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A *Rhizobium leguminosarum* AcpXL Mutant Produces Lipopolysaccharide Lacking 27-Hydroxyoctacosanoic Acid

Vinata Vedam,[1] Elmar L. Kannenberg,[1,2] Janine G. Haynes,[3] D. Janine Sherrier,[3] Anup Datta,[1] and Russell W. Carlson[1]*

Carbohydrate Research Center, University of Georgia Complex, Athens, Georgia 30602[1]; Department of Microbiology and Biotechnology, University of Tübingen, D072076 Tübingen, Germany[2]; and Department of Plant and Soil Sciences, University of Delaware, Newark, Delaware 19717[3]

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The structure of the lipid A from *Rhizobium etli* and *Rhizobium leguminosarum* lipopolysaccharides (LPSs) lacks phosphate and contains a galacturonosyl residue at its 4′ position, an acylated 2-aminogluconate in place of the proximal glucosamine, and a very long chain ω-1 hydroxy fatty acid, 27-hydroxyoctacosanoic acid (27OHC28:0). The 27OHC28:0 moiety is common in lipid A's among members of the *Rhizobiaceae* and also among a number of the facultative intracellular pathogens that form chronic infections, e.g., *Brucella abortus*, *Bartonella henselae*, and *Legionella pneumophila*. In this paper, a mutant of *R. leguminosarum* was created by placing a kanamycin resistance cassette within *acpXL*, the gene which encodes the acyl carrier protein for 27OHC28:0. The result was an LPS containing a tetraacylated lipid A lacking 27OHC28:0. A small amount of the mutant lipid A may contain an added palmitic acid residue. The mutant is sensitive to changes in osmolarity and an increase in acidity, growth conditions that likely occur in the nodule microenvironment. In spite of the probably hostile microenvironment of the nodule, the *acpXL* mutant is still able to form nitrogen-fixing root nodules even though the appearance and development of nodules are delayed. Therefore, it is possible that the *acpXL* mutant has a host-inducible mechanism which enables it to adapt to these physiological changes.

Lipopolysaccharides (LPSs) constitute the outer leaflet of the outer membrane of gram-negative bacteria and are important virulence factors for both animal- and plant-pathogenic bacteria as well as for rhizobia, the nitrogen-fixing symbionts of legumes. It has been shown that the O-chain polysaccharide portion of the LPS is absolutely required for establishing nitrogen-fixing symbioses, including that between *R. leguminosarum* bv. *viciae* and pea (for a review, see reference 29). Furthermore, it has been shown that subtle structural changes, e.g., addition of methyl groups, occur to the O-chain polysaccharide during symbiosis (3, 16, 17, 24–29, 32, 33, 45). In the case of *R. leguminosarum* bv. *viciae*, important structural alterations also occur to the lipid A portion of the LPS during symbiosis (26). This change involves a unique fatty acyl component found in the lipid A of *R. leguminosarum* bv. *viciae* as well as in the lipid A of all members of the *Rhizobiaceae* with the possible exception of *Azorhizobium caulinodans* (6) and in the lipid A of some intracellular pathogens, e.g., *Brucella abortus* and *Bartonella henselae* (i.e., the causal agent of cat scratch disease) (4). This fatty acyl component is a very long chain ω-1 fatty acid, 27-hydroxyoctacosanoic acid (27OHC28:0), which doubles in amount in *R. leguminosarum* bv. *viciae* lipid A during pea symbiosis (26). Also during symbiosis, and during growth under conditions that mimic those that occur during symbiosis, both the LPS and the entire bacterial cell become more hydrophobic (26). Thus, the increase in 27OHC28:0, together with increased O-chain methylation, may be responsible for this increase in hydrophobicity of the LPS and bacterial cell.

The initial stages of symbiosis include root hair deformation and the stimulation of root cortical cell division, resulting in nodule formation. These events are induced via an exchange of signal molecules; flavonoids produced by the host plant induce expression of nodulation (*nod*) genes in the *Rhizobium* strain (for recent reviews, see references 14 and 44). The *nod* gene products produce an acylated chitin oligosaccharide, the Nod factor, which stimulates these initial events. Rhizobia adhere to the emerging root hairs of the host, and an infection conduit known as the infection thread is formed by invagination of the plasma membrane and deposition of plant extracellular matrix material. Bacteria spread into the nascent nodule tissue though these infection threads. This is followed by intracellular invasion of the root nodule tissue in an endocytosis-like process, resulting in an intracellular compartment known as a symbiosome, in which the invading *Rhizobium* cells are surrounded by a plant membrane. In this process, the infection thread penetrates the nodule cortical cell and expands, forming an infection droplet that contains a number of symbiont bacteria. Bacteria are released from the droplet into the host cell cytoplasm.

With indeterminate nodules, such as those formed by pea, the bacteria become singly surrounded by the host-derived membrane known as the symbiosome membrane via a synchronous bacterium-symbiosome membrane division process. Electron micrographs suggest that during this process, there are actual points of contact between the bacterial membrane and the symbiosome membrane (8). Inside the symbiosome, *Rhi-*

* Corresponding author. Mailing address: University of Georgia, Complex Carbohydrate Research Center, 220 Riverbend Rd., Athens, GA 30602. Phone: (706) 542-4439. Fax: (706) 542-4412. E-mail: rcarlson@ccrc.uga.edu.

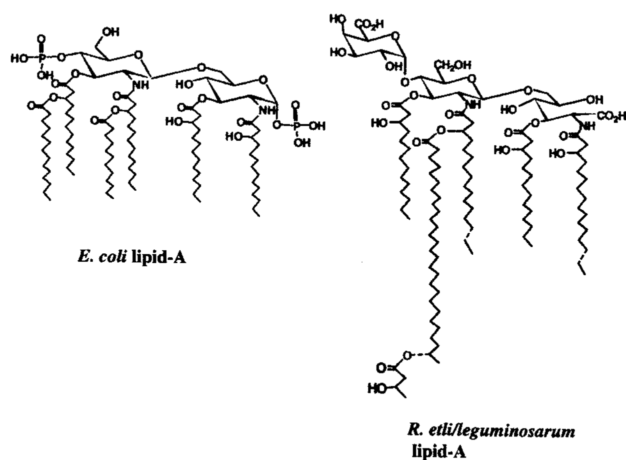

FIG. 1. Structures of lipid A from *E. coli* and lipid A found in *R. etli* and *R. leguminosarum* bv. *viciae* LPSs. It should be noted that in the case of *R. etli* and *R. leguminosarum* bv. *viciae*, the lipid A preparation exists as a mixture of several structures due to variation in both fatty acyl chain length and the nature of the proximal glucosaminosyl residue (5, 39, 40).

zobium differentiates into a bacterial form known as the bacteroid, which produces nitrogenase.

It has been hypothesized that the presence of 27OHC28:0 in lipid A is required for maintaining the stability of the bacterial membrane during the endocytotic invasion process and also for survival of the bacterium within the plant-derived symbiosome compartment. Increased membrane stability could be provided by the 27OHC28:0 residue because the alkyl chain of this fatty acid is long enough to span the entire outer membrane bilayer.

