

# Elucidation of the 3-O-Deacylase Gene, *pagL*, Required for the Removal of Primary $\beta$ -Hydroxy Fatty Acid from the Lipid A in the Nitrogen-fixing Endosymbiont *Rhizobium etli* CE3\*<sup>§</sup>

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**Background:** *R. etli* lipid-A de-O-acylation occurs during symbiosis.

**Results:** *R. etli* produces PagL, which de-O-acylates lipid A, and is active on *E. coli* lipid A, and a *pagL* mutant has a symbiotic defect.

**Conclusion:** *R. etli* CE3 contains PagL; PagL acts on diverse lipid A structures and may be needed for proper *R. etli*-bean symbiosis.

**Significance:** PagL may be important for proper infection of the bean host plant.

Until now, the gene responsible for the 3-O-deacylation of lipid A among nitrogen-fixing endosymbionts has not been characterized. Several Gram-negative animal pathogens such as *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Bordetella bronchiseptica* contain an outer membrane 3-O-deacylase (PagL) that has been implicated in host immune evasion. The role of 3-O-deacylated lipid A among nitrogen-fixing endosymbionts, plant endophytes, and plant pathogens has not been studied. However, D'Haese *et al.* (D'Haese, W., Leoff, C., Freshour, G., Noel, K. D., and Carlson, R. W. (2007) *J. Biol. Chem.* 282, 17101–17113) reported that the lipopolysaccharide from *Rhizobium etli* CE3 bacteroids isolated from host bean root nodules contained exclusively tetraacylated lipid A that lacked a lipid A  $\beta$ -hydroxymyristyl residue, an observation that is consistent with the possibility of PagL activity being important in symbiosis. A putative *pagL* gene was identified in the *R. etli* genome sequence. With this information, we created a *pagL*<sup>−</sup> mutant strain derived from *R. etli* CE3. Using mass spectrometry, we demonstrated that the mutant lacks 3-O-deacylated lipid A. The parent and mutant LPS were very similar as determined by gel electrophoresis and glycosyl composition analysis using gas chromatography/mass spectrometry. However, fatty acid analysis showed that the mutant lipid A contained larger amounts of  $\beta$ -hydroxypentadecanoic acid than that of the parent. Furthermore, the mutant was adversely affected in establishing symbiosis with its host, *Phaseolus vulgaris*.

*garis* (common black bean). Nitrogen-fixing endosymbiosis is initiated through chemical signaling between the symbiont (bacteria) and legume host, which leads to organogenesis of root nodules and the internalization of the bacteria. The invading symbionts travel through infection threads to the root nodule where they are endocytosed. After endocytosis, the bacteria develop into bacteroids within plant-derived peribacteroid membranes and form nitrogen-fixing symbiosomes. For a current review on legume symbiosis, refer to Oldroyd and Downie (1).

Surface and secreted bacterial polysaccharides are necessary for complete symbiosis (2, 3). LPS, a surface polysaccharide located in the outer membrane, is the focus of this study. Partial disruption of LPS biosynthesis (*e.g.*, O-chain biosynthesis and lipid A fatty acid acylation) leads to disruption of proper symbiosis in many nitrogen-fixing symbiotic systems (4–8). For a detailed review on the role of LPS in symbiosis, see Carlson *et al.* (4). The general structure of *R. etli* CE3 LPS has been extensively studied (4, 9–11). D'Haese *et al.* (9) determined that subtle structural changes occurred to the O-chain polysaccharide of *R. etli* CE3 bacteroid LPS when compared with that of free living CE3 whereby a single methyl group was added to the 2-position of a fucosyl residue. In addition, the lipid A of CE3 bacteroid LPS was exclusively tetraacylated as compared with the free living lipid A, which contained a mixture of penta- and tetraacylated lipid A. It was speculated that tetraacylated lipid A resulted from the removal of a primary  $\beta$ -hydroxymyristoyl residue located at position 3 of the proximal glucosamine (or 2-aminogluconoate) residue by a 3-O-deacylase. Indeed, Basu *et al.* (12) reported 3-O-deacylase activity in the membranes of a number of symbionts including *R. etli* CE3. The outer membrane 3-O-deacylase enzyme PagL has been reported in a number of Gram-negative pathogens including *Salmonella typhimurium*, *Salmonella enterica*, *Pseudomonas aeruginosa*, and *Bordetella bronchiseptica* (13) and has been implicated in the evasion of the host immune response (14, 15). It is possible that plant symbionts, endophytes, and pathogens modify their lipid A in a similar way to promote infection.

The Gram-negative endosymbiont *Rhizobium etli* forms nitrogen-fixing symbiosis with the legume host *Phaseolus vul-*

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**TABLE 1**  
Strains and plasmids

Strains or plasmid	Characteristics	Source
<i>E. coli</i>		
Top 10	F <sup>-</sup> <i>mcrAΔ (mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(araleu) 7697 galU galK rpsL (Strr) endA1 nupG</i>	Invitrogen
Top 10/pRePagL	<i>E. coli</i> Top 10 containing plasmid pRePagL, Tc <sup>r</sup>	This study
<i>R. etli</i> bv. <i>phaseoli</i>		
CE3	Fix <sup>+</sup> , Nod <sup>+</sup> , Str <sup>r</sup>	Ref. 46
ODB31	<i>pagL::aacCI</i> , Fix <sup>+</sup> , Nod <sup>+</sup> , Str <sup>r</sup> , Gm <sup>r</sup>	This study
ODB31/pRePagL	Strain ODB31 harboring plasmid pRePagL, Fix <sup>+</sup> , Nod <sup>+</sup> , Str <sup>r</sup> , Gm <sup>r</sup> , Tc <sup>r</sup>	This study
<b>Plasmids</b>		
pUC18	Cloning vector, Am <sup>r</sup>	Fermentas
pMS255	Source of <i>Gm</i> cassette, Gm <sup>r</sup>	Ref. 20
pRK2013	Mobilizing plasmid for pEX18-Tc, Col E1 replicon, Kan <sup>r</sup>	Ref. 34
pEX18-Tc	Suicide vector, allows positive selection for integration, Tc <sup>r</sup>	Ref. 21
pRK404E1	Broad host range shuttle vector for, Tc <sup>r</sup>	Ref. 22
pPagL-Up	pUC18 containing the upstream region and 5' end of <i>pagL</i> , Am <sup>r</sup>	This study
pPagL-Dwn	pUC18 containing the downstream region and 3' end of <i>pagL</i> , Am <sup>r</sup>	This study
pPagL-UpDwn	pUC18 containing the inserts from pPagL-Up and pPagL Dwn, ( <i>pagLΔ100bp</i> ), Am <sup>r</sup>	This study
pPagL-UpGmDwn	<i>Gm</i> cassette ( <i>aacCI</i> ) from pMS255 cloned into pPagL-UpDwn, Am <sup>r</sup> , Gm <sup>r</sup>	This study
pRePagL-KO	Insert from pPagL-UpGmDwn cloned into pEX18-Tc, Tc <sup>r</sup> , Gm <sup>r</sup>	This study
pRePagL	<i>pagL</i> region from strain CE3 PCR cloned into pRK404E1, Tc <sup>r</sup>	This study

