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The Pea Nodule Environment Restores the Ability of a *Rhizobium leguminosarum* Lipopolysaccharide *acpXL* Mutant To Add 27-Hydroxyoctacosanoic Acid to Its Lipid A

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Members of the *Rhizobiaceae* contain 27-hydroxyoctacosanoic acid ($27OHC_{28:0}$) in their lipid A. A *Rhizobium leguminosarum 3841 acpXL* mutant (named here Rlv22) lacking a functional specialized acyl carrier lacked $27OHC_{28:0}$ in its lipid A, had altered growth and physiological properties (e.g., it was unable to grow in the presence of an elevated salt concentration [0.5% NaCl]), and formed irregularly shaped bacteroids, and the synchronous division of this mutant and the host plant-derived symbiosome membrane was disrupted. In spite of these defects, the mutant was able to persist within the root nodule cells and eventually form, albeit inefficiently, nitrogen-fixing bacteroids. This result suggested that while it is in a host root nodule, the mutant may have some mechanism by which it adapts to the loss of $27OHC_{28:0}$ from its lipid A. In order to further define the function of this fatty acyl residue, it was necessary to examine the lipid A isolated from mutant bacteroids. In this report we show that addition of $27OHC_{28:0}$ to the lipid A of Rlv22 lipopolysaccharides is partially restored in Rlv22 *acpXL* mutant bacteroids. We hypothesize that *R. leguminosarum* bv. viciae 3841 contains an alternate mechanism (e.g., another *acp* gene) for the synthesis of $27OHC_{28:0}$, which is activated when the bacteria are in the nodule environment, and that it is this alternative mechanism which functionally replaces *acpXL* and is responsible for the synthesis of $27OHC_{28:0}$-containing lipid A in the Rlv22 *acpXL* bacteroids.

Rhizobium leguminosarum cells have an envelope similar to that of other gram-negative bacteria. Lipopolysaccharide (LPS) is the primary component of the bacterial outer leaflet and is comprised of three structural regions: the O-chain polysaccharide, the core oligosaccharide, and lipid A. The lipid A region is anchored in the bacterial outer membrane, and the carbohydrate portion projects from the outer surface into the surrounding milieu and is the primary immunogenic determinant. Correlating LPS structure with function has been difficult as LPS is a very complex molecule and LPS preparations consist of structurally heterogeneous mixtures of molecules. There are pronounced variations in LPS structure from strain to strain, and even within a strain there are different sizes and compositions of LPS (23). Interestingly, there are marked differences between LPSs from free-living cultures and LPSs from nitrogen-fixing bacteroids in terms of size, composition, and antigenic properties (10, 32, 33). It has been shown that the rhizobial LPS undergoes structural modifications during the formation of bacteroids and that there are composition differences between the bacterial and bacteroid LPSs (10, 32).

Variation in LPS structure due to environmental changes has been studied in cultured rhizobia by altering the growth conditions, such as lowering the oxygen level, lowering the pH, altering the carbon source, or adding plant-derived compounds (2, 14, 21, 23, 27). Such studies have shown that cues from the environment play an important role in LPS composition. The results led to the hypothesis that the bacterial LPS structure inside legume root nodules is probably controlled, to a large extent, by the in planta microenvironmental conditions. In the study of Kannenberg and Carlson (22), *Rhizobium leguminosarum* bv. viciae 3841 (named here Rlv3841) was cultured under various growth conditions, and the LPS structural modifications were analyzed chemically and immunochemically. It was observed that the LPS extracted from nodule bacteria or from laboratory-grown bacteria cultured under low-oxygen conditions was much more hydrophobic than the LPS from bacteria grown under normal laboratory conditions. Chemical analysis of the LPS derived from bacteria grown under low-oxygen conditions indicated that changes occurred in both the polysaccharide and lipid A portions of the LPS; the polysaccharide was affected in the extent of methylation and acetylation, while the lipid A showed an increase in a unique very-long-chain fatty acyl component, 27-hydroxyoctacosanoic acid ($27OHC_{28:0}$). This suggested that the low-oxygen conditions in nodule cells may cause similar structural changes to the LPS.