The structure of the lipid A's from *R. etli* (formerly *R. leguminosarum* biovar phaseoli) and *R. leguminosarum* bv. *viciae* (5, 39, 40) is shown in Fig. 1 together with the structure of *Escherichia coli* lipid A. The *R. leguminosarum* bv. *viciae* and *R. etli* lipid A's have identical structures with unique features compared to those of *E. coli* lipid A. These unique structural features include the replacement of the 4′ phosphate with a galacturonosyl residue, the oxidation of the proximal glucosaminosyl residue to 2-amino-gluconate, the lack of acyloxyacyl lauryl and myristyl residues, and the presence of a very long chain acyloxyacyl moiety, 27OHC28:0. A similar lipid A occurs in the LPS from *Rhizobium* sp. strain Sin-1 (which nodulates the roots of *Sesbania* spp.) but differs slightly from that of *R. leguminosarum* bv. *viciae* in that it lacks the galacturonic acid residue and, while containing 27OHC28:0, has an altered fatty acylation pattern (22).

It has been shown that *R. leguminosarum* bv. *viciae* (and *R. etli)* contains the same enzymes as does *E. coli* to convert UDP-GlcNAc into a common lipid A precursor known as Kdo_2lipid-IVA (37) and that *R. leguminosarum* bv. *viciae* has additional enzymes that process this precursor into the unique lipid A structure shown in Fig. 1 (10, 23, 36, 40). In *E. coli* (and in *Salmonella* spp.), the secondary acyloxyacyl lauryl and myristyl fatty acids are added by LpxL and LpxM, respectively (for a review on enteric lipid A biosynthesis, see reference 41). In *R. leguminosarum* bv. *viciae*, the sole secondary acyloxyacyl residue is 27OHC28:0. This fatty acid residue requires a unique acyl carrier protein, AcpXL (10), and a transferase, LpxXL (1). It is not clear whether the additional amounts of 27OHC28:0 fatty acid residues observed in the lipid A's of *R. leguminosarum* bv. *viciae* bacteroids involve additional biosynthetic enzymes, e.g., additional transferases.

The focus of the work presented in this paper is the unusual 27OHC28:0 acyl residue of the *R. leguminosarum* bv. *viciae* lipid A because it increases in the lipid A during symbiosis between *R. leguminosarum* bv. *viciae* and pea and its presence in the lipid A's of the *Rhizobiaceae* as well as in the lipid A of several intracellular pathogens suggests a functional importance in symbiosis as well as in pathogenicity. As a first step towards elucidating the function of this remarkable acyl component of the lipid A, a mutant of *R. leguminosarum* bv. *viciae* that is unable to incorporate 27OHC28:0 fatty acid into its lipid A was prepared. In order to eliminate the presence of 27OHC28:0 in *R. leguminosarum* bv. *viciae* LPS lipid A's, it was decided to target *acpXL* for mutational inactivation. The *acpXL* gene was cloned and partially sequenced as *orf** by Colonna-Rommano et al. (12), and the AcpXL protein was characterized by Brozek et al. (10). As described by Colonna-Rommano et al. (12), the gene is localized on cosmid pCS115, and this plasmid provided the starting point for the generation of *acpXL*::*kan R. leguminosarum* bv. *viciae* mutants. Here we report the construction and characterization of the *acpXL*::*kan* mutant of *R. leguminosarum* bv. *viciae* 3841.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this work are listed in Table 1. All the *E. coli* strains were grown at 37°C on Luria-Bertani (LB) medium. *R. leguminosarum* 3841 was used as the parental strain for this study. All rhizobia were grown on tryptone-yeast extract (TY) medium with added calcium (34) or on Vincent's minimal medium (48). Media were supplemented with the following concentrations of antibiotics where mentioned: ampicillin (Amp, 50 μg ml^{-1}), kanamycin (Kan, 50 μg ml^{-1}), gentamicin (Gm, 40 μg ml^{-1}), and tetracycline (Tet, 10 μg ml^{-1}). The β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and inducer isopropylthiogalactopyranoside (IPTG) were incorporated into solid Luria broth medium at 50 μg ml^{-1} and 100 μg ml^{-1}, respectively. Peas were grown on N_2-free Fahraeus medium (35) at 22°C with 14 h of light, 18°C with 6 h of dark, and 55% humidity.

Generation of *acpXL*::*kan* mutants and complements. Plasmid pCS115, containing *acpXL* (formerly known as *orf** [12]), was digested with restriction enzymes *Hin*dIII and *Psh*AI to isolate a 3.9-kb fragment containing *acpXL* (327 bp) with flanking regions (about 1.8 kb on either side of *acpXL*). All restriction endonucleases were purchased from New England Biolabs. This fragment was subcloned into cloning vector pBluescript pBST SK(+) that had been digested with *Hin*dIII and *Sma*I, yielding pVV1. Plasmid pVV1 was transformed into *E. coli* XL1 Blue. A number of white colony transformants were selected on LB agar plates containing ampicillin, X-Gal, and IPTG. Plasmid DNA was extracted from the transformants and digested with *Hin*dIII and *Eco*RI, and the resulting DNA fragments were analyzed by agarose gel electrophoresis to determine if the correct construct was present. Colonies that had the correct construct were preserved in glycerol stocks.

To inactivate *acpXL*, a kanamycin resistance (*kan*) cassette from pUC4K (Amersham BioLabs) was isolated by digestion with *Eco*RI, and the resulting 1.2-kb *kan* cassette was inserted into pVV1 digested with *Eco*RI to generate pVV2. Plasmid pVV2 was transformed into *E. coli* XL1 Blue, and transformants were selected on LB agar plates containing ampicillin and kanamycin. The plasmid DNA from each transformant was analyzed for orientation of the *kan* cassette by restriction analysis (*Eco*RI) of their plasmid DNA.

A 4.8-kb fragment containing the interrupted gene from pVV2 was released by partial digestion with *Xho*I and cloned into suicide plasmid pJQ200uc1 at a unique *Xho*I site in the multiple cloning site of this vector. Plasmid pJ200uc1 carries the conditionally lethal gene *sacB*, allowing discrimination between integration of the vector and double recombination events (38). The resulting plasmid, pVV4, was transformed into *E. coli* JM109, and transformants were selected

TABLE 1. Bacterial strains and plasmids used in this report

Strain or plasmid	Characteristics	Source
E. coli		
XL1 Blue	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44 lac* [F′ *proAB*$^+$ *lacI*^{q}ZΔM15 Tn*10*(Tet^r)]	Stratagene Corp.
JM109	*endA1 recA1 gyrA96 thi hsdR17*(r_K^- m_K^+) *relA1 supE44* Δ(*lac-proAB*) [F′ *traD36 proAB lacI*^{q}ZΔM15]	Promega Corp.
DH5α	ϕ80d*lacZ* Δ*M15 recA1 endA1 gyrA96 thi-1 hsdR17*(r_K^- m_K^+) *supE44 relA1 deoR* Δ(*lacZYA-argF*)*U169*	Stratagene Corp.
R. leguminosarum bv. *viciae*		
3841	Strain 300 Str^r Fix$^+$	52
22	Strain 3841 *acpXL*::*kan* Str^r Kan^r Fix$^+$	This study
26	Strain 3841 *acpXL*::*kan* Str^r Kan^r Fix$^+$	This study
pBS II SK+	2.96-kb phagemid derived from pUC19, *lacZ* Amp^r	Stratagene Corp.
Plasmids		
pCS115	pWKR56 carrying *Hin*dIII fragment from pRIA76, Tet^r	12
pUC4K	Source of *kan* cassette, Kan^r	Pharmacia Biotech.
pJQ200uc1	Suicide vector, allows positive selection for integration, Gm^r *sacB*	38
pRK600	Mobilizing plasmid for pJQ200uc1, pRK2013 Nm^r::Tn*9* Cm^r	19
pBBR1MCS-3	pBBR1MCS derivative, Tet^r	30
pVV1	pB*S* SK derivative with 3.9-kb insert from pCS115 containing *acpXL*	This study
pVV2	pVV1 containing *kan* from pUC4K	This study
pVV4	pVV2 derivative cloned into pJQ200uc1	This study
pVV5	pVV1 derivative cloned into pBBR1MCS-3	This study

on LB agar plates containing kanamycin. The construction of the pVV4 plasmid is schematically depicted in Fig. 2A.