To understand the role of PagL concerning symbiosis, pathogenesis, and endophytic association with agriculturally and economically significant host plants, it is important to locate and characterize the gene that encodes PagL in these organisms. Although the membranes from *R. etli* CE3 demonstrated 3-*O*-deacylase activity *in vitro* (12), the isolated enzyme has not been characterized, and consequently, the amino acid sequence and the gene encoding PagL are not known. Furthermore, *R. etli* PagL could not be identified by amino acid sequence alignment with known PagL proteins because of low sequence identity. The crystal structure of the *P. aeruginosa* PagL was solved (16), and a conserved domain was elucidated that directly enabled us to identify a putative *pagL* gene in the *R. etli* CE3 genome (17). Therefore, we hypothesized that this gene is responsible for 3-*O*-deacylation of *R. etli* CE3 lipid A. In addition, we have identified *R. etli* CE3 PagL homologues in the genomes of a number of endosymbionts including *Sinorhizobium meliloti*, *Sinorhizobium medicae*, *Sinorhizobium fredii*, *Rhizobium leguminosarum*, *R. etli*, and *Methylobacterium nodulans*, as well as the plant pathogens of the genus *Agrobacterium* and the poplar tree endophyte *Methylobacterium populi* (18) (supplemental Fig. S1).

In this study, we mutated the *pagL* gene via insertion mutagenesis in the model organism *R. etli* CE3 and determined its functional involvement in the 3-*O*-deacylation of lipid A. We further characterized the effect of the mutation on LPS synthesis and symbiosis with the host plant *P. vulgaris*.

## EXPERIMENTAL PROCEDURES

**Bacterial Strain, Plasmids, and Growth Conditions**—Bacterial strains and plasmids are described in Table 1. *Escherichia coli* strains were grown on LB medium at 37 °C. *Rhizobium* strains were grown on tryptone yeast medium containing 10 mM CaCl<sub>2</sub> or Y minimal medium containing 2% sucrose at 30 °C (19). Antibiotics were added in the following concentrations where indicated: for *E. coli*, 15 μg/ml gentamicin, 10 μg/ml tetracycline, or 50 μg/ml ampicillin; and for *R. etli*, 30 μg/ml gentamicin or 20 μg/ml tetracycline.

**Mutagenesis of the 3-*O*-Deacylase Gene**—The putative 3-*O*-deacylase (*pagL*) gene from *R. etli* bv. *phaseoli* CE3 was mutated

using insertion mutagenesis. PCR cloning was used to create a DNA fragment with the disrupted *pagL* gene and homologous flanking regions to be cloned into a suicide plasmid. Conjugal transfer of the constructed plasmid into *R. etli* CE3 was performed, and double recombinants were selected. Plasmid extractions, gel extractions, and PCR/enzyme cleanup was performed using Qiagen mini prep kits. Restriction enzymes were purchased from Promega. PCR reagent (iProof<sup>TM</sup> high fidelity polymerase) was purchased from Bio-Rad.

A primer pair was engineered with XbaI (underlined) and BamHI (underlined) restriction sites (AGTCCCTCAGACCGT-TATTGCCGCTGT and AGTCGGATCCAGCTGTTGCTGCCAGC) to construct an ~1.1-kb PCR product containing 225 bp of the *pagL* gene (5' region) and 873 bp DNA upstream of the *pagL* gene. The PCR product was cloned into pUC18 to create plasmid pPagL-Up. A second PCR product was constructed using a primer pair engineered with BamHI (underlined) and XmaI (underlined) cloning sites to create an ~1.5-kb product containing 239 bp of the *pagL* gene (3' region) and 916 bp of downstream DNA. The product was cloned into pUC18 to create the plasmid pPagL-Dwn. The two inserts were tethered together by subcloning the insert from pPagL-Up into pPagL-Dwn (double digest with XbaI and BamHI) to create pPagL-UpDwn, which contained an insert region with upstream DNA, the *pagL* coding region (~100-bp deletion near the center of the *pagL* gene), and downstream DNA. The center of the *pagL* region was engineered with a BamHI cloning site. The gentamicin-resistant cassette *aacCI* from plasmid pMS255 (20) was cloned into the *pagL* region at the BamHI cloning site to create plasmid pPagL-UpGmDwn. The insert from pPagL-UpGmDwn was subcloned into the suicide vector pEX18-Tc (21), which contains the *sacB* lethal gene (double digest with XbaI and XmaI) to create pPagL-KO. The plasmid pPagL-KO was moved into *R. etli* CE3 via tri-parental mating as described previously (6). A *pagL*<sup>-</sup> mutant was obtained and was given the strain number *R. etli* ODB31. A construct containing the *pagL* gene was created to complement the mutant strain. The *pagL* gene was PCR-cloned into plasmid pRK404E1 (22) to create plasmid pRePagL using a forward primer engineered with a

## Rhizobium Lipopolysaccharide Modification

BamHI cloning site (underlined) and homologous DNA starting 14 bp upstream of the *pagL* gene (ATCGATGGATCCGC-TTGGTGC GAATCAAAG). The reverse primer was engineered with an EcoRI cloning site (underlined) that recognized homologous DNA 30 bp downstream of the *pagL* coding region (ATCGATGAAATTCGCAGGCTTTTCTCGTAAAG). The insert was oriented in parallel with the lac promoter of pRK404E1 to promote transcription. Plasmid pRePagL was moved into strain ODB31 via tri-parental mating as described above. All of the strains were verified by PCR with the above primers that were used to create pRePagL (see Fig. 1). In addition, the *pagL* gene containing the native promoter was PCR-cloned into the EcoRI and HindIII cloning sites of pRK404E1 using the primer set AAGTCAGAGAATTCCAGGAATTGACCGC-AGGC and CATTAGGCAAGCTTGTGATCGGGATGGTG-TTTGAA. The *pagL* gene was oriented anti-parallel to the pRK404E1 lac operon.

**Lipopolysaccharide Isolation and Analysis**—Briefly, LPS was extracted by the hot water phenol extraction method (23) and dialyzed against deionized water using 12,000–14,000 molecular weight cutoff dialyzer bags and subsequently lyophilized. Composition of LPS was determined by GC/MS of trimethylsilyl-derived methyl glycosides and fatty acid methyl esters as described previously (10, 24) using a Hewlett Packard 5890 Series II GC/MS analyzer fitted with a 30-m DB-1 column (J&W Scientific).

**Matrix-assisted Desorption Ionization of Lipid A**—Lipid A was isolated by mild acid hydrolysis as described previously (25). Briefly, ~5 mg of extracted LPS was treated with 1% acetic acid (100 °C, 1 h) to partition the lipid A. Partitioned Lipid A was separated by 3× chloroform/methanol/water (2:2:1.8, v/v/v) extraction. The lipid A containing chloroform layers were pooled and dried under a stream of air. Isolated lipid A was dissolved in a 3:1 (v/v) chloroform/methanol mixture, mixed 1:1 (v/v) with 2,4,6-trihydroxyacetophenone matrix and spotted on a MALDI plate. Spectra were acquired by a MALDI-TOF analyzer (Applied Biosystems, 5800) in the positive reflectron mode operating at a 20 kV extraction voltage.

