3-O deacylase from *Rhizobium etli* is not essential for plant nodulation

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Abstract

The lipid A component of lipopolysaccharide from the nitrogen-fixing plant endosymbiont, *Rhizobium etli*, is structurally very different from that found in most enteric bacteria. The lipid A from free-living *R. etli* is structurally heterogeneous and exists as a mixture of species which are either pentaacylated or tetraacylated. In contrast, the lipid A from *R. etli* bacteroids is reported to consist exclusively of tetraacylated lipid A species. The tetraacylated lipid A species in both cases lack a β -hydroxymyristoyl chain at the 3-position of lipid A. Here, we show that the lipid A modification enzyme responsible for 3-O deacylation in *R. etli* is a homolog of the PagL protein originally described in *Salmonella enterica* sv. *Typhimurium*. In contrast to the PagL proteins described from other species, *R. etli* PagL displays a calcium dependency. To determine the importance of the lipid A modification catalyzed by PagL, we isolated and characterized a *R. etli* mutant deficient in the *pagL* gene. Mass spectrometric analysis confirmed that the mutant strain was exclusively tetraacylated and radiochemical analysis revealed that 3-O deacylase activity was absent in membranes prepared from the mutant. The *R. etli* mutant was not impaired in its ability to form nitrogen-fixing nodules on *Phaseolus vulgaris* but it displayed slower nodulation kinetics relative to the wild-type strain. The lipid A modification catalyzed by *R. etli* PagL, therefore, is not required for nodulation but may play other roles such as protecting bacterial endosymbionts from plant immune responses during infection.

Keywords

Gram-negative bacteria; lipopolysaccharide; Lipid A; *Rhizobium etli*; PagL

INTRODUCTION

The outer membrane of Gram-negative bacteria is asymmetric and is composed of an inner leaflet of glycerophospholipids and an outer leaflet of lipopolysaccharide (LPS). LPS is a

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unique glycolipid composed of three covalently linked domains: 1) the lipid A moiety, 2) a non-repeating core domain, and 3) a distal O-antigen domain [1]. Lipid A is the hydrophobic moiety that anchors LPS to the outer leaflet of the outer membrane. Lipid A is also the “endotoxin” component of LPS and is associated with the complications of severe Gram-negative sepsis [2]. The lipid A of pathogenic bacteria triggers an immune response in mammalian hosts by activating the Toll-like receptor-4/MD-2 (TLR4/MD-2) complex [3].

The structure of lipid A is relatively conserved among Gram-negative bacteria. In *E. coli* and *Salmonella typhimurium*, lipid A is a β -1',6-linked disaccharide of glucosamine, phosphorylated at positions 1 and 4', and acylated with R-3 hydroxymyristate groups at positions 2, 3, 2', and 3'. Two additional fatty acid groups, a laurate residue, and a myristate residue, are present as acyloxyacyl moieties at the 2' and 3'-positions (Fig. 1A) [1]. These relatively conserved structural characteristics have been found to be important for full activation of the TLR4/MD-2 complex [4]. The lipid A structure-based innate immune response of host cells, however, can be overcome with alterations in lipid A structure. Indeed, many animal pathogens have developed strategies for modifying lipid A in an attempt to evade detection and clearance [5].

Lipid A diversity however is not limited to pathogenic bacteria. Significant structural variation of lipid A occurs in the bacterial endosymbiont *R. etli* (Fig. 1B) [5]. This bacterial strain is of interest because it elicits nodule formation on leguminous plants and in doing so provides its host with a reduced nitrogen source. When *R. etli* bacteria establish a symbiosis with their host and live intracellularly they are known as bacteroids [6]. The lipid A from free-living *R. etli* is heterogeneous and exists as a mixture of species (components B, C, D, and E) (Fig 1B) [7, 8]. All lipid A species from *R. etli* lack the 1 and 4'-position phosphates found in most other Gram-negative bacteria. The 4'-position phosphate is replaced with a galacturonic acid moiety and the proximal glucosamine residue can exist in the hemiacetal form (components B and C) or as an aminogluconate moiety (components D and E). *R. etli* synthesizes pentaacylated molecules with a peculiar 28 carbon fatty acid at the 2'-position. A portion of the lipid A molecules are deacylated at the 3-position (components C and E) [7–10]. There is also considerable heterogeneity with regard to acyl chain lengths.

Despite its structural differences, the unique lipid A species found in *R. etli* is initially synthesized in a manner that is similar to that of *E. coli* [1]. The endosymbiont bacteria contain orthologues of the first seven *lpx* genes of the constitutive lipid A biosynthetic pathway except for *LpxH*. *LpxH* activity in *R. etli* is catalyzed by a transformation analog termed *LpxI* [11]. The resulting lipid A intermediate, Kdo₂-lipid IV_A intermediate is then processed by enzymes that are absent in *E. coli*. The *R. etli* lipid A modification enzymes include a 4'-phosphatase (*LpxF*) [12], a 1-phosphatase (*LpxE*) [13], a C28-specific long-chain acyltransferase (*LpxXL*) [14, 15], a galacturonosyltransferase (*RgtD*) [16–18], a lipid A oxidase (*LpxQ*) [19, 20], and an unidentified 3-*O*-deacylase [21].