Plasmid pVV4 was introduced into *R. leguminosarum* bv. *viciae* 3841 by triparental mating with *E. coli* JM109/pVV4 as the donor, *R. leguminosarum* bv. *viciae* 3841 as the recipient, and *E. coli*/pRK600 (19) as the helper strain. Transconjugants were selected on Vincent's minimal medium agar plates containing gentamicin and kanamycin. Double recombinants were selected on TY agar plates containing gentamicin, kanamycin, and 10% sucrose. Two *acpXL*::*kan* mutants were isolated (*R. leguminosarum* bv. *viciae* 22 and *R. leguminosarum* bv. *viciae* 26) and verified by PCR with primers to *acpXL* (GAGGGGGTTTAAATAGTCA and AGGCCTTGGCCGCTTTGA) and by sequencing the resulting PCR products. All DNA sequencing was done by the Molecular Genetics Instrumentation Facility at the University of Georgia.

A complement of the *acpXL*::*kan* mutant was constructed to confirm that any

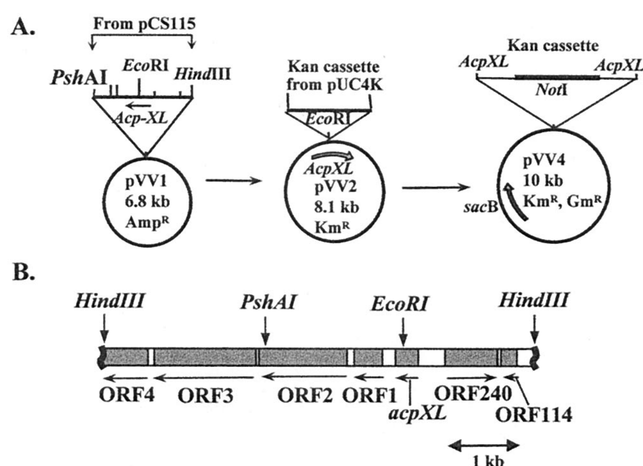

FIG. 2. (A) Diagram showing the construction of plasmid pVV4, which contains the *kan* insert within the *acpXL* gene, *acpXL*::*kan*, and was used to create the *acpXL*::*kan* mutants of *R. leguminosarum* bv. *viciae* strain 3841, strains 22 and 26. (B) The arrangement of the genes within the *R. leguminosarum* bv. *viciae* VF39 DNA that is present within cosmid pCS115. The *R. leguminosarum* bv. *viciae* VF39 nucleotide sequence is colinear to the recently described gene region from *R. leguminosarum* bv. *viciae* strain 3841 (1).

observable physiological differences in the mutant were a result of the *acpXL*::*kan* mutation. For this purpose, a 2.5-kb fragment containing the parental *acpXL* gene was isolated from pVV1 (see above) with the restriction enzyme *Kpn*I. This fragment was cloned into pBBR1MCS3, a broad-host-range vector (38) that had been digested with *Kpn*I. The resulting construct, pVV5, was transformed into *E. coli* JM109, and transformants were selected on LB plates containing tetracycline. This strain was used as the donor for triparental mating into *R. leguminosarum* bv. *viciae* 22 with *E. coli* pRK600 as the helper strain. Transconjugants were selected on TY plates containing tetracycline and kanamycin. Confirmation of *acpXL* (plasmid-borne) and *acpXL*::*kan* (chromosomal) in the complemented strains was done by PCR with primers to *acpXL* (described above) with plasmid and chromosomal DNA of the complements, respectively.

LPS extraction and purification. The wild-type and mutant LPSs were first extracted by the TEA/EDTA/ϕ procedure as previously described (43). For each strain, the bacterial pellet (approximately 10 g wet weight) from 8 liters of culture was extracted with 40 ml of TEA/EDTA/ϕ (0.25 M EDTA containing 5% phenol and titrated to pH 6.9 with triethylamine [TEA]) with constant stirring at 37°C for 1 h. The extract was then centrifuged at 10,000 rpm for 1 h, and the supernatant was collected and dialyzed (2,000 molecular weight cutoff; Spectrapor) against deionized water. This material was further purified by polymyxin affinity chromatography with polymyxin B-Sepharose (Pierce Chemical Company) as previously described (20, 43, 46). Briefly, after sample application in 50 mM NH_4HCO_3, the column (10-ml bed volume) was sequentially eluted with 30 ml of a solution of 0.3 M TEA adjusted to pH 6.4 with acetic acid plus 10% ethylene glycol, followed by 30 ml of a solution of 2.0 M urea in 0.1 M NH_4HCO_3 to elute the non-LPS components. The LPS was eluted from the column with 20 ml of 2.5% deoxycholate in 0.1 M NH_4HCO_3. The LPS fractions were pooled and dialyzed extensively (2,500 molecular weight cutoff) against a solution of 50 mM TEA brought to pH 8.5 with acetic acid and containing 10% ethanol and then against deionized water, after which the fractions were lyophilized.

Analytical procedures. Gel electrophoresis in the presence of deoxycholate was performed as described before (42). Typically, 1 μl of LPS sample (this volume includes dye, loading buffer, and 2 μg of LPS) was loaded onto the gels. The gels were silver stained as described previously (42). Analysis of the lipid A fatty acids was accomplished as previously described (5, 53) by methanolysis of the purified LPS samples in methanolic 1 M HCl, followed by trimethylsilylization of the hydroxyl groups and analysis by combined gas chromatography-mass spectrometry (GC/MS) with a 30-m DB-5 column from J&W Scientific.

Lipid A was isolated from the LPS preparation by mild acid hydrolysis (11). The LPS was dissolved in 1% sodium dodecyl sulfate in 20 mM sodium acetate, adjusted to pH 4.5 with 4 M HCl, and then placed in an ultrasound bath until the sample was dissolved. This solution was then heated at 100°C for 1 h, followed by lyophilization. The sodium dodecyl sulfate was removed by washing the lyophilized residue with a solution of 2:1 deionized H_2O-acidified ethanol (100 μl of 4

M HCl in 20 ml of 95% ethanol). The residue was collected by centrifugation, washed again with 95% ethanol (nonacidified), and collected by centrifugation (200 × *g* for 15 min). The washing and centrifugation steps were repeated. Lastly, the residue was lyophilized to give a white, solid, fluffy lipid A preparation.

Matrix-assisted laser desorption ionization time of flight–mass spectrometry (MALDI-TOF MS) was performed with a Kratos Analytical Kompact SEQ MALDI-TOF spectrometer system (Kratos, Manchester, United Kingdom) in the negative-ion reflectron mode with a 337-nm nitrogen laser operating at a 20-kV extraction voltage and with time-delayed extraction. Approximately 2 μl of a 1-mg/ml lipid A solution in chloroform-methanol (3:1, vol/vol) was mixed with 1 μl of trihydroxyacetophenone matrix solution (≈93.5 mg of trihydroxyacetophenone per ml of methanol) and applied to the probe for mass analysis. Spectra were calibrated externally with *E. coli* lipid A (Sigma).