**Deoxycholic Acid-PAGE and Silver Stain of Lipopolysaccharide**—Polyacrylamide gel electrophoresis in the presence of deoxycholic acid (DOC)<sup>3</sup> was adapted from Reuhs *et al.* (26). A loop full of bacteria grown on TY agar medium was dissolved in 100 µl of lysis buffer (1% SDS, 50 mM Tris, and 10 mM EDTA) and incubated at 100 °C for 10 min to release LPS from the cell envelope. The sample was microcentrifuged (14,000 rpm) to pellet cell debris. The supernatant (containing LPS) was mixed 1:1 with sample loading buffer, and 2 µl was loaded for DOC-PAGE. The gel was silver-stained as described previously (27).

**Plant Growth and Inoculation**—Black turtle beans (lot HP-BTB-04) from Wheatgrasskits.com (Springville, UT) were surface-sterilized in 95% ethanol for 1 min followed by three rinses in sterile deionized water. The beans were further sterilized in 5% hypochlorite (2 min) followed by 10 rinses in sterile deionized water. Then the beans were transferred to 0.8% tap water agar plates and germinated in the dark for 3 days at room tem-

perature. The sprouts were transferred to sterile 500-ml Erlenmeyer flasks containing a foam top, hydroponic beads immersed in defined, nitrogen-free liquid plant medium (Fahreus medium, (28)) and surrounded with a brown paper bag. The plants were kept in a Conviron growth chamber and were maintained at 24 °C with 14 h of light and 20 °C with 10 h of darkness. Bacteria used as inoculants were grown to mid/late log phase in liquid TY medium containing appropriate antibiotics. The bacteria were pelleted, washed in sterile phosphate-buffered saline (pH 7.4), and resuspended to an approximate A of 0.15 (600 nm wave length). Then 1 ml of inoculate was added to the plants 11 days after germination. Plant phenotypes were observed and scored. The experiment was repeated three times. 30 plants per inoculate strain was used in one experiment, and 15 plants per inoculate strain was used in subsequent experiments.

**Acetylene Reduction Assay**—The plants were inoculated with appropriate strains as described above and assessed for their ability to fix nitrogen by the acetylene reduction assay as previously described (6). Statistical analysis was performed using the Student's *t* test.

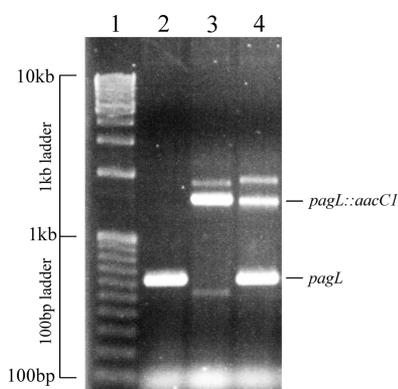
## RESULTS

**Creation of the *pagL*<sup>-</sup> Mutant in *R. etli* CE3**—To identify the gene responsible for 3-*O*-deacylase activity in *R. etli* and study its function, the putative *pagL* gene was mutated. The putative *pagL* gene (accession number ABC91065.1) in *R. etli* CE3 (isogenic to *R. etli* CFN 42) is located on the chromosome and contains the conserved PagL catalytic domain (16, 17) and predicted outer membrane protein structure. The *pagL*<sup>-</sup> mutant was created via insertion mutagenesis as described under “Experimental Procedures” and was given the strain name *R. etli* ODB31. To validate phenotypic differences observed between the mutant and parent strain, the mutant ODB31 was complemented with the native *pagL* gene from parent strain CE3 (Fig. 1).

**Lipopolysaccharide Composition of the *pagL*<sup>-</sup> Mutant**—To study differences between parent and mutant strain LPS, the composition of each LPS was determined. The LPS of *R. etli* CE3 has been studied extensively, and its structure is known (4, 9, 29). When compared with parent strain CE3, the mutant ODB31 contained the known glycosyl residues found in the O-chain polysaccharide (GlcA, *N*-acetylquinovosamine, 3-*O*-methyl-6-deoxytalose, Man, and fucose), the core (Man, Gal, galacturonic acid, and 3-deoxy-*D*-manno-2 octulosonic acid), and lipid A (GlcN and galacturonic acid). In addition, the DOC-PAGE profiles of parent and mutant LPS displayed similar banding patterns (Fig. 2). These results suggest that the mutant maintained the general carbohydrate structure as the parent strain LPS. The removal of fatty acids by PagL produces LPS with a lower molecular weight. Because the mutant does not have a functional PagL, the LPS molecules migrate at a slightly higher molecular weight when compared with parent and complemented strain (Fig. 2).

**Mass Spectroscopic Analysis of the *pagL*<sup>-</sup> Mutant Lipid A**—Mass spectroscopic analysis of mutant ODB31 lipid A was performed to characterize the loss of 3-*O*-deacylase activity on its structure. The spectra are shown in Fig. 3, and proposed com-

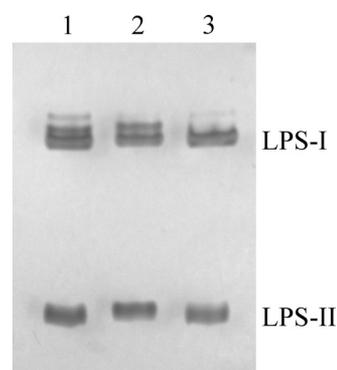
<sup>3</sup> The abbreviations used are: DOC, deoxycholic acid; dpi, days post-inoculation; LRR, leucine-rich repeat; TLR, Toll-like receptor.



**FIGURE 1. PCR analysis of the *pagL*<sup>-</sup> mutant and complemented strains.** Lane 1, DNA ladder. Lane 2, parent strain CE3. Lane 3, mutant strain ODB31. Lane 4, complemented mutant strain. The predicted size of the native PCR product (lane 2) is 635 bp. The predicted size of the mutant PCR product (lane 3) containing the *aacC1* insert is 1531 bp. The mutant complemented strain (lane 4) contains both PCR native and mutated PCR product.