A 3-*O* deacylase activity was previously demonstrated in *Rhizobium leguminosarum* extracts. The *R. leguminosarum* enzyme was shown to be capable of removing the 3-*O* acyl chain of lipid A precursors including lipid IV_A and Kdo₂-lipid IV_A and its activity was dependent on the presence of both detergent and divalent cations. [21]. The identification of the gene responsible for the enzymatic activity however has remained undetermined. A 3-*O*-deacylase activity similar to that described in *R. leguminosarum*, however, known to be catalyzed by the enzyme PagL, has been described in *S. typhimurium* and *Pseudomonas aeruginosa* [22, 23]. Interestingly, homologs of the *pagL* gene can be found in both pathogenic and nonpathogenic bacterial species. All predicted PagL proteins, like other classical serine esterases, contain a catalytic triad made up of a serine, a histidine, and an acidic residue, most often present as an aspartic acid or glutamic acid. In distantly related

PagL homologs, only four residues are completely conserved: the serine and histidine of the catalytic triad, a phenylalanine residue and an asparagine residue thought to be part of a functional oxyanion hole [23, 24]. An x-ray crystal structure of PagL from *P. aeruginosa* was recently solved confirming the role of many of these residues [24].

The biological role of the PagL-catalyzed modification of lipid A is unknown in many organisms. In *S. typhimurium*, PagL expression has been shown to be regulated by the two-component PhoP/PhoQ regulatory system, which is induced by low Mg^{2+} concentrations [22]. PagL was also found to have greater activity in *P. aeruginosa* strains isolated from infants with cystic fibrosis [25]. Other reports have indicated that PagL expression leads to decreased endotoxic activity of LPS [26, 27]. These results suggest that lipid A 3-*O*-deacylation could confer a selective advantage under certain growth conditions. Recently it was reported, that *R. etli* bacteroids isolated from nodules were exclusively 3-*O*-deacylated [28]. This result suggests that the *R. etli* *pagL* gene could be up-regulated during symbiosis. In an effort to determine the importance of *R. etli* PagL activity in both free-living and *R. etli* bacteroids, we identified the *R. etli* PagL gene and constructed a relevant knockout strain. In this article, homologs of PagL are described in the endosymbionts *R. etli* and *Sinorhizobium meliloti*. We show that both homologs are functional 3-*O*-deacylases. The *R. etli* PagL homolog is shown to have the same properties as the protein characterized from *R. leguminosarum* membranes. Finally, we describe the characterization of the *R. etli* mutant deficient in *pagL*.

2. MATERIALS AND METHODS

2.1 Materials

[γ - ^{32}P] ATP was obtained from PerkinElmer Life And Analytical Sciences, Inc. (Waltham, MA). Acetic acid, ammonium acetate, chloroform, formic acid, and glass-backed 0.25-mm Silica Gel-60 thin layer chromatography (TLC) plates were from EMD Chemicals, Inc. (Gibbstown, NJ). Pyridine and methanol were from Mallinckrodt Baker, Inc. (Phillipsburg, NJ). Yeast extract, agar, and tryptone were from Becton, Dickinson, and Company (Franklin Lakes, NJ). Sodium chloride and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from VWR International (West Chester, PA). Bicinchoninic (BCA) protein assay reagents and Triton X-100 were from Thermo Fisher Scientific (Waltham, MA). Diethylaminoethyl (DEAE) cellulose DE52 was from Whatman, Inc. (Florham Park, NJ). PCR reagents were purchased from EMD and Stratagene (La Jolla, CA). IPTG was from Invitrogen (Carlsbad, CA). All other chemicals were reagent grade and were purchased from either Sigma-Aldrich (St. Louis, MO) or VWR International (West Chester, PA).

2.2 Bacterial strains, growth conditions and molecular biology protocols

The bacterial strains and plasmids used and their relevant characteristics are shown in Table I. *R. etli* strains were grown at 30 °C on complex tryptone yeast extract medium (TY) supplemented with 10 mM $CaCl_2$ [29]. *R. etli* strains were grown in media supplemented with the following concentrations of antibiotics where appropriate: neomycin (200 μ g/mL), nalidixic acid (20 μ g/mL) streptomycin (200 μ g/mL), and spectinomycin (200 μ g/mL). *E. coli* strains were cultured on LB broth at 37 °C. Growth media were supplemented with the following concentrations of antibiotics where appropriate: kanamycin (50 μ g/mL), carbenicillin (100 μ g/mL), spectinomycin (200 μ g/mL), or ampicillin (100 μ g/mL). Derivatives of the suicide plasmid pK18*mobsacB* were mobilized into *R. etli* strains by triparental mating using the mobilizing plasmid pRK2013, as described previously [30]. Recombinant DNA techniques were performed according to standard protocols [31] using *E. coli* DH5 α as a host strain [32]. The XL-PCR kit (Applied Biosystems) was used for amplifying DNA sequences from *R. etli* genomic DNA.

2.3 Construction of plasmids RePagL and SmPagL

The primers RePagLfor (5'-CGCGCCATATGAGGGTTCGAGTTCGGAAAG ATCG-3') and RePagLrev (5'-GCGCGAAGCTTTCAGAACTTGTAGCCGATCTGG ATGCCG-3') were used to amplify the *R. etli* *pagL* gene from genomic DNA. The primers SmPagLfor (5'-CGCGCCATATGAGAGATTTCGGCTCGGTTGCAGG-3') and SmPagLrev (5'-GCGCGAAGCTTTTAGAACGTGTAGCCGAGCATCAACC-3') were used to amplify the *S. meliloti* *pagL* gene from genomic DNA. The coding region of *pagL* in the two primers is indicated in bold type, and the restriction enzyme sites are italicized. Both of the PCR products were digested with NdeI and HindIII and ligated into pET23a(+), yielding plasmids RePagL and SmPagL. The plasmids were transformed into competent *E. coli* DH5 α cells and their sequence was confirmed by DNA sequencing. The plasmids were then transformed into BLR(DE3) cells.