Study of physiological properties. Growth rates of the wild-type and mutant rhizobia were compared by standard methods at 30°C (spectrophotometer readings of liquid cultures in TY/calcium broth at 600 nm and plating dilution series). Three replicate flasks of bacterial cultures were initiated with starter cultures, and samples were collected at multiple time points between 16 and 96 h for spectrophotometric analysis and serial dilution plating. For both the mutant and the parent strain, 5-ml cultures from single colonies were prepared. In the case of the *R. leguminosarum* bv. *viciae* 3841 parent, 50 μl of the 5-ml culture having an optical density at 600 nm of 0.103 (1.96×10^6 cells/ml, as determined by colony counting of serial dilutions) was placed in 500 ml of medium, and the growth curve was monitored by periodic removal of 1-ml samples for measurements of both optical density and cell count. In the case of the *R. leguminosarum* bv. *viciae* 22 mutant, 50 μl of the 5-ml culture, which had an optical density at 600 nm of 0.168 (2.09×10^6 cells/ml), was placed in 500 ml of culture, and the growth curve was monitored as for *R. leguminosarum* bv. *viciae* 3841.

In order to assess the osmotic tolerance of the mutant, *R. leguminosarum* bv. *viciae* 22, its complement, and *R. leguminosarum* bv. *viciae* 3841 were grown at 30°C on TY/calcium agar containing various concentrations of NaCl (0.0%, 0.25%, 0.5%, 0.75%, and 1.0%). Sensitivity to pH was determined by growth on TY plates at various pHs (7.0, 6.0, 5.5, 5.0, and 4.8), with PIPES (piperazine-*N,N′-bis*[2-ethanesulfonic acid]) and piperazine as buffering agents. PIPES was used at a final concentration of 40 mM to buffer the medium at pH 7, and a final concentration of 40 mM piperazine was used in the medium while adjusting the acidity of the medium to the various lower pHs.

Symbiotic phenotype characterization. The effect of the *R. leguminosarum* bv. *viciae acpXL*::*kan* mutation on nodulation of pea, *Pisum sativum* bv. Early Alaska, was examined by inoculating pea seedlings with 0.5 ml of late-log/early-stationary-phase liquid cultures of wild-type and mutant strains. The seedlings were grown in Erlenmeyer flasks with defined, nitrogen-free plant agar (Fahraeus medium) in growth chambers. The temperature was maintained at 22°C with 14 h of daylight, at 18°C with 10 h of dark, and at 55% humidity. Nodule formation was monitored throughout the growth period.

Nitrogenase activity was measured with the acetylene reduction assay as previously described (51). Three weeks after inoculation, roots with attached nodules were transferred into glass bottles (20 ml) with screw caps and fitted rubber membranes. Then 1 ml of acetylene was added to the roots and incubated for 1 h at room temperature. The amounts of ethylene generated from the nitrogenase were measured through GC analysis (isotherm at 70°C, with a 50 m by 0.53 mm, 10-μm Al_2O_3-KCl column [Chrompack]).

RESULTS

Characterization of gene region harboring *acpXL*. The nucleotide sequence of the cloned DNA in plasmid pCS115, which harbors the *acpXL gene* from *R. leguminosarum* bv. *viciae* VF39, was determined. Compared with the recently published sequence for this region from *R. leguminosarum* bv. *viciae* 3841, which contains ORF114, ORF240, *acpXL*, ORFs 1 through 4, and *lpxXL* (the gene encoding the acyltransferase for 27OHC28:0) (1), the pCS115 *R. leguminosarum* bv. *viciae* VF39 fragment was 100% identical to that published for strain *R. leguminosarum* bv. *viciae* 3841 ending midway through ORF4. The arrangement of the genes and ORFs in the pCS115 *R. leguminosarum* bv. *viciae* VF39 fragment, based on its identity with the published sequence for *R. leguminosarum* bv. *viciae* 3841 (1), is shown in Fig. 2B.

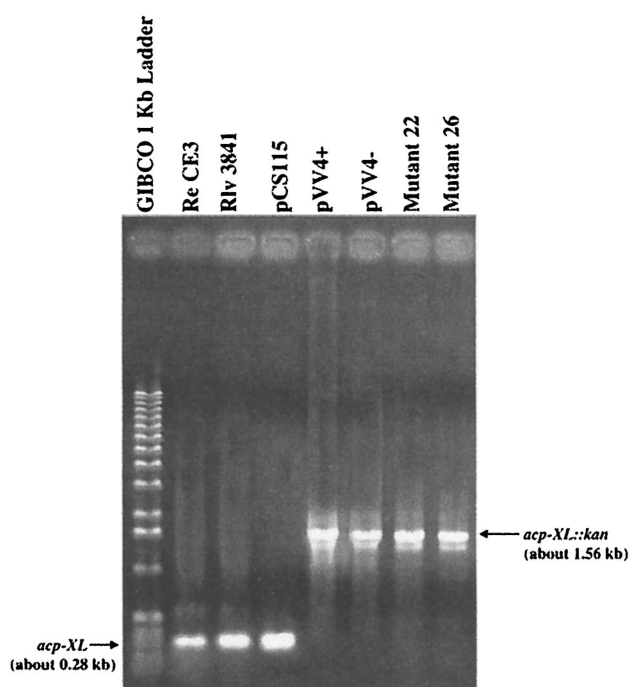

FIG. 3. Agarose gel showing the *acpXL* PCR products obtained from *R. etli* (*Re*) CE3, *R. leguminosarum* bv. *viciae* (*Rlv*) 3841, pCS115, pVV4+, pVV4−, mutant 22, and mutant 26. Plasmids pVV4+ and pVV4− are constructs in which the *kan* insert was placed within *acpXL* in opposite directions.

Isolation of *R. leguminosarum* bv. *viciae* 3841 *acpXL*::*kan* mutants. The *acpXL*::*kan* mutants were isolated as described in Materials and Methods (depicted in Fig. 2A). Figure 3 shows the PCR DNA fragments isolated from the parent strain 3841 and the two mutants, *R. leguminosarum* bv. *viciae* 22 and 26, with *acpXL*-specific primers. For comparison, DNA from *R. etli* CE3 was also included.

The *R. etli* CE3 and *R. leguminosarum* bv. *viciae* 3841 strains had the expected 0.28-kb *acpXL* fragment, while *acpXL*-containing PCR fragments from both mutant strains were approximately 1.56 kb in size, which is consistent with the combined size of the *kan* cassette and *acpXL*. The insertion of the *kan* cassette into the *Eco*RI site of the *acpXL* gene was confirmed for both mutants by sequencing the *acpXL*::*kan* PCR products. The *kan* cassette in both mutants showed a parallel orientation to the *acpXL* gene. In mutant strains complemented with a plasmid harboring the *acpXL* gene, PCR analysis of the plasmid and chromosomal DNA with the *acpXL*-specific primers gave a 0.28-kb fragment, indicative of the wild-type gene in the plasmid DNA, and a 1.56-kb fragment from the chromosomal DNA, indicative of *acpXL*::*kan* (data not shown).