positions for the various ions are listed in Table 2. The spectrum obtained from parent strain lipid A (Fig. 3, panel I) contains two major ion groups, each displaying a mixture of microheterogeneous ions. Microheterogeneity occurs because of variation in acyl chain length ( $\pm 28$  mass units [ $C_2H_4$ ] or  $\pm 14$  mass units [ $CH_2$ ]), the presence of  $\beta$ -hydroxybutyryl group ( $\pm 86$ ) at the C27 position of 27-hydroxyoctacosanoic acid, and the oxidation of the proximal glucosamine to 2-aminogluconate ( $\pm 16$ ). The higher molecular weight ion group,  $m/z$  1914–2022, represents penta-acylated lipid A, whereas the lower molecular weight ion group,  $m/z$  1624–1774, represents tetra-acylated lipid A. Two forms of tetraacylated lipid A are present. One form is an artifact of the isolation process caused by the acid-catalyzed  $\beta$ -elimination of the 3-*O* linked primary fatty acid during the mild acid hydrolysis procedure as reported by Que *et al.* (30). This occurs on lipid A species in which the proximal 2-aminogluconate residue forms a lactone during mild acid hydrolysis of the LPS. The resulting structure(s) on MS analysis, structure(s) C, show ions of  $m/z$  1738.7, 1710.6, 1652.5, and 1624.4 (Fig. 3). The second tetraacylated lipid A is the product of PagL activity that results from the hydrolysis of the 3-*O*-linked hydroxyl fatty acid by 3-*O*-deacylase (PagL) leaving a hydroxyl group at the C3 position of the proximal glucosamine/2-aminogluconate (Fig. 3, structures D and E). The resulting ions for D and E structures are  $m/z$  1644.5, 1660.6, 1672.5, 1688.7, 1700.5, 1728.7, 1730.5, 1746.7, 1758.7, 1768.7, 1786.7, 1774.7, 1796.7, and 1802.7.

The parent strain CE3 produced a mixture of ions consistent with A, B, C, and D structures, whereas ions caused by E structures were low in intensity (Fig. 3, panel I, and Table 2). The lipid A from the mutant ODB31 did not contain detectable ions consistent with structures D or E (Fig. 3, panel II, and Table 2). However, structures A, B, and C were present. The lipid A from the complemented mutant recovered the presence of D and E structures (Fig. 3, panel III, and Table 2). Because the expected product resulting from PagL activity is structure D, these results strongly suggest that the *R. etli* CE3 *pagL* gene product is responsible for the removal of a primary  $\beta$ -hydroxy fatty acid from the lipid A.



**FIGURE 2. DOC-PAGE analysis of mutant LPS.** Lane 1, parent strain CE3. Lane 2, mutant strain ODB31. Lane 3, complemented mutant strain. LPS-I, high molecular weight LPS species containing lipid A, core, and O-chain. LPS-II, low molecular weight LPS species containing lipid A and core.

It was noticed that the relative intensities of the D and E to the A, B, and C ions for the complemented mutant lipid A were significantly greater than for the parent lipid A, suggesting that the PagL activity in the complemented mutant may be greater than in the parent. In fact the D:B ion intensity ratio for the parent lipid A was 1.4, whereas this ratio for the complement lipid A was 3.1, indicating that the PagL activity in the complement was two to three times greater than in the parent. It was thought that this may be due to the fact that the complement PagL was under lac promoter control rather than the *R. etli* native promoter. Therefore, a complement was prepared using a plasmid that contained *pagL* with its native promoter. This complement also gave results indicating that it had more PagL activity than the parent, *i.e.*, repeated isolation of the parent and mutant lipids A, and comparison with the native promoter *pagL* complement showed a D:B ratio of 1.1, 0.0, and 3.8, respectively, indicating that this complement had 3-fold greater PagL activity than the parent (data not shown). It seems possible that for both complements the increase in PagL activity may be due to the presence of multiple copies of the *pagL*-containing plasmid rather than due to a difference in the lac versus native promoter.

*E. coli* LPS Is Deacylated by the *R. etli* PagL Enzyme—The *Rhizobium* 3-*O*-deacylase, PagL, has less than 20% amino acid sequence similarity when compared with the PagL of enteric bacteria *Salmonella* spp. and *P. aeruginosa*. Enteric bacteria share a similar general lipid A structure defined by a bis-phosphorylated diglucosamine backbone containing four  $\beta$ -hydroxymyristic primary fatty acids at the 2', 3', 2, and 3 positions of the distal and proximal GlcN residues, respectively. The 2' and 3' primary fatty acids contain ester-linked myristoyl and lauroyl substituents, respectively, at their  $\beta$ -OH groups creating the acyloxyacyl lipid A moieties (Fig. 4, structure A). A 3-*O*-deacylase has not been detected in *E. coli*, and this is consistent with the fact that the *E. coli* genome does not contain a PagL homologue. Therefore, the *R. etli* PagL was introduced into the *E. coli* Top 10 cloning strain (*E. coli* Top 10/pRePagL) to determine whether *R. etli* PagL can de-*O*-acylate *E. coli* lipid A.

Isolated *E. coli* LPS was subjected to the mild acid hydrolysis procedure as described under "Experimental Procedures" and subjected to MALDI-TOF MS analysis. The mass spectrum of the lipid A from the parent *E. coli* strain (Fig. 4, panel I) contains