2.4 Expression of *R. etli* *pagL* in *E. coli*

Single colonies of BLR(DE3)/RePagL and BLR(DE3)/SmPagL were used to inoculate 5 mL of LB supplemented with 100 μ g/mL ampicillin. The cultures were grown overnight at 37 °C with shaking at 225 rpm and used to inoculate 200 mL of LB broth supplemented with ampicillin (100 μ g/mL) to a starting A_{600} of ~ 0.01. The cells were grown with shaking at 37 °C until the A_{600} reached 0.6, at which time 1 mM IPTG was added. The cells were grown for 3 additional hours and harvested by centrifugation at 4000 $\times g$ for 20 min at 4 °C. The cells were washed with 50 mM HEPES, pH 7.5. The washed cell pellet was stored frozen at -80 °C.

2.5 Preparation of membranes and substrates

All enzyme preparations were carried out at 0–4 °C. The bicinchoninic acid method [33] was used to determine protein concentrations, using bovine serum albumin as a standard. Cell-free extracts and washed membranes were prepared as described previously [21] and stored in aliquots at -80 °C. The preparation of carrier Kdo₂-lipid IV_A [34] and Kdo₂-[4'-³²P]-lipid IV_A was described previously [35]. The substrates were stored as aqueous dispersions in 25 mM Tris-HCl, pH 7.8, containing 1 mM EDTA, 1mM EGTA, and 0.1% Triton X-100. Prior to use, the lipid substrates were subjected to sonication for 1 min in a bath sonicator.

2.6 In vitro assay of 3-O-deacylase activity

The 3-*O*-deacylase activity of *R. etli* membranes was determined as previously described [21]. Standard assay conditions were as follows: the reaction mixture (10–20 μ L) contained 50 mM MES, pH 6.25, 1.0% Triton X-100, 2 mM dithiothreitol, 2 mM EDTA, 20 mM CaCl₂, and 10 μ M [4'-³²P] lipid IV_A (1,000 cpm/ μ L). The *E. coli* membranes expressing RePagL and SmPagL were assayed under different conditions where noted. In these instances, 3-*O* deacylase activity was assayed in a buffer containing 50 mM HEPES, pH 8, 0.1% Triton, 0.5 M NaCl, and 10 μ M [4'-³²P] lipid IV_A (1,000 cpm/ μ L). CaCl₂ was added to this assay buffer as indicated. Reactions were initiated with the enzyme source and were incubated at 30°C for the indicated times. The reactions were terminated by spotting 3 μ L samples onto a TLC plate, which was developed in the solvent chloroform/pyridine/88% formic acid/water (30:70:16:10, v/v). After drying and overnight exposure of the plate to a PhosphorImager screen (GE Healthcare), product formation was detected and quantified with a Storm 840 PhosphorImager equipped with ImageQuant software (GE Healthcare).

2.7 Exchange of the rhizobial *pagL* gene with a spectinomycin resistance cassette

A *pagL* deletion mutant in *R. etli* was constructed using a procedure previously described [36]. PCR and molecular cloning methods were utilized to generate a plasmid containing a

spectinomycin cassette flanked on each side by ~1 kb of the genomic DNA found upstream and downstream of the *R. etli* *pagL* gene. The cassette and flanking regions were subcloned from the plasmid into the suicide vector pK18*mobsacB* and introduced by triparental mating into wild-type *R. etli* CE3 using pRK2013 as a helper plasmid. Transconjugants were selected on TY medium containing neomycin and spectinomycin to select for single recombinants in a first step. The single recombinants were grown under nonselective conditions in complex medium for one day before being plated on TY medium containing spectinomycin and 10% sucrose (w/v) to select for the loss of the vector backbone of pK18*mobsacB* from the bacterial genome. Several candidates grew after 5 days. Southern Blot analysis confirmed that the putative mutant strains were double recombinants in which the *pagL* gene was replaced with a spectinomycin cassette (data not shown). One of these confirmed mutants was named CS4 and was used throughout this study.

2.8 Lipid A extraction

Lipid A from 1 L *R. etli* cultures was isolated as described previously using 1% acetic acid [36]. After TLC in a chloroform:methanol:water:ammonium hydroxide system (40:25:4:2, v/v), the lipids were visualized by sulfuric acid charring.

2.9 Normal phase liquid chromatography/mass spectrometry (LC/MS) of lipid A species

Lipid A species recovered from the cells were analyzed by normal phase LC/MS using a TripleTOF 5600 quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Foster City, CA). Normal phase liquid chromatography (LC) was performed on an Agilent 1200 Quaternary LC system equipped with an Ascentis Silica HPLC column, 5 μ m, 25 cm $\times$ 2.1 mm (Sigma-Aldrich). Chromatography was carried out as described previously [37]. Mobile phase A consisted of chloroform/methanol/aqueous ammonium hydroxide [800:195:5 (v/v)]; mobile phase B consisted of chloroform/methanol/water/aqueous ammonium hydroxide [600:340:50:5 (v/v)]; and mobile phase C consisted of chloroform/methanol/water/aqueous ammonium hydroxide [450:450:95:5 (v/v)]. The elution program consisted of the following: 100% mobile phase A was held isocratically for 2 min and then linearly increased to 100% mobile phase B over 14 min and held at 100% B for 11 min. The LC gradient was then changed to 100% mobile phase C over 3 min and held at 100% C for 3 min and finally returned to 100% A over 0.5 min and held at 100% A for 5 min. The total LC flow rate was 300 μ L/min. Data analysis was performed using Analyst TF1.5 Software (Applied Biosystem).

2.10 Assay of polymyxin sensitivity

The outer membrane integrity of wild-type *R. etli* and the *pagL*-deficient *R. etli* mutant strain was evaluated by examining the sensitivity of the strains to the cationic antimicrobial peptide polymyxin B sulfate (Sigma). The lipid A phosphatase-deficient mutant CS513 (Δ *lpxE lpxF*) was included as a positive control strain in this assay because it is known to show an increased susceptibility to polymyxin B [36]. A disk diffusion test was performed. *R. etli* cells (100 μ L) from an A₆₂₀ suspension of 0.2 were plated on TY agar. Sterile 6-mm filter disks were placed onto the lawn of cells. Polymyxin (10 μ L of a 1, 2, 10, or 20 mg/mL stock solution) was then applied to the disks. After 48 hours of growth at 30 °C, the diameters of the halos were measured, thereby providing an assessment of the relative polymyxin sensitivity.