LPS isolation and characterization. We employed two standard protocols for LPS extraction, hot phenol-water and TEA/EDTA/ϕ. With both methods, the yield of LPS from the mutant was less than that obtained from the *R. leguminosarum* bv. *viciae* 3841 parent; however, the LPS yields from both parent and mutants were increased with TEA/EDTA/ϕ extraction. In the case of the phenol-water extraction, the LPS from the *R. leguminosarum* bv. *viciae* 3841 parent extracted into both the phenol and water phases, as described previously (26). How-

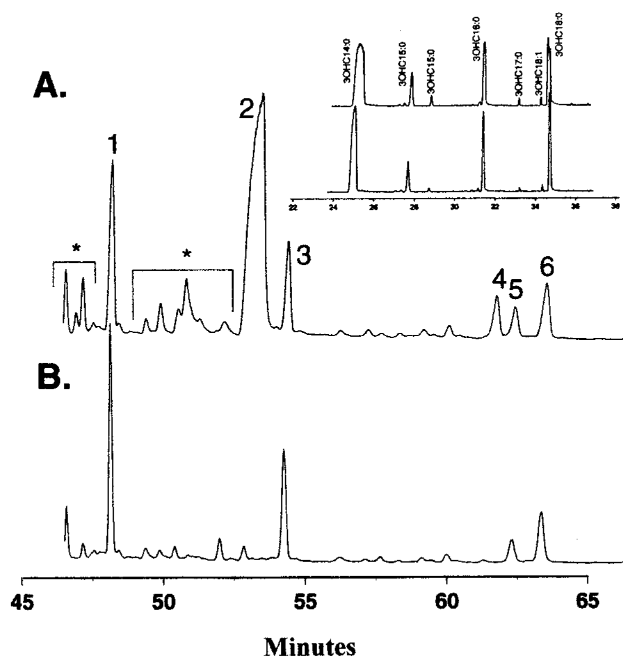

FIG. 4. GC-MS profiles showing the fatty acids present in lipid A from *R. leguminosarum* bv. *viciae* 3841 (A) and mutant 22 (B). The insert shows the β-hydroxy fatty acyl components (scan of the diagnostic *m*/*z* 175 ion) for the lipid A from *R. leguminosarum* bv. *viciae* 3841 (top) and mutant 22 (bottom). The peaks are: 1, the trimethylsilyl (TMS) methyl glycoside of *N*-3OHC14:0-GlcN; 2, TMS fatty acid methyl ester of 27OHC28:0; 3, TMS methyl glycoside of *N*-3OHC16:0; 4, TMS fatty acid methyl ester of 29OHC30:0; 5, TMS methyl glycoside of *N*-3OHC18:1-GlcN; and 6, TMS methyl glycoside of *N*-3OHC18:0-GlcN. *, contaminating deoxycholate (DOC).

ever, with the *acpXL*::*kan* mutants, the LPS largely extracted into the water phase, with only small amounts found in the phenol phase. This result suggested that the content of hydrophobic LPS in the mutant was much less than that found in the *R. leguminosarum* bv. *viciae* 3841 parent. For chemical analysis, the TEA/EDTA/ϕ extraction method was used because this procedure resulted in increased yields of LPS compared to hot phenol-water extraction. The mutant LPS yield per gram (wet weight) of bacterial pellet from the TEA/EDTA/ϕ procedure was approximately one-third of that obtained from the *R. leguminosarum* bv. *viciae* 3841 parent.

In order to determine if mutation of *acpXL* resulted in an LPS that lacked 27OHC28:0, the LPS preparations from the two *acpXL*::*kan* mutants, 22 and 26, were analyzed for their fatty acid content and compared with that of the parent strain. The GC/MS profiles for the fatty acids of the lipid A from the parent and mutant 22 are shown in Fig. 4. The parent LPS contained 27OHC28:0 as well as a small amount of 29OHC30:0. The latter fatty acid is often observed in small amounts in this *R. leguminosarum* bv. *viciae* lipid A, and its presence is consistent with the report that AcpXL exists in an acylated form in which the acyl group can be either 27OHC28:0 or 29OHC30:0 (1). The presence of the N-acylated GlcN residues (*N*-3OHC14:0-, *N*-3OHC16:0-, *N*-3OHC18:1-, and *N*-3OHC18:0-GlcN) is consistent with previous reports (5) showing the heterogeneity of the N-acyl

TABLE 2. Relative fatty acid compositions of lipid A's from strain 3841 and its *acpXL*::*kan* mutant, strain 22[a]

Strain	Relative mol %				
	3OHC14:0	3OHC15:0	3OHC16:0	3OHC18:0	27OHC28:0
3841	40	6.0	13	15	25
22	55	7.5	17	20	<1.0

[a] The compositions are given as relative mole percents. Small amounts of 3OHC17:0, 3OHC18:1, and 29OHC30:0 were also detected but not quantified. Also, small amounts of palmitic (C16:0), stearic (C18:0), and vaccenic (C18:1) acids were present. Mutant 22 lipid A appeared to have slightly more C16:0 than did the strain 3841 lipid A. These straight-chain fatty acids are common components of phospholipids, which occasionally contaminate LPS preparations.

substituents and the resistance of the N-acyl residues to the methanolysis procedure (2).

Neither mutant 22 (Fig. 4B) nor mutant 26 had LPS with appreciable levels of 27OHC28:0 or 29OHC30:0. Selective ion scanning (i.e., *m*/*z* 117) of the mutant LPS indicated the possibility of a slight amount of 27OHC28:0 at less than 1% of the parental LPS level. However, the lipid A's from both mutants had the same level of *N*-acyl-GlcN residues as the parent lipid A. The insert in Fig. 4 shows that the GC/MS profiles for the remaining β-hydroxy fatty acids of the parent and mutant lipid A's were essentially identical. The fatty acyl compositions are given in Table 2. Other than the lack of 27OHC28:0 and 29OHC30:0, the relative amounts of the remaining fatty acids found in the mutant LPSs were essentially the same as in the parent LPS. Analysis of the LPS from the complemented mutant showed that the 27OHC28:0 content of its lipid A was restored (data not shown). In addition to the fatty acyl components just described, both the parent and the mutant lipid A molecules contained galacturonic acid, glucosamine (as indicated above), and 2-aminogluconate.

Lipid A preparations from the parent and mutant strains were isolated and analyzed by MALDI-TOF/MS. The results are shown in Fig. 5. The mass spectrum of the lipid A from the parent 3841 strain (Fig. 5A) shows two clusters of ions that are consistent with the composition described above and with the previously published *R. leguminosarum* bv. *viciae*/*R. etli* lipid A structures (5, 39, 40). In one cluster, the ions ranged from *m*/*z* 1,887.6 to 2,058.0, with the most intense ion at 1,915.4, and in the second cluster, the ions ranged from *m*/*z* 1,625.7 to 1,738.9, with the most intense ion at *m*/*z* 1,653.5. The ion at *m*/*z* 1,915.4 in the first cluster of ions is consistent with the published structure (5, 39, 40), in which lipid A has a disaccharide backbone consisting of a distal glucosaminosyl residue β-1,6-linked to a proximal 2-aminogluconic acid residue. This disaccharide is substituted at the 4′ position of the distal glucosamine with an α-galacturonosyl residue and is fatty acylated with β-hydroxy fatty acyl moieties at the 2, 3, 2′, and 3′ positions (e.g., 3OHC14:0, 3OHC16:0, 3OHC14:0, and 3OHC16:0, respectively), with the 27OHC28:0 moiety present as a secondary acyloxyacyl residue ester linked to the hydroxyl group of the 3′-β-hydroxy fatty acyl residue.