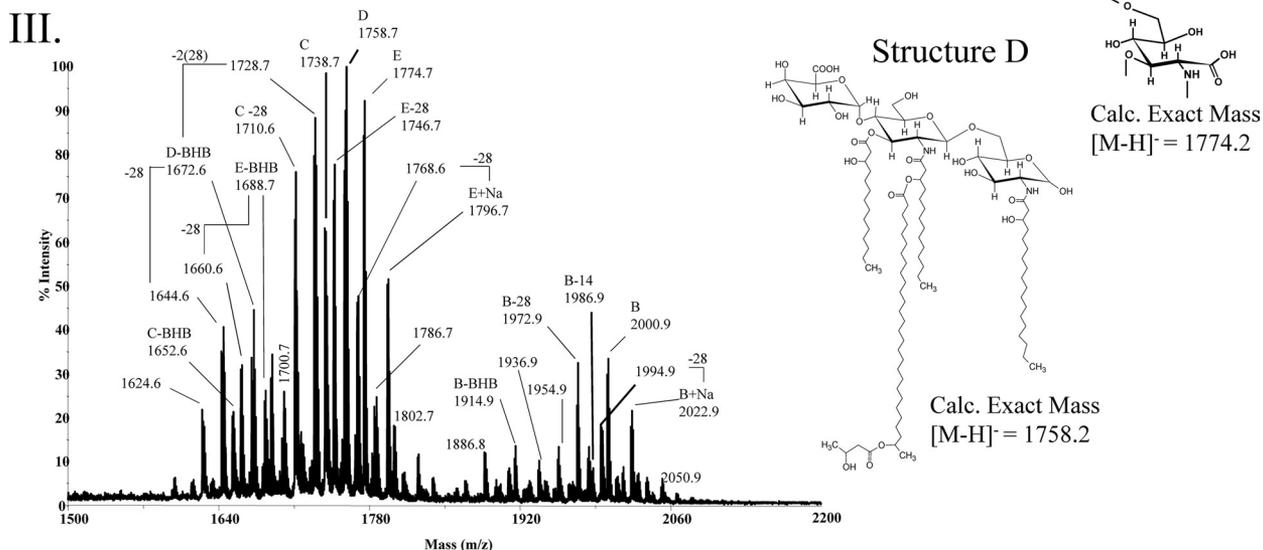
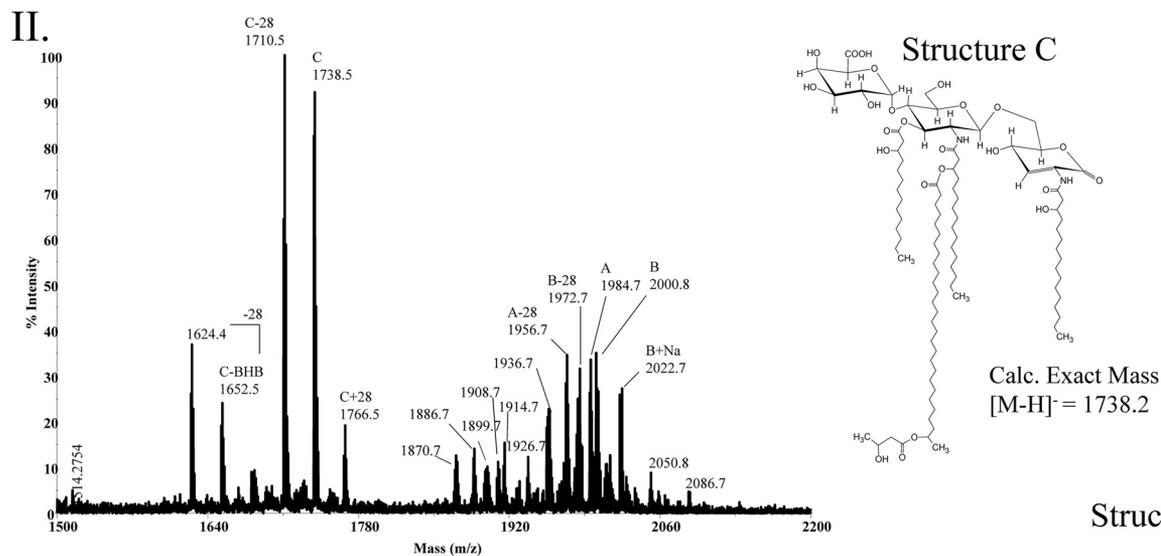
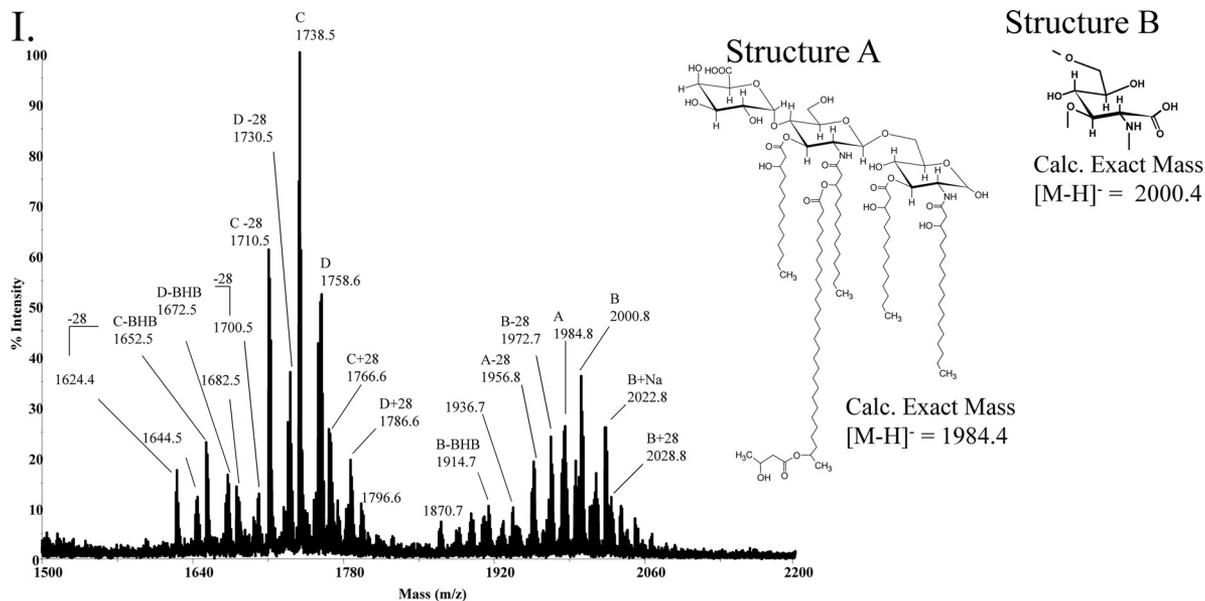


TABLE 2

Lipid A ions ( $[M-H]^-$ ) observed in the MALDI-TOF-MS spectra and their predicted compositionsCalibration was done using exact mass values, and the calculated values are exact mass values. BHB,  $\beta$ -hydroxybutyrate.

Observed	Calculated	Type <sup>a</sup>	Proposed compositions	CE3	ODB31
1984.8	1984.4	A <sup>a</sup>	GalA <sub>1</sub> GlcN <sub>2</sub> BOHC14:0 <sub>3</sub> BOHC18:0 <sub>1</sub> BHBC28:0 <sub>1</sub>	+	+
2000.8	2000.4	B <sup>a</sup>	GalA <sub>1</sub> GlcN <sub>1</sub> GlcNonate <sub>1</sub> BOHC14:0 <sub>3</sub> BOHC18:0 <sub>1</sub> BHBC28:0 <sub>1</sub>	+	+
1738.7	1738.2	C <sup>a</sup>	GalA <sub>1</sub> GlcN <sub>1</sub> 2,3-unsaturated GlcN(acyl)onolactone <sub>1</sub> BOHC14:0 <sub>2</sub> BOHC18:0 <sub>1</sub> BHBC28:0 <sub>1</sub>	+	+
1758.7	1758.2	D <sup>a</sup>	GalA <sub>1</sub> GlcN <sub>2</sub> BOHC14:0 <sub>2</sub> BOHC18:0 <sub>1</sub> BHBC28:0 <sub>1</sub>	+	-
1774.7	1774.2	E <sup>a</sup>	GalA <sub>1</sub> GlcN <sub>1</sub> GlcNonate <sub>1</sub> BOHC14:0 <sub>2</sub> BOHC18:0 <sub>1</sub> BHBC28:0 <sub>1</sub>	+	-
1956.7	1956.4	A	A - 28 (C <sub>2</sub> H <sub>4</sub> )	+	+
1936.9	1936.4	B	B - 86 (BHB) + 22 (Na); i.e. an [MNa-H] <sup>-</sup> ion.	+	+
1926.7	1926.8	A	A - 86 (BHB) + 28 (C <sub>2</sub> H <sub>4</sub> )	+	+
1914.9	1914.4	B	B - 86 (BHB)	+	+
1886.8	1886.3	B	B - 86 (BHB) - 28 (C <sub>2</sub> H <sub>4</sub> )	+	+
1802.7	1802.2	E	E + 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1796.7	1797.2	E	E + 22 (Na)	+	-
1786.7	1786.3	D	D + 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1768.6	1769.2	E	E - 28 (C <sub>2</sub> H <sub>4</sub> ) + 22 (Na)	+	-
1746.7	1746.2	E	E - 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1730.5	1730.3	D	D - 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1728.7	1728.2	D	D - 86 (BHB) + (2) 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1710.6	1710.2	C	C - 28 (C <sub>2</sub> H <sub>4</sub> )	+	+
1700.5	1700.2	D	D - 86 (BHB) + 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1672.5	1672.2	D	D - 86 (BHB)	+	-
1688.7	1688.2	E	E - 86 (BHB)	+	-
1660.6	1660.2	E	E - 86 (BHB) - 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1652.5	1652.4	C	C - 86 (BHB)	+	+
1644.5	1644.2	D	D - 86 (BHB) - 28 (C <sub>2</sub> H <sub>4</sub> )	+	-
1624.4	1624.1	C	C - 86 (BHB) - (3) 28 (C <sub>2</sub> H <sub>4</sub> )	+	+