2.11 Plant test for symbiotic phenotype characterization

Phaseolus vulgaris cv. *Negro Jamapa* seeds were surface-sterilized with 1.2% sodium hypochlorite and germinated on 1% (w/v) water agar as described previously [38]. Seedlings were transferred to 250 mL flasks filled with vermiculite and nitrogen-free nutrient solution

[39], and inoculated with about 10^5 CFU of bacteria per plant. Plants were grown in a controlled growth chamber at 28 °C with a 15 h day/9 h night cycle and harvested 21 d after inoculation. Nitrogenase activity of nodulated roots was determined by the acetylene reduction assay as described previously [40]. Nitrogen fixation activity per plant was normalized with respect to the nodule fresh weight per plant. For the nodulation kinetics experiment seeds were sterilized and germinated as described above. Seedlings were then transferred to 250 mL flasks filled with 200 mL nitrogen-free nutrient solution solidified with 0.75% agar. Conditions of inoculation and plant growth were identical to as described above. Roots were scored for nodule formation after 7, 10, 12, 14, 16, 19, and 21 days. Nodules were classified as either white, beige, red or senescent. When nodules start to form on the inoculated roots they are white. They keep growing and developing and turn beige and finally red when they are fully functional, that is fixing molecular nitrogen. The red color of the nodules is due to the accumulation of leghemoglobin which is important for oxygen transport inside the nodule. When senescent, the red color of the nodules is lost and the nodules turn greenish or dark brown.

3. RESULTS

3.1 Identification of pagL homologs in *R. etli* and *S. meliloti*

The amino acid sequence of the *Pseudomonas aeruginosa* *pagL* gene (accession number NP_253350) [23, 24] was used as a probe to identify putative *pagL* homologs in members of the *Rhizobiaceae* family. Homologs containing a PagL domain (pfam09411) with E-values ranging from 0.023 to 1.6 were found in *Rhizobium leguminosarum* bv. *trifolii* WSM1325, *Sinorhizobium fredii* NGR234, *Agrobacterium vitis* S4 and *Sinorhizobium meliloti* Rm41 in the NCBI data base. A consensus sequence was derived from these PagL homologs using the ClustalW multiple sequence alignment program available at <http://www.ebi.ac.uk/tools/clustalw2> [41, 42]. The consensus *rhizobial* PagL sequence was subsequently used as a probe to identify other *rhizobial* PagL homologs in the *Rhizobiaceae* family. Using this approach, additional PagL homologs were found in both *R. etli* CFN42 and *S. meliloti*. A focus was placed on the *R. etli* homolog because 3-*O*-deacylase activity in *R. etli* is presumed to account for some of the heterogeneity found in its lipid A structure (Fig. 1B) [7, 8, 21]. The *S. meliloti* gene, which had been identified as a possible homolog previously [24], was of interest because *S. meliloti* extracts are reported to lack 3-*O*-deacylase activity [21].

The protein sequences of the putative *R. etli* and *S. meliloti* PagL homologs were aligned against the *P. aeruginosa* PagL protein sequence using ClustalW (Fig. 2). The 192-amino acid PagL homolog from *R. etli* (accession number ABC91065) and the 196-amino acid PagL homolog from *S. meliloti* (accession number NP_385884) are 20% identical on the amino acid level to *P. aeruginosa* PagL. The two *Rhizobium* homologs are predicted to have signal peptide sequences as predicted by the SignalP server [43], consistent with the proposed outer membrane localization of PagL [22, 24]. The homologs also contain conserved histidine and serine residues that make up part of the active site of *P. aeruginosa* PagL. In addition, an asparagine residue thought to comprise the oxyanion hole in *P. aeruginosa* is present. The glutamate residue proposed to serve as the acidic residue of the catalytic triad in *P. aeruginosa* PagL, however, is substituted with threonine residues in the *rhizobial* homologs. The corresponding amino acids are indicated with Xs in the alignment shown in Fig. 2.

3.2 In vitro activity and catalytic properties of heterologously expressed RePagL and SmPagL

The *R. etli* and *S. meliloti* homologs were cloned from genomic DNA and overexpressed heterologously in *E. coli* as described in sections 2.3 and 2.4. The membranes from these over-expressing strains were assayed in parallel with membranes from wild-type *R. etli* CE3 and *E. coli* W3110, which lacks 3-*O*-deacylase activity. In this assay system, the substrate [4'-³²P]-lipid IV_A is deacylated and the more hydrophilic product migrates more slowly. As shown in Fig 3A, membranes overexpressing the PagL homologues generated a product with the same mobility as that generated with the *R. etli* membranes. This activity was absent in the *E. coli* W3110 membranes and from *E. coli* cells expressing the vector control.

In Fig. 3A, the *rhizobial* PagL homologs were assayed using conditions previously optimized for measuring 3-*O*-deacylase activity in *R. leguminosarum* membranes [21]. This assay condition included 20 mM calcium, as it was found that 3-*O*-deacylase activity in *R. leguminosarum* membranes was inhibited by EDTA and stimulated by divalent cations [21]. A metal requirement, however, has not been reported for other PagL homologs such as that from *S. typhimurium* or *P. aeruginosa* [22, 24]. In order to investigate the significance of the apparent differences in metal-stimulation between the enzymes, the 3-*O*-deacylase activity of the *E. coli* membranes over-expressing the *R. etli* PagL homolog was assessed under assay conditions with variable calcium chloride concentrations. When the *E. coli* membranes expressing RePagL were assayed using the assay conditions previously optimized for measuring *S. typhimurium* PagL activity [22], a robust calcium-stimulated activity was observed (Fig. 3B). Increasing concentrations of calcium chloride had a similar effect on the *E. coli* membranes over-expressing SmPagL (data not shown).