The functions of LPS structural changes that occur during symbiosis are not known. These changes may be needed for the increased membrane stability and barrier properties that are required for the bacteroid to persist and function within the symbiosome compartment. One of the LPS components that may be essential in the symbiotic process is the very-long-chain

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fatty acyl component $27OHC_{28:0}$ in lipid A. This fatty acyl component is present in the LPS of members of the *Rhizobiaceae* (5, 11, 23). In addition, a number of facultative intracellular pathogenic bacterial species that cause chronic infections also contain this lipid A fatty acyl residue or orthologs of *acpXL* or *lpxXL'* genes that are required for its synthesis and transfer to lipid A. These species include *Brucella abortus* (3), *Brucella melitensis* (1), *Legionella pneumophila* (39, 40), and *Bartonella henselae* (3). Thus, it is possible that the $27OHC_{28:0}$ residue may be required for endosymbiotic rhizobia and these intracellular pathogens to persist and function within their host cells.

In order to examine the symbiotic function of the $27OHC_{28:0}$ lipid A component, we prepared and characterized an LPS mutant that is defective in the acyl carrier protein (ACP) required for its synthesis, AcpXL (34, 35). Laboratory-grown cultures of this mutant, Rlv22, produced an LPS that did not contain $27OHC_{28:0}$ in its lipid A and was unaffected in its O-chain polysaccharide and core oligosaccharide structures (35). The Rlv22 mutant was unable to grow under laboratory conditions at a low pH (pH 5.0) or with 0.5% NaCl added to the medium, and nodule development was delayed, although eventually nitrogen-fixing nodules were formed. Similar results were reported by Sharypova et al. for an *acpXL* mutant of *Sinorhizobium meliloti* (31). The ability of Rlv22 to form nitrogen-fixing nodules in spite of its inability to adjust to changes in osmotic strength or pH ex planta suggested that it may adapt in a specific manner to the in planta conditions (34, 35). Microscopic examination revealed that the Rlv22 mutant formed large irregularly shaped bacteroids and that multiple bacteroids were often surrounded by a single symbiosome membrane (34). However, it was also observed that a significant number of normal bacteroids were present, and, as previously observed, nitrogen-fixing nodules formed, although the level of nitrogenase in Rlv22-induced nodules was significantly lower than the level in normal nodules (34). Thus, it was concluded that depletion of $27OHC_{28:0}$ in the lipid A of the Rlv22 mutant disrupted bacteroid development and the synchrony between bacterial and symbiosome membrane division. However, the eventual appearance of some normal bacteroids and nitrogen-fixing nodules suggested that the mutant, once it was in its host, was able to partially compensate in some manner for the loss of $27OHC_{28:0}$ in its lipid A. Investigation of the nature of this compensation required isolation and characterization of the lipid A from mutant bacteroids. In this paper, we report the results of this analysis.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains used are listed in Table 1. Strains were cultured on solid or in liquid tryptone yeast extract (TY) medium (Difco Laboratories), as previously described (35). Cultures were supplemented with kanamycin (Kan) (50 μg ml^{-1}) and streptomycin (Str) (100 μg ml^{-1}), when appropriate. The osmotic tolerance of bacterial isolates was studied by growing two strains on TY containing 0.5% NaCl at 30°C, along with the appropriate controls. The pH tolerance of the bacterial isolates was examined at pH 5, using piperazine-buffered TY plates as described by Vedam et al. (35).

Growth of peas. Peas (*Pisum sativum* cv. Early Alaska) were surface sterilized by soaking them in 95% ethanol for 30 s, rinsing them three times with sterile water, washing them with bleach for 3 min, and rinsing them 10 times with sterile water. Seeds were placed in sterile petri dishes with a small amount of sterile water and allowed to imbibe and germinate in the dark for 48 h. Pea seedlings were sown in "caissons" and supplied with Lullien's nutrient solution (25) lacking ammonium nitrate. Caissons are aeroponic growth systems that provide controlled conditions for the growth of peas. After 1 week of germination and stabilization of the seedlings, the peas were inoculated with either Rlv3841 (wild type) or Rlv22 (*acpXL* mutant). The pea plants were allowed to nodulate for 3 weeks, and the caissons were periodically refreshed with nutrient solution. At the end of the growth period, the nodules were harvested for preparation of bacteroids.

TABLE 1. Bacterial strains used in this study

Strain	Characteristics	Reference
R. leguminosarum bv. *viciae* 3841 (Rlv3841)	300 Str^r Fix^+ $NaCl^r$	37
R. leguminosarum bv. *viciae* 22 (Rlv22)	Rlv3841 *acpXL*::*kan* Str^r Km^r Fix^+ $NaCl^s$	35
R. leguminosarum bv. *viciae* 22[a] (EN2)	Rlv22 Str^r Km^r Fix^+ $NaCl^r$	This study
R. leguminosarum bv. *viciae* 22[a] (EN4)	Rlv22 Str^r Km^r Fix^+ $NaCl^r$	This study

[a] Ex nodule isolate.