<sup>a</sup> Structure types A–E are shown in Fig. 3. Structure types D and E are those that result from PagL activity. Structure type C results from acid catalyzed  $\beta$ -elimination of the 3-O-acyl residue from the proximal GlcNonolactone during mild acid hydrolysis of the LPS, which converts that residue into 2,3-unsaturated GlcN(acyl)onolactone.

three major ion groups. The *E. coli* lipid A structure and the source of the observed ions are as indicated and are consistent with the normal hexaacylated lipid A observed in *E. coli* (31). The lower molecular weight ions are a result of dephosphorylation ( $-80$  mass units) and deacylation ( $-210$  [C14] and  $-226$  [ $-\beta$ -OHC14]), likely artifacts that occur during the mild acid hydrolysis procedure used to isolate the lipid A. When the *pagL* gene is introduced into *E. coli*, a clear shift of  $-226$  ( $\beta$ -hydroxymyristate, OHC14:0) mass units occurs for all three groups of ions (Fig. 4, panel II), e.g.,  $1796-226 \rightarrow 1570$ ,  $1586-226 \rightarrow 1360$ , and  $1360-226 \rightarrow 1134$  (this latter ion is not shown in Fig. 4), consistent with lipid A, which lacks a  $\beta$ -OHC14:0 residue. Only extremely low intensity ions caused by residual amounts of hexaacylated lipid A were observed in the *E. coli* Top 10/pRePagL lipid A (1795 mass units), indicating that the *R. etli* PagL was very active on the *E. coli* lipid A. Taken together with the lipid A analysis of the mutant strain ODB31, these results suggest that the *R. etli* PagL has a rather loose substrate specificity in that it can act on the structurally diverse *E. coli* and *R. etli* lipid A.

*The pagL<sup>-</sup> Mutant Displays an Aberrant Symbiotic Phenotype with the Host Plant P. vulgaris*—The ability of mutant ODB31 to form proper symbiosis with the host plant *P. vulgaris* was assayed by inoculating plants with bacteria as described under “Experimental Procedures.” Between 10 and 14 dpi, even though nodules were present, the mutant inoculated plants showed yellowing leaves indicative of nitrogen starvation, whereas plants inoculated with the parent strain maintained green leaves, as well as having root nodules. At 21 dpi, the leaves of the mutant inoculated plants maintained their

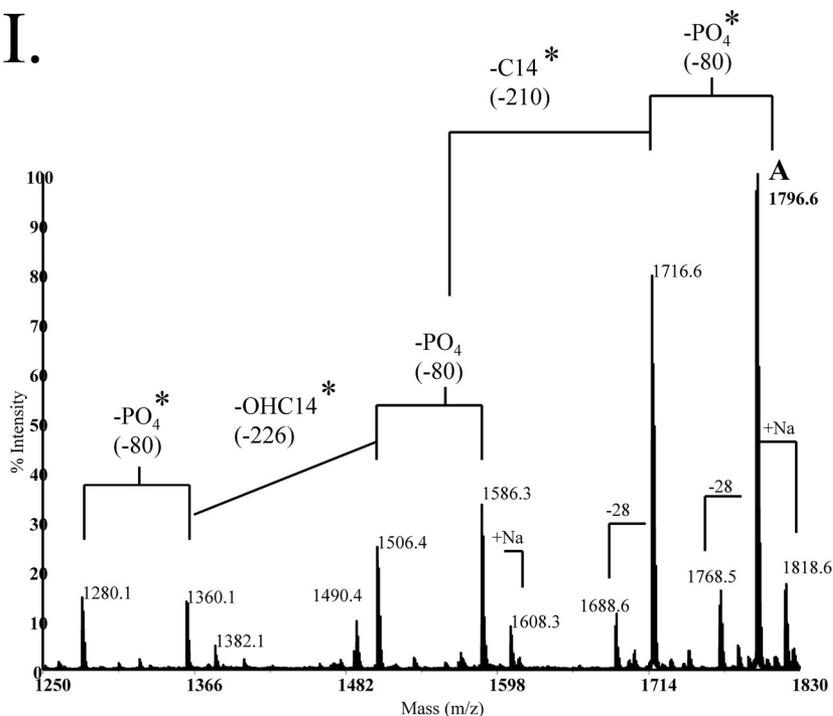
yellow color (Fig. 5), and although pink in color, the root nodules were considerably smaller compared with those found on plants inoculated with the parent strain (Fig. 5). The acetylene reduction assay was employed to determine how efficiently strain ODB31 fixed nitrogen during symbiosis (Fig. 6). At 15 dpi, the ODB31-inoculated plants were significantly impaired in nitrogen fixation compared with parent strain CE3-inoculated plants. By 22 dpi, the ODB31-inoculated roots fixed nitrogen at the same level as the CE3-inoculated roots and maintained comparable levels at 31 dpi. Unexpectedly, plants inoculated with the ODB31 *pagL* complemented strain maintained a similar phenotype as the plants inoculated with the mutant strain ODB31 (data not shown).

## DISCUSSION

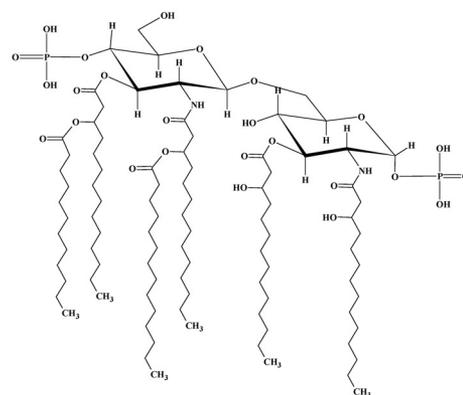
The lipid A 3-O-deacylase, PagL, has been characterized in a number of Gram-negative animal pathogens (13) and has been implicated in the evasion of the host immune response and resistance to antimicrobial peptides (14, 15, 32). It was determined *in vitro* that the membranes of several nitrogen-fixing endosymbionts contained 3-O-deacylase activity able to remove the 3-O linked  $\beta$ -hydroxymyristoyl moiety from the lipid IV<sub>A</sub> precursor molecule (12). In addition, mass spectrometric evidence suggests that the endosymbionts *R. etli* CE3 (9) and *S. fredii* NGR 234 (33), when grown under laboratory conditions, produce 3-O-deacylated lipid A molecules. Furthermore, *R. etli* CE3 bacteroids exclusively produce tetraacylated lipid A, presumably because of 3-O-deacylation (9). Therefore, we hypothesized that *R. etli* CE3 produces a PagL enzyme that may play a role in symbiotic infection.