3.3 Determination of lipid A 3-O deacylase activity in wild-type *R. etli* and the PagL mutant

A deletion mutant of the *pagL* gene was constructed in *R. etli* as described in section 2.7. The *in vitro* 3-*O*-deacylase activity of the mutant and the parental wild-type strain was measured using washed membranes as the enzyme source. As shown in Fig. 4, the 3-*O*-deacylase activity characteristic of wild-type *R. etli* cells is absent in the *pagL* mutant (CS4). The membranes from *E. coli* over-expressing the two *rhizobial* PagL homologues are included as positive controls. This result demonstrates that the putative *R. etli* gene is responsible for all of the 3-*O*-deacylase activity observed in *R. etli* extracts

3.4 Lipid A from wild-type *R. etli* and the *pagL* knockout strain

The lipid A components released from wild-type *R. etli* cells migrated as multiple species, when analyzed by TLC (Fig. 5) [7, 8]. The structures of the major lipid A components (B, C, D, and E) (Fig 1B) from the wild type strain have been described previously [7, 8]. The TLC pattern of the lipid A species from the *pagL* mutant strain differed from the wild-type in that it was less heterogeneous. The mutant contained components B and D but lacked the normal 3-*O*-deacylated species (components C and E) (Fig. 1B). Both strains contained component A (Supplementary Fig. 1), a tetra-acylated artifact arising from the mild acid hydrolysis procedure utilized to isolate *R. etli* lipid A. Lipid A components with a proximal aminogluconate residue (component D, Fig 1B) are subject to the acid catalyzed β -elimination of the 3-*O* linked ester chain and an additional water molecule during the lipid A extraction procedure [7, 44].

3.5 ESI-MS analysis of the lipid A species

The negative ion ESI mass spectra of the lipid A species extracted from wild-type *R. etli* and the *pagL* mutant strain are shown in Fig. 6. Both samples were analyzed by normal phase LC/MS. Under the chromatography conditions described in section 2.9, all *R. etli* lipid A

species eluted between 16 and 21.5 min. The predicted masses of the major molecular species are listed in Table 2. In panel A of Figure 6, the lipid A species from the wild type strain eluting between 16–21.5 minutes are shown. There are three major lipid A species (components B, C, and A) seen between m/z 1600 and 2000. The m/z of these major lipid A species are 1984.46, 1758.26, and 1738.24, respectively. There is significant additional micro-heterogeneity associated with all these lipid A species because of acyl chain length differences (difference of ± 28 amu) and/or the presence of a β -hydroxybutyryl moiety at the 27:OH position of the 2'-acyloxyacyl chain (difference of 86 amu) [7, 8, 10]. These additional differences are annotated in the figure. Component B is a tetraacylated lipid A species containing a glucosamine sugar at the 1-position of lipid A and component C is the 3-*O*-deacylated species derived from component B, which presumably is formed enzymatically by PagL (Fig. 1B). The oxidized lipid A species with an aminogluconate sugar at the 1 position of lipid A, components D and E (Fig 1B) are in minor abundance relative to the other lipid A components. As described in section 3.4, lipid A species with a proximal aminogluconate residue such as component D are subject to elimination of their 3-*O* ester chain and an additional water molecule ($244 + 18 = 262$ atomic mass units) during lipid A hydrolysis. Component A (Supplementary Fig. 1), the most abundant peak observed in Fig. 6A, therefore, is proposed to be an artifact derived from component D (molecular weight of 2002.8). Non-enzymatic modifications can also occur with oxidized lipid A species (components D and E) under the experimental conditions required for mass analysis [44]. Component E, the oxidized tetraacylated lipid A component derived from component D (Fig 1B), therefore, may also be degraded to component A during mass analysis. In panel B of Fig. 6, the same mass region is shown for the *pagL* mutant. Components B and A are seen, but the deacylated lipid A corresponding to component C is absent, consistent with the TLC analysis (Fig. 5).

The relative intensity of the ion corresponding to component C is low in comparison to the other lipid A species shown in the elution profile for the wildtype strain in the interval between 16 and 21.5 min (Fig. 6A). Because the lipid A species were separated by normal phase chromatography, the different lipid A components elute at times based on differences in polarity. In order to selectively examine only the differences in the levels of component C between the wildtype and the *pagL* mutant strain the elution profile was examined at a different time interval. Under the chromatography conditions employed component C eluted later than components A and B. Component C and all of its acyl chain variants eluted between 19 and 21.5 min. In panel C of Fig. 6, the lipid A species eluting for the wildtype strain between the interval of 19 and 21.5 min are shown. In panel D of Fig. 6, the lipid A species eluting between 19 and 21.5 min are shown for the *pagL* mutant. Component C, the enzymatically-formed tetra-acylated lipid A component proposed to be formed by PagL, cannot be detected.