The *m*/*z* 2001.4 ion is the $[M-H]^-$ ion for this structure in which the 27-hydroxyl group of 27OHC28:0 is esterified with β-hydroxybutyrate, as previously reported for the *R. leguminosarum* bv. *viciae* lipid A (5, 39, 40). The other ions in this cluster are due to variations in these structures as a result of various

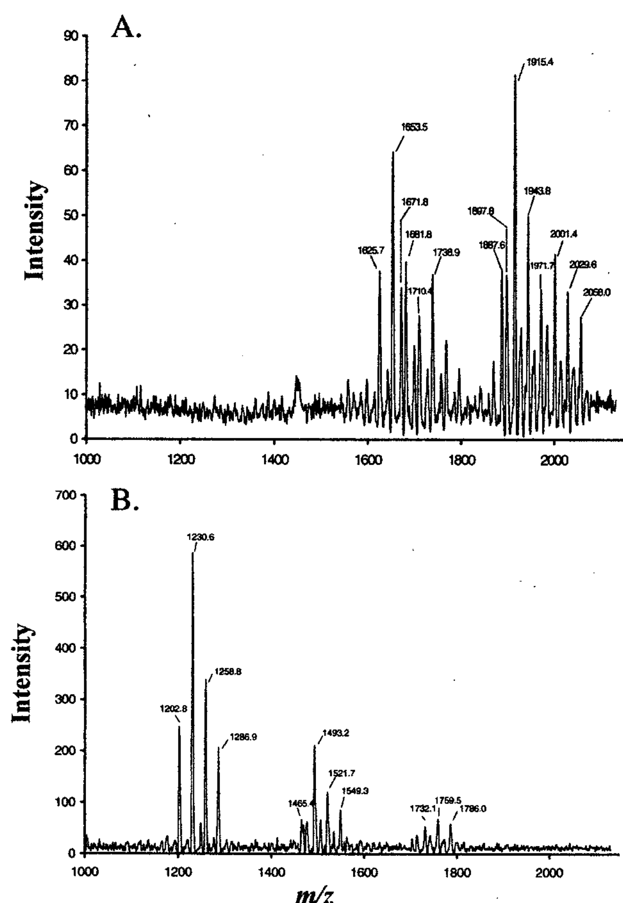

FIG. 5. MALDI-TOF/MS spectra of lipid A from *R. leguminosarum* bv. *viciae* 3841 (A) and mutant 22 (B). The identity of each ion is described in the text and shown in Fig. 6.

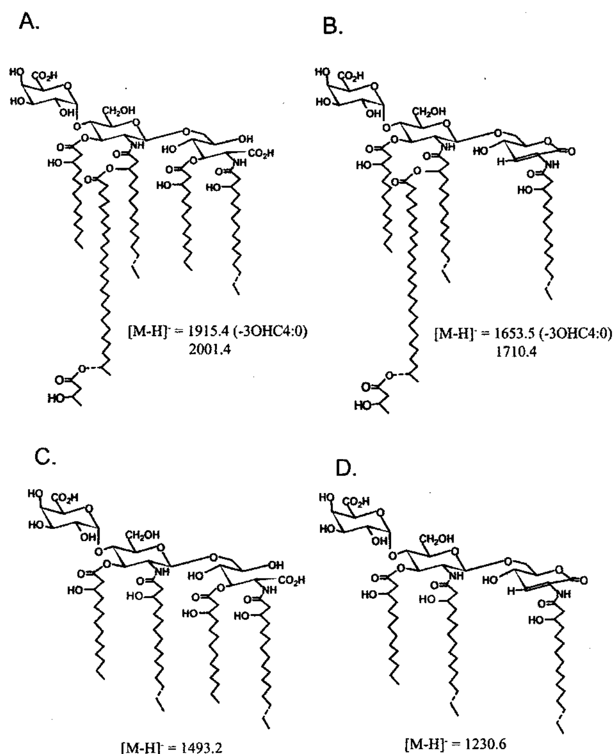

FIG. 6. The lipid A structures proposed for the indicated ions observed for the lipid A from *R. leguminosarum* bv. *viciae* 3841 (A and B), and for the lipid A from mutant 22 (C and D).

fatty acyl chain lengths. In the second ion cluster, the ion at *m/z* 1,653.5 is due to lactonization of the proximal 2-aminogluconate residue and the subsequent elimination of a 3OHC14:0 moiety from the β position of that residue. The result is a loss of 262 mass units (minus 244 mass units due to loss of 3OHC14:0 and minus 18 mass units due to loss of water; 1,915 − 262 = 1,653) and the formation of a 2,3-unsaturated 2-aminogluconolactone proximal residue, i.e., 2-amino-2,3-dideoxy-D-*erythro*-hex-2-enono-1,5-lactone. This structure was reported to be present in *R. etli* lipid A (39, 40) and has also been reported as a component in the lipid A from *Rhizobium* sp. strain Sin-1 (a symbiont of *Sesbania* spp.) (22).

As previously reported (22, 40), this structure is most likely an artifact of the lipid A isolation procedure, which promotes both lactonization of the 2-aminogluconate residue and the subsequent acid-catalyzed β-elimination of the 3-hydroxy fatty acyl moiety (22). The most intense ion in this cluster, *m/z* 1,653.5, is due to this structure, which lacks a β-hydroxybutyrate group, and the ion at *m/z* 1,738.9 is due to the molecule containing the β-hydroxybutyrate group. Again, the other ions in this cluster are due to variations of this structure as a result of various fatty acyl chain lengths. The structures for the parent *R. leguminosarum* bv. *viciae* 3841 lipid A molecules are shown in Fig. 6A and B.

The mass spectrum of *R. leguminosarum* bv. *viciae* mutant 22 (Fig. 5B) shows two main clusters of ions; one cluster is centered on ion *m/z* 1,493.2, and another around the ion at *m/z* 1,230.6. A minor third ion cluster is present centered on an ion of *m/z* 1759.5. An identical mass spectrum was observed for the lipid A from *R. leguminosarum* bv. *viciae* mutant 26 (data not shown). The two main ion clusters are due to lipid A structures analogous to those of the parent described above but lacking 27OHC28:0 (i.e., less 422 mass units; 1,915 − 422 = 1,493 and 1,653 − 422 = 1,231) or lacking both β-hydroxybutyrate and 27OHC28:0 (i.e., less 508 mass units; 2,001 − 508 = 1,493, and 1,739 − 508 = 1,231). These structures are shown in Fig. 6C and D. These results are consistent with the composition analysis showing that the mutant lipid A lacks 27OHC28:0.

The ions in the minor ion cluster, ranging from *m/z* 1,732.1 to 1,786.0, are approximately 238 mass units larger than the corresponding ions in the *m/z* 1,493.2 ion cluster, i.e., 1,493.2 + 238 = 1,731.2, 1,521.7 + 238 = 1,759.7, and 1,549.3 + 238 = 1,787.3. The additional 238 mass units could be accounted for by the addition of a palmitoyl residue to the mutant lipid A. Small amounts of palmitic acid were observed in both the parent and mutant lipid A preparations, with increased amounts in the mutant lipid A. However, this fatty acid is normally one of the major components of phospholipids and therefore its presence in the lipid A preparation is usually due to some contamination by phospholipids. Thus, confirmation of palmitic acid as a component of the mutant lipid A will require enrichment of that minor lipid A species and further structural characterization.