FIGURE 3. MALDI-TOF mass spectrometric analysis of mutant lipid A. The spectra were obtained in the negative reflectron mode. Panel I, parent strain CE3. Panel II, mutant strain ODB31. Panel III, complemented mutant strain. The peaks are labeled with the predicted structures A–D. The general structures of A–D vary because of the presence or absence of  $\beta$ -hydroxybutyrate (BHB,  $\pm 86$  mass units) and variation in acyl chain length ( $\pm 28$  or 14 mass units). Calc., calculated.

I.

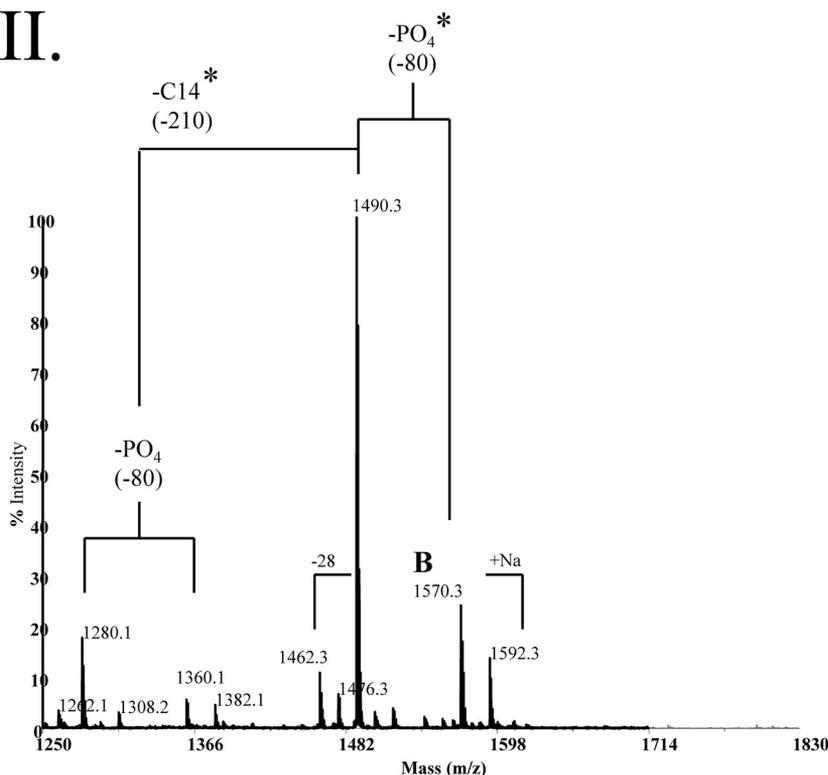


Structure A

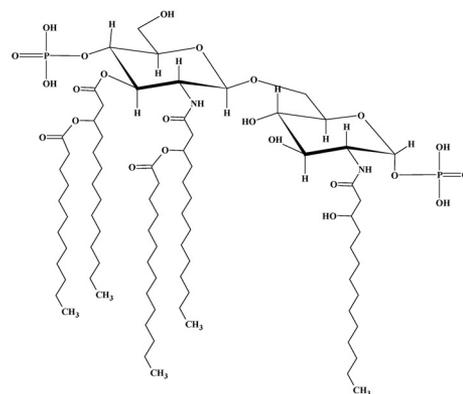


Calc. Exact Mass  
M-H = 1796.2

II.



Structure B

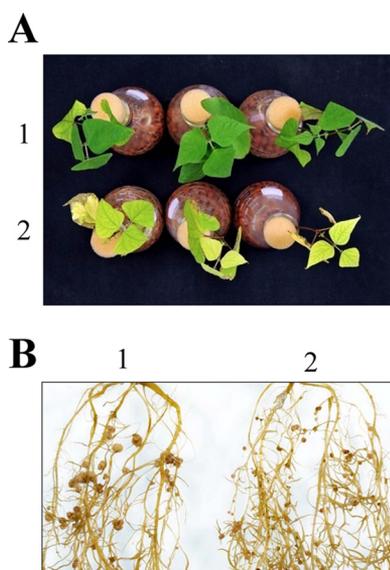


Calc. Exact Mass  
M-H = 1570.0

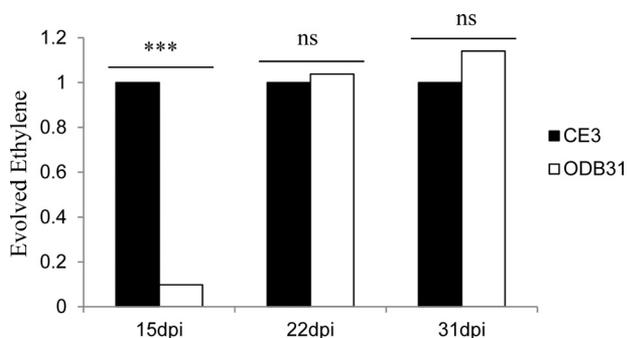
FIGURE 4. MALDI-TOF MS analysis of *E. coli*/pRePagL lipid A. The spectra were obtained in the negative reflectron mode. Panel I, parent strain *E. coli* Top 10. Panel II, *E. coli* Top 10 containing plasmid pRePagL. The structures are labeled A or B (de-O-acylated). Microheterogeneity occurs as a result of deacylation and dephosphorylation of *E. coli* lipid A during the mild acid hydrolysis procedure and variation in acyl chain length ( $\pm 28$  mass units). \*, these structures are present because of the chemical deacylation of lipid A during mild acid hydrolysis of the LPS. *E. coli* does not normally contain 3-O-deacylase activity. Calc., calculated.

In this study we describe the preparation of a mutant that lacks a functional *pagL* gene in *R. etli* bv. *phaseoli* CE3. The *pagL*<sup>-</sup> mutant does not produce tetraacylated lipid A caused by the action of an acylase, indicating that enzymatic de-O-acylation of lipid A has been disrupted. In addition, the mutant lipid A contained a larger amount of  $\beta$ -OHC15:0 than that of the

parent strain. When the native *pagL* gene is introduced into the mutant, the enzymatically tetraacylated lipid A structure was recovered, and the amount of  $\beta$ -OHC15:0 decreased to parent strain levels. Based on these results, we conclude that the putative *pagL* gene described in this study is indeed responsible for the deacylation of lipid A and that when  $\beta$ -OHC15:0 is present



**FIGURE 5. Plant phenotype of mutant inoculated *P. vulgaris* (black bean).** Pictures of representative plants were taken 21 dpi. *A*, row 1, top view of parent strain CE3-inoculated plants. Row 2, top view of mutant ODB31-inoculated plants. *B*, row 1, a representative picture of root nodules from parent strain CE3-inoculated plant. Row 2, a representative picture of root nodules from mutant strain ODB31-inoculated plant.