3.6 Polymyxin sensitivity of the *pagL*-deficient *R. etli* strain

Modifications of the lipid A structure such as the elimination of phosphate groups by the phosphatases LpxE and LpxF have been shown to increase resistance to the cationic antimicrobial peptide polymyxin B [36]. D'Haeze et al had demonstrated that bacteroid-derived *R. etli* are more susceptible to cationic peptides than broth cultured cells [28]. Bacteria that are resistant to cationic peptides usually acquire this resistance by modifying their LPS structure. As D'Haeze et al. had shown that lipid A isolated from bacteroids is tetraacylated, the deacylation of lipid A by PagL might be responsible for the observed increase in sensitivity [28]. To determine if the lipid A deacylation catalyzed by PagL causes a change in polymyxin B sensitivity, disk diffusion assays were performed. No significant difference in polymyxin B sensitivity was observed between the *R. etli* wild-type and the *pagL*-deficient mutant. In contrast, the *R. etli lpxE lpxF* double mutant included in this assay

showed a drastic increase in sensitivity to the cationic peptide. Thus, it seems that the presence or absence of PagL and therefore the presence of tetraacylated lipid A is not directly responsible for the increased sensitivity to polymyxin B observed in bacteroid-derived *R. etli*.

3.7 Symbiotic phenotype of the *R. etli* pagL mutant

Phaseolus vulgaris roots were inoculated with the *R. etli* wild-type strain or the *pagL* mutant strain, CS4. 21 days after inoculation the plant roots were analyzed for nodule formation. No nodule formation was detected on non-inoculated control plants, but both bacterial strains caused the formation of root nodules on the host plant. The total number of nodules per plant was similar in plants inoculated with either strain, and no major difference in nitrogen fixing activities of the wild-type and mutant strain (data not shown) could be observed.

We also studied the kinetics of nodulation of bean plants inoculated with the *R. etli* wild-type or the *pagL* mutant. The roots of the plants were analyzed 7, 10, 12, 14, 16, 19, and 21 d after inoculation (Fig. 7). Both rhizobial strains caused nodule formation, but it appeared as if the *pagL* mutant developed nodules slightly later than the wild-type. Functional nitrogen-fixing nodules that are red due to the accumulation of leghemoglobin appeared later on plants inoculated with the *pagL* mutant strain and the number of senescent-looking nodules appeared to be increased.

4. DISCUSSION

Given the structural diversity of lipid A structures found in *R. etli* (Fig. 1B), it has been hypothesized that such unique lipid A structures may be important in the establishment of nitrogen-fixation. The heterogeneous lipid A structures of *R. etli* lipid A arise from a group of lipid A modification enzymes that have been extensively described over the last two decades [5]. The importance of rhizobial lipid A modifications, however, in plant nodulation has only recently been investigated. It was found that an *R. etli* mutant deficient in both the 1 and 4'-phosphatases (LpxE and LpxF) accumulated bis-phosphorylated lipid A species [36]. Despite the altered lipid A structure, the *lpxE/lpxF* mutant did not display an altered nodulation phenotype. This result indicated that the aminogluconate and galacturonic acid residues that normally replace the phosphates at the 1 and 4'-positions of *R. etli* lipid A (Fig. 1B) are not essential for symbiosis. In contrast to this result, a *R. leguminosarum* lipid A mutant deficient in *acpXL*, the acyl carrier protein involved in acylation of lipid A with a 27-hydroxyoctacosanoid acid moiety (Fig. 1B), was reported to accumulate irregular shaped bacteroids and to be delayed in nodule formation [45, 46].

To date, the only *R. etli* lipid A modification enzyme yet to be identified or characterized in a symbiotic strain is the 3-*O*-deacylase. To address the functional importance of the 3-*O*-deacylated lipid A species observed *R. etli* lipid A structures, we used the *P. aeruginosa* PagL protein sequence as a probe to search for homologs of the protein in members of the *Rhizobiaceae* family. Homologs were found in several rhizobial species including *R. etli* and *S. meliloti* (Fig. 2). The C-terminus of the rhizobial proteins was most homologous and contained conserved serine and histidine residues previously confirmed as active site residues in *P. aeruginosa* [23]. To address if the putative *R. etli* PagL homolog was responsible for generation of the deacylated lipid A species observed in *R. etli* lipid A we cloned the gene and expressed it recombinantly in *E. coli*. In addition, we made the respective deletion in *R. etli* and assessed the importance of 3-*O*-deacylation in nodulation.

Consistent with all other reported PagL activities, we found the activity of the overexpressed *R. etli* and *S. meliloti* PagL homologs to be localized to the membrane fractions of *E. coli*. Both overexpressed proteins displayed activity under conditions described previously for

analyzing *R. leguminosarum* 3-*O*-deacylase activity (Fig. 3A) [21]. The conditions previously described for assaying *R. leguminosarum* activity included calcium chloride as the activity was shown to be stimulated by divalent cations [21]. Consistent with this observation, the activity of the recombinantly expressed *R. etli* PagL protein was found to be stimulated by the presence of divalent cations (Fig. 3B). This property of *R. etli* PagL is different relative to the other described PagL proteins. Both the *S. typhimurium* and *P. aeruginosa* PagL proteins are active even in the presence of EDTA [24, 47]. This difference could reflect differences in catalysis or regulation between the proteins. The lipid A from free-living *R. etli* is typically isolated as a mixture of penta- and tetraacylated forms. Intriguingly, the results of D'Haeze et al suggest that the PagL activity of *R. etli* may be upregulated *in planta*. While outside the scope of this work, it will be of future interest to further determine the mechanisms governing PagL activity both in free-living bacteria and in bacteroids.

The role of the PagL enzyme, like other lipid A modification enzymes in *R. etli* is unclear. A deletion mutant of the putative *pagL* gene was made in *R. etli* in order to address its functional importance in nodulation. The *R. etli* PagL mutant lacked *in vitro* deacylase activity and its lipid A was primarily pentaacylated (Figs. 4–6). The PagL mutant was able to form nitrogen-fixing nodules, however, nodule development varied amongst the two bacterial strains. The PagL mutant strain displayed slower nodulation kinetics (Fig 7). Although it appears that the 3-*O* deacylation reaction is not critical for symbiosis, it remains a possibility that 3-*O* deacylation of lipid A could be achieved by an alternative mechanism in the nodular environment.