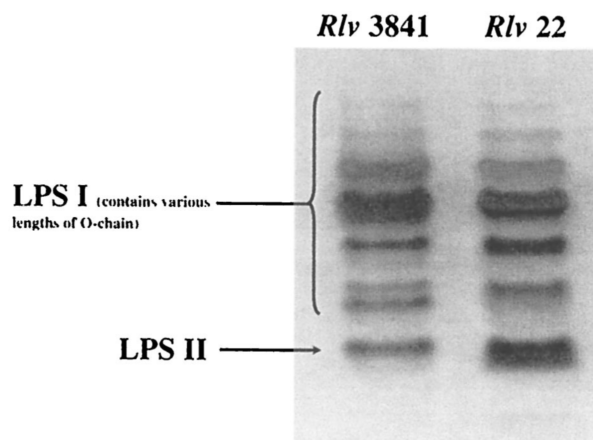

FIG. 7. Deoxycholate-PAGE profiles of LPSs from *R. leguminosarum* bv. *viciae* 3841 and mutant 22. The amount of LPS used in each case was 1 μl of a 2-μg/μl solution. The bands in the LPS I region contain lipid A, core oligosaccharide, and various lengths of O-chain polysaccharide. The band labeled LPS II lacks O-chain polysaccharide.

The LPSs from the mutants and the parent strain were also compared by deoxycholate-polyacrylamide gel electrophoresis (PAGE) analysis to determine if there were any differences in the LPS banding patterns. The banding patterns for the *R. leguminosarum* bv. *viciae* 3841 parent and *R. leguminosarum* bv. *viciae* mutant 22 (*R. leguminosarum* bv. *viciae* mutant 26 LPS gave the same results as shown for *R. leguminosarum* bv. *viciae* mutant 22) are shown in Fig. 7. Some alterations in both intensities and mobilities of the *R. leguminosarum* bv. *viciae* mutant 22 LPS bands were observed compared to *R. leguminosarum* bv. *viciae* 3841 LPS. The LPS I banding region of the mutant LPS was missing one band, and the LPS II of the mutant was somewhat greater in intensity, with a slightly faster electrophoretic mobility than that of the parent LPS II, a result indicating a slightly lower molecular weight for the mutant LPS II. However, it is somewhat surprising that the mobility of the mutant LPS II, while slightly increased compared to that of the parent, is still so similar, because if the mutant lacks 27OHC28:0, its LPS II should have a molecular weight that is 422 mass units less than that of the parent LPS II. It is possible that an answer to this apparent discrepancy lies in some slight structural difference in the core oligosaccharide of the molecule. Further LPS structural work is in progress.

Physiological properties of *acpXL*::*kan* mutants. The growth rates of the *acpXL*::*kan* mutants were compared with that of the parent. The doubling times of the parent and mutant 22 were not significantly different. In spite of the fact that both the parent and mutant cultures started out with the same number of logarithmically growing cells, the mutant appeared to have a significant lag phase compared to the parent; the parent strain began active growth at about 16 h postinoculation, while the mutant began growth at about 33 h. The reasons for the increased lag phase of the mutant are not known. Also, the stationary phase of the parent had a higher cell density than that of the mutant, 1.85 optical density units (1.8×10^9 cells/ml) versus 1.48 optical density units (1.45×10^9 cells/ml).

Despite considerable gaps in our understanding of the physiological conditions inside nodules, it is increasingly clear that rhizobia are required to adjust to changes within the nodule microenvironment. Besides the low oxygen conditions of the central nodule tissue and cells, increased osmolarity is likely to exist in the infection threads and the symbiosomes (31). In addition, acidic conditions are likely to occur within the infection thread and symbiosome microenvironments. The rhizobial symbiont would have to adapt to these changes. Therefore, the *acpXL*::*kan* mutants were examined for their sensitivity to salt and acidic pHs. The growth of the mutant was monitored on solid medium at added salt concentrations of 0.0%, 0.25%, 0.5%, 0.75%, and 1.0%. Its growth was impaired at 0.25% and abolished at 0.5% salt. Figure 8B shows the results at 0.5% salt. Both the parent and the complemented mutant grew well in 0.5% salt. The pH sensitivity was examined by growing the strains on solid medium at pH 7.0, 6.0, 5.5, 5.0, and 4.8. The mutants were unable to grow at pH 5.0 and below, while the parent and the complemented mutant grew well at pH 4.8 and above. The results at pH 5.0 are shown in Fig. 8C.

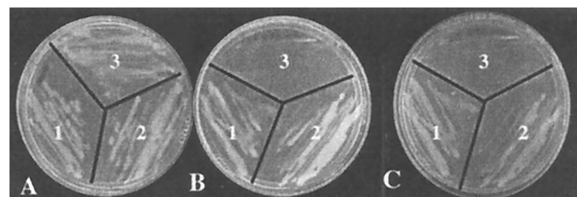

FIG. 8. Growth of *R. leguminosarum* bv. *viciae* 3841 (sector 1), mutant 22 (sector 3), and a complemented mutant 22 (sector 2) in normal medium (A), with 0.5% NaCl (B), and at pH 5.0 (C). The media are described in Materials and Methods.

Symbiotic phenotype. In order to determine the nodulation characteristics of the mutants, pea plants were inoculated with either the *acpXL*::*kan* mutant or parent bacteria, and nodule development was followed. One week postinoculation, the peas inoculated with the parent showed small pinkish nodules. On peas inoculated with *R. leguminosarum* bv. *viciae* mutant 22, at 1 week postinoculation, there were small whitish nodules which were clearly delayed in development compared to those resulting from the parent inoculation. After 3 weeks, the nitrogen-fixing ability of the mutant nodules was compared with that of the parent nodules, and no significant differences were observed. The delayed nodule development by the mutant may result from the fact that the mutant grows more slowly inside the nodule. On the other hand, the delayed nodule development may also result from the fact that the mutant is severely stressed (e.g., osmotically) in the nodule environment but is somehow able to compensate for this defect. A detailed analysis of nodule formation and nodule morphology is under investigation.

The rhizobia recovered from mutant nodules were genetically stable, and no revertants were observed, as indicated by the presence of expected antibiotic resistance and agarose gel electrophoretic analysis of the *acpXL*::*kan* PCR fragments (data not shown).

DISCUSSION

The LPSs from members of the *Rhizobiaceae* and from a number of gram-negative facultative intracellular pathogens contain 27OHC28:0 in their lipid A's (4), suggesting conserved

and important cellular functions for growth and survival in their natural environments. In particular, both members of the *Rhizobiaceae* and the pathogens have the ability to survive within special intracellular compartments of their host cells.

There is a great deal of precedent in the literature for the ability of bacteria to modify their lipid A in response to a changing environment, which illustrates the functional importance of lipid A changes in the adaptation of bacteria to their changing environments. It is known that enteric bacteria modify their lipid A in response to changes in environmental conditions such as those within the host intracellular "spacious phagosomes," i.e., low pH and/or low Mg^{2+} concentration (for reviews, see references 18 and 41). These modifications, such as the addition of 4-aminoarabinose, phosphoethanolamine, 2-hydroxylation of myristic acid, and the addition of a palmitoyl residue, are mediated by the global regulatory gene products PhoP and PhoQ and are important for protecting the bacteria from the host defenses.

In symbiotic bacteria, the reported doubling of 27OHC28:0 in the lipid A of *R. leguminosarum* bv. *viciae* during symbiosis (26) probably reflects one of the ways in which this bacterium adapts to the changing environment of its host. However, the exact function of this structural modification has not yet been determined. For other intracellular pathogens, e.g., *Brucella abortus*, which survive within phagosomes and cause chronic infections, it is not known whether the lipid A 27OHC28:0 component contributes to virulence.