**FIGURE 6. The ability of the *pagL*<sup>-</sup> mutant to fix nitrogen during symbiotic infection.** Dinitrogenase activity was assessed by the acetylene reduction assay at 15, 22, and 31 dpi. Displayed are the relative amounts of ethylene produced by roots inoculated with mutant strains ODB31 compared with roots inoculated with parent strain CE3. \*\*\*, extremely significant difference ( $p < 0.0001$ ). ns, no significant difference.  $n = 3$  for each experiment.

it is located almost exclusively at position 3 of the lipid A, *i.e.*, the target of PagL activity.

The *P. aeruginosa* PagL crystal structure has been solved (16), and a catalytic domain was proposed to contain a HXSSX<sub>(n+18)</sub>E motif by which the histidine, serine, and glutamate residues form a classical serine esterase catalytic triad. The *R. etli* PagL contains the HXS motif, but instead of glutamic acid, a threonine was observed (supplemental Fig. S1). The threonine residue is conserved in PagL homologues from organisms found in the *Rhizobiales* order (supplemental Fig. S1) and can theoretically perform a similar function as glutamate (*i.e.*, stabilization of the deprotonated catalytic histidine by hydrogen bonding). In addition, the *R. etli* PagL contained the conserved phenylalanine hydrophobic residue (supplemental Fig. S1) previously implicated in positioning the lipid A molecule for hydrolysis by the *P. aeruginosa* PagL (13). However, a lipid A-binding motif has not been fully characterized for PagL enzymes. The substrate specificity for the *R. etli* PagL enzyme

extends to *E. coli* lipid A (Fig. 4). *E. coli* and *R. etli* lipid A structures are quite different, and our results suggest that the *R. etli* PagL has a broad substrate specificity that may extend to a variety of Gram-negative lipid A structures.

The *pagL*<sup>-</sup> mutant formed abnormal symbiosis with the host plant *Phaseolus* (Figs. 5 and 6), indicating that disruption of *pagL* adversely influenced symbiotic infection. However, when complemented with the constitutively expressed native *pagL* gene via a plasmid vector, the complemented mutant displayed the same symbiotic phenotype as the mutant when inoculated on *Phaseolus*. We had thought perhaps this may be due to overexpression of PagL activity in the complement, possibly caused by the fact that *pagL* expression is under control of a lac promoter. However, a second complement prepared using a plasmid containing *pagL* under the control of its native promoter gave the same result; namely two to three times greater PagL activity and a failure to complement the symbiotic phenotype. The common feature of both complements is this apparent overexpression of PagL activity, likely because of multiple copies of the complementing plasmid in both cases. Although the role of PagL in symbiosis remains to be determined, we suggest from these results that both over- and underexpression of *pagL* is deleterious to symbiosis; that is, we hypothesize that the regulation of PagL activity is important for this *R. etli* CE3-bean symbiosis. This hypothesis is consistent with the work of D'Haese *et al.* (9), who reported the lipid A structure of *R. etli* CE3 bacteroids and demonstrated that these bacteroids produced exclusively tetraacylated lipid A (*i.e.*, the PagL lipid A structure D), whereas free living bacteria produced a mixture of penta and tetraacylated lipid A. However, the mechanism of PagL regulation is unknown, and until this report, the *pagL* gene in *R. etli* was unknown. It is possible that the *pagL* gene is subject to transcriptional regulation via an as yet unidentified upstream promoter region. Another possibility is that the PagL protein is subject to post-translational regulation or substrate specific latency as is the case with the *Salmonella* PagL enzyme (15, 35). In any case, the results by D'Haese *et al.* strongly suggest that regulation of PagL is important for proper symbiotic infection, and therefore, overexpression (*i.e.*, lack of regulation) in the complemented mutants (as indicated by the mass spectrometry results) may explain its inability to recover normal symbiotic infection. Although elucidating the regulatory mechanisms underlying PagL expression and/or enzyme activity is beyond the scope of this manuscript, it is important to devote attention to this area in future studies to fully understand the role PagL plays in nitrogen fixing endosymbiosis.

In mammalian systems, LPS is recognized by the Toll-like receptor 4 (TLR-4), which in turn induces a proinflammatory response (36, 37). The TLR-4 ectodomain contains leucine-rich repeats (LRRs), a common feature of the TLR receptor family responsible for the perception of a wide variety of conserved microbial associated molecular patterns. The modification or synthesis of unique lipid A structures has been implicated in the evasion of the animal LPS recognition system (13, 38–40). Although it is unknown how LPS is recognized in plants, there are numerous transmembrane pattern recognition proteins containing an LRR ectodomain and an intracellular receptor-like kinase domain (LRR-RLK proteins) that are

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involved in plant innate immunity and cell signaling (41). Some of these have been shown to recognize two highly conserved microbial associated molecular patterns: flagellin (flg22) and elongation factor (EF-Tu) (42). Furthermore, LRR-RLKs are crucial for bacterial infection and nodule organogenesis in legume symbiosis (16, 35–45). It is possible that plant-associated bacteria modulate their lipid A similar to mammalian-associated bacteria to modulate plant signaling through LPS-LRR-RLK interactions. Elucidating the LPS receptor(s) in plants, as well as possible LPS-associated polycationic antimicrobial peptides/nodule-specific cysteine-rich repeats, will aid in our higher understanding of the role LPS has in symbiotic, pathogenic, and endophytic relationships.

It is important to note that the *pagL* gene is not present or expressed in all symbionts and is not absolutely required for complete symbiosis in these systems. Recent work by Brown *et al.* (6) showed that the bean nodulating strain *R. leguminosarum* bv. *phaseoli* 8002 does not produce deacylated lipid A under laboratory or symbiotic conditions and likely does not contain the *pagL* gene (the genome of strain 8002 is not published). The genome of the closely related organism *R. leguminosarum* bv. *viciae* 3841 has been published and does not contain the *pagL* gene (supplemental Fig. S2). However, strain 3841 contains remnants of the *pagL* gene, suggesting that these organisms may have lost the *pagL* gene possibly through the loss of selective pressure in their natural environment or in the laboratory. Another closely related clover-nodulating strain, *R. leguminosarum* bv. *trifolii* WSM 1325 does contain the *pagL* gene. It is not known whether or not strain WSM 1325 produces 3-*O*-deacylated lipid A. As stated above, *S. meliloti* contains both deacylated lipid A and the *pagL* gene. The absence of the *pagL* gene from some endosymbionts suggests that *pagL* is not absolutely required for complete symbiosis. However, the results presented in this study supports the conclusion that *R. etli* CE3 requires *pagL* expression for normal symbiosis (Fig. 5).

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