In conclusion, we have shown that *R. etli* possesses a PagL homolog that is responsible for generating the tetraacylated species of lipid A. The *R. etli* homolog shares distant homology with other PagL homologs. It is, however, unique among the described PagL proteins in its requirement for divalent cations. Despite the apparent up-regulation of PagL in *R. etli* bacteroids, the modification catalyzed by PagL does not appear to be essential for nodule development and nitrogen-fixation. The 3-*O* deacylation reaction, like other lipid A modification reactions, could play a role in protecting bacterial symbionts from the plant immune response during pathogenic infections [18, 36, 48]. PagL activity in Gram-negative pathogens has been linked to an increase in resistance to antimicrobial peptides [49]. In this study, however, we did not observe any differences in polymyxin sensitivities between wildtype *R. etli* CE3 and the *pagL* mutant strain when the strains were grown as free-living bacteria. More significant differences between the two strains in terms of antimicrobial resistance may be seen in the *in planta* environment. Future work will address this issue.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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We dedicate this paper to the memory of coauthor Christian R. H. Raetz (1946–2011). We thank Dr. Ziqiang Guan for assistance with mass spectrometry.

Abbreviations

The abbreviations used are

BCA	bicinchoninic acid
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
Kdo	3-deoxy-D- <i>manno</i> -oct-2-ulosonic acid
LC	liquid chromatography
LPS	lipopolysaccharide
MES	2-(N-morpholino)-ethanesulfonic acid
MS	mass spectrometry
PBS	phosphate-buffered saline
TLC	thin layer chromatography
TLR-4	Toll-like receptor-4

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version.

Highlights

- The gene coding for the *R. etli* lipid A 3-*O* deacylase is a homolog of PagL
- The function of *R. etli* PagL is confirmed using *in vitro* assays
- *R. etli pagL* mutants are exclusively pentaacylated
- *R. etli pagL* mutants display a slower nodulation phenotype relative to wildtype

Fig 1. Structures of the major lipid A species present in *E. coli* and *R. etli* and proposed reaction catalyzed by *R. etli* PagL

Panel A. The predominant lipid A moiety of *E. coli* LPS consists of a hexa-acylated disaccharide of glucosamine, substituted with monophosphate groups at the 1- and 4'-positions. **Panel B.** The major lipid A species in *R. etli* lack phosphate substituents and are more heterogeneous with respect to fatty acyl chain length [7, 8]. Components B and C are constructed around the typical glucosamine disaccharide found in the lipid A of other Gram-negative organisms. Components D and E feature an aminogluconate unit in place of the proximal glucosamine residue, derived by LpxQ-catalyzed oxidation of B and C respectively [19, 20]. All *R. etli* lipid A species contain a galacturonic acid moiety at position-4' in place of the more typical monophosphate group, and they also contain an unusual C28 secondary acyl chain that may be further derivatized at the 27-OH moiety with a β -hydroxybutyrate group [10]. Components C and E differ from B and D by the absence of a hydroxyacyl chain at position 3, which is proposed to be removed by the deacylase PagL [21]. Dashed bonds highlight the most prominent micro-heterogeneity of *R. etli* lipid A with respect to its acyl chains and the presence of the β -hydroxybutyrate substituent.

Fig 2. Alignment of selected PagL sequences

Comparison of the *P. aeruginosa* 3-*O*-deacylase (PagL) protein sequence with the PagL sequences from *Rhizobium etli* and *Sinorhizobium meliloti*. The protein sequences were aligned using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Black and gray boxes indicate conserved and similar residues, respectively. Asterisks indicate amino acid residues conserved in all known PagL sequences. The conserved residues proposed to make up the active site (Ser-His-Glu) and oxyanion hole (Asn) of *P. aeruginosa* PagL are indicated by Xs. The GenBankTM protein accession numbers for the genes are as follows: *S. meliloti*, NP_385884; *R. etli*, ABC91065; *P. aeruginosa* NP_253350.

Fig 3. 3-O-deacylase activity and calcium dependency of *R. etli* and *E. coli* membranes expressing *R. etli* PagL and *S. meliloti* PagL

Panel A. Membranes of the indicated strains were assayed for 3-O deacylase activity. The assay was carried out in 50 mM MES, pH 6.25, 1.0% Triton X-100, 2 mM dithiothreitol, 2 mM EDTA, 20 mM CaCl₂, and 10 μ M [4'-³²P]lipid IV_A (1,000 cpm/ μ L), and the membranes were used at 1 mg/mL. After incubation for 1 h at 30 °C, the products were separated by thin layer chromatography and detected with a PhosphorImager. NE: no enzyme; R. etli: *R. etli* membranes; *E. coli*: W3110 membranes; BLR pET23: BLR(DE3)/pET23(a)+ membranes; BLR RePagL: BLR(DE3)/RePagL membranes; BLR Sm PagL: BLR(DE3)/SmPagL membranes. **Panel B.** *E. coli* cells expressing RePagL were assayed at 0.01 mg/mL in 50 mM HEPES, pH 8, 0.1% Triton, 0.5 M NaCl, and 10 μ M [4'-³²P] lipid IV_A (1,000 cpm/ μ L). The concentration of calcium chloride was varied as indicated. Reaction mixtures were incubated for 30 min at 30 °C, after which product formation was analyzed by thin layer chromatography and PhosphorImager analysis.

Fig 4. Absence of 3-*O* deacylase activity in the PagL deletion strain CS4

Membranes of the indicated strains (1.0 mg/mL) were assayed for 3-*O* deacylase activity at 30 °C with 10 μM [4'-³²P]-lipid IV_A (1,000 cpm/μL) under the assay conditions described in Fig 3A. The products were separated by TLC followed by detection with a Phosphorimager. NE: No enzyme; *R. etli*: *R. etli* wild-type; CS4 ΔpagL: *R. etli* ΔpagL; BLR *Re* PagL: BLR(DE3)/RePagL membranes; BLR *Sm* PagL: BLR(DE3)/SmPagL membranes.