The first step that was necessary to characterize the symbiotic function of this unusual lipid A 27OHC28:0 fatty acyl component was to isolate a *Rhizobium* mutant that was unable to incorporate this very long chain fatty acid into its lipid A. In this report, we described the preparation and partial characterization of such a mutant. In summary, we have shown that (i) an *acpXL* mutant of *R. leguminosarum* bv. *viciae* 3841 has been generated, (ii) this mutant does not incorporate significant levels of 27OHC28:0 into its lipid A, resulting in a tetraacylated lipid A molecule, (iii) the mutant has altered growth properties (increased lag time and lower stationary-phase cell density), (iv) the mutant is sensitive to both salt and low pH, (v) it forms nodules more slowly than the parent but these mutant nodules are able to fix nitrogen, and (vi) when the mutant is complemented with normal *acpXL,* it is corrected in both its lipid A structure and its physiological characteristics.

It is not yet known if the *acpXL*::*kan* mutation and resulting lack of 27OHC28:0 on the lipid A affects the biosynthesis of the polysaccharide portion of the *R. leguminosarum* bv. *viciae* 3841 LPS. It is clear from the deoxycholate-PAGE analysis that the *acpXL*::*kan* mutant is able to produce LPS that contains O-chain polysaccharide, because it has a banding pattern that, while slightly altered, includes the presence of LPS I, which contains O-chain polysaccharide. Detailed structural work on the carbohydrate portions of the LPSs from the parent and mutant is in progress.

Analysis of the mutant lipid A showed that it has the same structure as the parental lipid A, the major difference being the lack of 27OHC28:0. As stated in the introduction, it has been shown that *R. leguminosarum* bv. *viciae* contains the same enzymes that convert UDP-GlcNAc into a common lipid A precursor known as Kdo_2lipid-IVA as *E. coli*, that *R. leguminosarum* bv. *viciae* has unique enzymes that process this precursor into its unique lipid A, and that AcpXL is required (as described in this paper) for acyloxyacylation of Kdo_2lipid-IVA with 27OHC28:0. The fact that the *acpXL*::*kan* mutant is able to produce a lipid A structure that is the same as that of the parent except for the missing 27OHC28:0 residue shows that the addition of this fatty acid is not required for processing the Kdo_2lipid-IVA precursor into the rhizobial structure, i.e., 27OHC28:0 acylation is not required for removal of the 4′- and 1-phosphates, addition of the galacturonosyl residue, or oxidation of the proximal glucosaminosyl residue to 2-aminogluconate.

The mutant lipid A preparation has a minor molecular species in which the mass of the molecular ion indicates that the 27OHC28:0 residue has been replaced with a palmitoyl residue. The addition of a straight-chain fatty acyl moiety to the rhizobial lipid A would be unusual because it has been reported that the transfer of straight-chain fatty acyl substituents to *R. etli* or *R. leguminosarum* bv. *viciae* lipid A does not occur (10), and, in fact, no such fatty acyl residues have been observed on these lipid A molecules (5, 39, 40). Changes in lipid A fatty acylation do occur in enteric bacteria. For example, under certain conditions, it has been reported that *Salmonella* spp. can transfer a palmitoyl residue from glycerophospholipids to its lipid A via the action of PagP, a protein that is under PhoPQ regulation (7). However, there is no evidence of a PagP homolog in either the *Mesorhizobium loti* or *Sinorhizobium meliloti* genome sequence (www.kazusa.or.jp/en/, http://sequence.toulouse.inra.fr/meliloti.html). The *R. leguminosarum* bv. *viciae* 3841 genome sequence is not yet finished; however, there is no PagP homolog in the sequence that is currently available (www.sanger.ac.uk/Projects/R_leguminosarum/). It will be necessary to isolate this minor lipid A species in larger amounts in order to determine its structure and verify whether or not it contains a palmitoyl residue and, if so, the location of that residue.

The increased lag phase and the lower LPS yield of the *R. leguminosarum* bv. *viciae acpXL*::*kan* mutant may be due, indirectly, to the production of tetraacylated lipid A. In the case of an *E. coli lpxL* mutant, tetraacylated lipid A accumulates at the inner membrane (49, 50), suggesting inefficient transport. It may be that the reduced yield of LPS from the *R. leguminosarum* bv. *viciae* 3841 *acpXL*::*kan* mutant and, perhaps, the increased lag time are due to poor transport of the tetraacylated lipid A. The inefficient transport of the tetracyl lipid A in the *E. coli lpxL* mutant can be suppressed by overexpression of the ABC transporter MsbA (15). Further investigation is required to determine if the *R. leguminosarum* bv. *viciae* 3841 *acpXL*::*kan* mutant accumulates tetraacyl lipid A at the inner membrane.

During symbiosis, the bacterial symbiont must adapt to the environment of its plant host. These environmental changes likely require that the bacteria adjust to changes in osmolarity, acidity, and O_2 tension. In view of these necessary adjustments, it is interesting that the *R. leguminosarum* bv. *viciae acpXL*::*kan* mutant is able to form nitrogen-fixing nodules even though it is unable to grow at pH 5.0 or in 0.5% NaCl. These results suggest that the mutant, in planta, is capable of compensating, at least in part, for the loss of *acpXL*. In a recent review by Geiger and Lopez-Lara (21), the presence of multiple types of acyl carrier protein in various bacterial species was discussed.

Some members of the *Rhizobiaceae* have several specialized types of acyl carrier protein, including AcpXL and NodF (required for acylation of the acylated chitin oligomers known as Nod factors) (21). Thus, it may be that an additional acyl carrier protein comes into play in the in planta environment.

In the case of *S. meliloti*, Geiger and Lopez-Lara reported that an acyl carrier protein is present on the symbiotic plasmid, and other workers have shown that various long-chain ω-1 hydroxy fatty acyl residues (C_{18} to C_{26}) can be incorporated into the Nod factors and that this incorporation is regulated by *nodD3* (13, 21). Thus, it may be that *R. leguminosarum* bv. *viciae* has an alternative mechanism for the addition of 27OHC28:0 to lipid A which is host inducible. The fact that the lipid A from *R. leguminosarum* bv. *viciae* 3841 isolated from bacteria grown under low O_2 has increased levels of 27OHC28:0 supports the possibility of such an additional and, perhaps, inducible mechanism for 27OHC28:0 acylation.

Further examination of this *R. leguminosarum* bv. *viciae* *acpXL*::*kan* mutant may also facilitate understanding of the virulence mechanism of several gram-negative pathogens. As mentioned in the introduction, long-chain ω-1 hydroxy or oxo-fatty acyl residues are present in a variety of gram-negative bacterial species in addition to members of the *Rhizobiaceae*. The presence of these lipid A long-chain ω-1 fatty acyl residues and/or *acpXL* or *lpxXL* homologs has been reported in a number of gram-negative facultative intracellular organisms, including *Brucella abortus* (4), *Brucella melitensis* (1), *Legionella pneumophila* (54, 55), *Bartonella henselae* (4), and *Rickettsia prowazekii* (1). Thus, the presence of the 27OHC28:0 in the lipid A of such pathogens may play a role in their ability to survive within the host cell.

As with the proposed function for 27OHC28:0 in *Rhizobium*-legume symbiosis, this very long chain fatty acyl component may increase the stability of the bacterial membrane. It is also possible that this fatty acyl residue results in a low endotoxic response in the host. In fact, the *R. etli* LPSs and the *Rhizobium* sp. strain Sin-1 LPS, which has a lipid A structure similar to that of *R. etli,* do not induce cytokines in human macrophages (monomac 6) cells and inhibit the ability of enteric LPSs to induce cytokines by interfering with endotoxin binding to both LPS binding protein and the cellular differentiation protein CD14 (47). It would be very instructive to characterize the effects of *acpXL*::*kan* mutations on the virulence of these intracellular pathogens.

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