Extraction of bacteroids from pea nodules. Nodules were harvested from pea plants that had been inoculated with either Rlv3841 or Rlv22 into an ice-cold solution of 0.5 M sucrose in 50 mM Tris-HCl (pH 7.4) plus a 1:100 dilution of protease inhibitor cocktail (Sigma P9599). For (bio)chemical analysis, bacteroids were isolated from these nodules and purified by a procedure involving a sucrose step gradient, as described previously (13). Briefly, pea nodules were washed with cold Tris-HCl/sucrose buffer (0.5 M sucrose–50 mM Tris-HCl [pH 8.0] at 4°C containing dithiothreitol, proteinase inhibitor, and polyvinylpolypyrrolidone), suspended in the same buffer, and ground up using a mortar and pestle. The initial steps of the isolation protocol were done with sucrose-containing buffer to osmotically stabilize symbiosomes and bacteroids. To remove tissue and cell debris, the crushed nodule material was filtered through miracloth and rinsed with the same solution. The rinse solution was added to the filtrate, and the resulting suspension was centrifuged for 1 min at 10,000 × *g*. The pellet, containing the symbiosomes, was resuspended in Tris-HCl/sucrose buffer. Portions of the suspension were distributed in several microcentrifuge tubes, overlaid onto sucrose cushions (composed of 1.5 M sucrose and 50 mM Tris-HCl [pH 8.0] at 4°C), and centrifuged at 5,000 × *g* for 30 s. The top phases of the different tubes, strongly enriched in the bacteroid-containing symbiosomes, were transferred to a clean tube and centrifuged at 10,000 × *g* for 90 s to collect the symbiosome fraction in the pellet. The supernatant was discarded, and the pellet was resuspended in Tris-HCl/sucrose buffer and again overlaid onto a sucrose cushion as described above; however, this time the preparation was centrifuged at 10,000 × *g* for 5 min, which sedimented the symbiosomes with the bacteroids in the pellet. To remove the peribacteroid membrane by osmotic shock from the symbiosomes and wash the bacteroids, the pellets were repeatedly (two or three times) resuspended, with vigorous mixing, in 500 µl of Tris-HCl buffer without sucrose (0.50 mM Tris-HCl [pH 8.0] with dithiothreitol and proteinase inhibitor) and centrifuged; the final pellet was suspended in 1,600 µl of the same buffer and stored at −20°C.

During this work, individual colonies obtained from Rlv22-induced nodules were examined for antibiotic resistance and the presence of *acpXL*::*kan*. Resistance to Kan and Str was measured for about 500 colonies using growth on solid agar with and without the antibiotics. The presence of *acpXL*::*kan* (or *acpXL*) was measured for 12 of the colonies described above using PCR and primers for *acpXL* (GAGGGGGTTTAAATAGTCA and AGGCTTGGCCGCTTTGA), as previously described by Vedam et al. (35).

To rule out the possibility that a small number of possible mutant revertants escaped the analysis described above and occupied nodules undetected, a PCR screening analysis was performed directly with bacteroid preparations from nodules using the primers specific for *acpXL* described above in order to determine if any of the wild-type *acpXL* gene was present. Preparations of bacteroids isolated from nodules of 10 pea plants induced by Rlv3841 or Rlv22 were PCR screened. Aliquots of the bacteroid preparations (2 to 5 µl from an approximately 500-µl dense suspension of bacteroids) were pipetted into the PCR mixture and lysed during an initial 15-min hot start in the assay. The PCR products were analyzed using 1% agarose gel electrophoresis.

Preparation of bacterial isolates from Rlv22-induced nodules (Rlv22 EN isolates). In order to fully examine the effect of passage of Rlv22 through the plant, it was necessary to isolate and characterize in more detail mutant isolates obtained from pea root nodules. Rlv22 ex nodule (EN) isolates were analyzed for