Fig 5. TLC analysis of lipid A species released by acetic acid hydrolysis from wild-type and *pagL* mutants

Approximately 5 μ g of the lipid A released from wild-type *R. etli* cells or the *pagL* mutant, CS4, was spotted onto a TLC plate, which was developed in the solvent system $\text{CHCl}_3/\text{MeOH}/\text{H}_2\text{O}/\text{NH}_4\text{OH}$ (40:25:4:2, v/v). The lipids were detected after chromatography by spraying with 10% sulfuric acid in ethanol and charring on a hot plate. The labeled lipid A species correspond with the structures shown in Fig 1B and Supplementary Fig 1.

Fig 6. Normal phase LC/MS of lipid A components from *R. etli* wild-type and the PagL knockout strain CS4

The lipid A from the wild-type and PagL deletion strain was extracted as described and analyzed by normal phase LC/MS. **Panel A.** In the spectrum of the wild-type strain, the major lipid A species eluting between 16 and 21 min is shown. The cluster of ions between 1860 amu and 2000 amu correspond to a pentaacylated form of lipid A (component B, Fig. 1B) and the cluster of ions between 1620 amu and 1800 amu correspond to tetraacylated lipid A species (components C and A). Component C (Fig. 1B) is derived from the PagL catalyzed deacylation reaction of component B. Component A (Supplementary Fig 1), a tetra-acylated form of lipid A that is formed non-enzymatically from components D during the lipid A extraction procedure is present in the same mass region. Heterogeneity in fatty acid chain length (14 or 28 atomic mass units) and partial substitution with β -hydroxybutyrate (86 atomic mass units) is observed for all lipid A components. **Panel B.** In the spectrum of the PagL mutant (CS4), the major lipid A species eluting between 16 and 21 min are shown. The mutant strain accumulates the cluster of ions between 1860 and 2000 corresponding to component B but only contains one tetraacylated species (component A) in the region between 1620 amu and 1800. The mutant strain lacks the enzymatically derived lipid A species, component C. **Panel C.** The lipid A species eluting between 19 and 21.5

min are shown for the wildtype strain. The cluster of ions shown correspond to component C, which is derived from PagL activity. **Panel D.** The lipid A species eluting between 19 and 21.5 min are shown for the *pagL* mutant strain. Component C does not accumulate in the mutant strain.

Fig 7. Nodule formation by *R. etli* CE3 and *PagL* mutant CS4

The nodules on *P. vulgaris* roots inoculated with wild-type CE3 and *pagL* deficient CS4 were scored at the indicated days after inoculation. The number of nodules and their color are indicated. The nodules normally start out as white, then transition to beige, then red, and finally turn senescent. Actively nitrogen-fixing nodules are red.

TABLE 1

Bacterial strains and plasmids used in this work

Strain or Plasmid	Genotype or Description	Source or reference
Strains		
<i>R. etli</i> CE3	Spontaneous Sm ^r derivative of wild-type strain CFN42, NaI ^r	[50]
<i>R. etli</i> CE3 derivatives		
CS4	<i>pagL</i> mutant, spectinomycin-resistant	this work
CS513	$\Delta lpxE/\Delta lpxF$ mutant, spectinomycin and gentamicin-resistant	[36]
<i>E. coli</i>		
DH5 α	<i>recA1</i> ϕ 80 <i>lacZ</i> Δ M15	[32]
BLR(DE3)	B strain, DE3 lysogen	EMD
W3110	Wild type, F ⁻ , λ -	CGSC
Plasmids		
pRK2013	helper plasmid, kanamycin/neomycin-resistant	[51]
pK18 <i>mobsacB</i>	suicide vector, kanamycin/neomycin-resistant	[52]
pUC18	cloning vector, carbenicillin-resistant	[53]
pHY109	spectinomycin resistance-conferring Ω interposon cloned in the <i>EcoRI</i> restriction sites of plasmid pCHO341	[54]
pET23(a)+	<i>T7lac</i> expression vector, Amp ^r	EMD
RePagL	<i>R. etli</i> PagL in pET23(a)+, Amp ^r	this work
SmPagL	<i>S. meliloti</i> PagL in pET23(a)+, Amp ^r	this work

Abbreviations: Sm^r-Streptomycin sulfate-resistant, NaI-Nalidixic acid resistant, Amp^r-Ampicillin resistant

Table 2Masses of lipid A components from *R. etli* wild-type and PagL knockout strain

Component	B	C	A
Molecular formula	C ₁₁₀ H ₂₀₄ N ₂ O ₂₇	C ₉₆ H ₁₇₈ N ₂ O ₂₅	C ₉₆ H ₁₇₄ N ₂ O ₂₄
Exact Mass	1985.47	1759.27	1739.25
Observed m/z [M-H] ⁻¹	1984.46	1758.26	1738.24

Table 3Polymyxin sensitivity of *R. etli* CE3 mutants

Strain	<u>Zone of clearing diameter (mm)^a</u>		
	Amount of Polymyxin per disk		
	(10 ug)	(20 ug)	(100 ug)
<i>R. etli</i> CE3	10 ± 0.6	11.6 ± 0.5	16.5 ± 0.5
CS4 ($\Delta pagL$)	9.6 ± 0.5	11.7 ± 0.3	16.1 ± 0.4
CS513 ($\Delta lpxE/\Delta lpxF$)	14.6 ± 0.3	15.8 ± 0.4	19.2 ± 0.3

^aThe diameter of the disk is 6 mm, and the diameter of the zone of clearing shown above includes the disk diameter. Results are the average of six replicate experiments.