antibiotic resistance, the presence of *acpXL*::*kan* or *acpXL*, and sensitivity to growth at a low pH (pH 5.0) and in the presence of 0.5% NaCl, and their LPSs were extracted and analyzed as described below. Seeds were germinated and grown in Erlenmeyer flasks containing solid Fahraeus medium. After surface sterilization (see above) and transfer into the flasks, pea seeds were immediately inoculated with the Rlv22 mutant, and plant growth and nodulation were evaluated as described by Brewin et al. (9). Root nodules from several pea plants were removed, briefly washed in 95% ethanol, and, by size, surface sterilized in diluted bleach for 30 to 60 s. The nodules were transferred through a series of washes with sterile water and finally crushed to squeeze the occupying bacteria out onto TY agar plates. The plates were developed, and three single colonies from each plate were streaked onto fresh plates. Care was taken to pick colonies of different sizes (small, medium, and large) in case the size differences represented different variants of the original mutant. Two or three nodules per plant were analyzed in this way. From the Rlv22 EN colonies (kept on TY plates), a total of 23 clones (derived from four plants and eight nodules) were analyzed for antibiotic resistance (resistance to Str and Kan). A random selection of 16 clones (two clones per nodule) were tested further for sensitivity to salt (0.5% NaCl) and to an acidic pH (pH 5). As a control and for comparison, strains Rlv3841 and Rlv22 were included in the antibiotic, saline, and low-pH sensitivity tests. Since all Rlv22 EN isolates behaved similarly in these tests, two random Rlv22 EN isolates, EN2 and EN4, were selected, and we verified that they contained the *acpXL*::*kan* mutation using PCR and the primers for *acpXL* as previously described by Vedam et al. (35).

Lipid A purification. LPSs were extracted by the triethylamine (TEA)/EDTA/phenol procedure as previously described (25). Briefly, for each strain, the LPS was extracted from the bacterial or bacteroid pellet using 3 volumes of TEA/EDTA/phenol (0.25 m EDTA, 5% phenol, titrated to pH 6.9 with TEA with constant stirring for 1 h at 37°C). The extract was centrifuged at 13,000 × *g* for 1 h, and the supernatant was collected and dialyzed (molecular weight cutoff, 10,000; Spectrapor) against deionized water. The bacterial and bacteroid LPSs were lyophilized for analysis. Lipid A was isolated from the LPS preparations by mild acid hydrolysis (12). Briefly, the LPS was dissolved in 1% sodium dodecyl sulfate in 20 mM sodium acetate, the pH was adjusted to 4.5 with 4 M HCl, and then the preparation was placed in an ultrasound bath until the sample was dissolved. The solution was then heated to 100°C for 1 h, which was followed by lyophilization. The sodium dodecyl sulfate was removed by washing the lyophilized residue with a 2:1 solution of deionized H_2O and acidified ethanol (100 μl of 4 M HCl in 20 ml of 95% ethanol). The residue was collected by centrifugation, washed with 95% ethanol (nonacidified), and collected by centrifugation (200 × *g* for 15 min). The washing and centrifugation steps were repeated. Finally, the residue was lyophilized to obtain a white, solid, fluffy lipid A preparation.

Analytical procedures. The composition was determined by gas chromatography-mass spectrometry (MS) analysis of trimethylsilyl derivatives of methyl glycosides and hydroxy fatty acid methyl esters as described previously (4, 38). Matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) MS was performed in the negative-ion reflectron mode with a 337-nm nitrogen laser operating at an extraction voltage of 20 kV 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/ml of methanol) and applied to the probe for mass analysis. Spectra were calibrated externally using *E. coli* lipid A (Sigma).

RESULTS

Stability of the *acpXL* mutation in Rlv22 during nodule development. In order to rule out the possibility of genetic reversion of the *acpXL* mutation in planta, bacterial colonies obtained from nodules infected by Rlv22 were tested for resistance to kanamycin by using an antibiotic marker cassette introduced into *acpXL* (35). Approximately 500 colonies were screened, and there was no loss of the resistance marker; i.e., all 500 colonies were resistant to both Kan and Str. In addition, genomic DNA from 12 of these colonies was tested by PCR, using primers for *acpXL*. Each of the 12 colonies showed only the presence of *acpXL*::*kan*, indicating that the mutation was intact. Furthermore, chromosomal DNA from two of these colonies was analyzed by Southern hybridization using amplified *acpXL* as the labeled probe, and the results showed that both colonies retained the mutation. In addition, bacteroids obtained from Rlv22-induced nodules were directly examined by PCR using primers for *acpXL*. As a control, PCR analysis was also performed with bacteroids from Rlv3841-induced nodules. The results (Fig. 1) showed that bacteroids from Rlv22-induced nodules contained only *acpXL*::*kan* and that there was no detectable evidence of intact *acpXL*. This finding confirmed the stability of the *acpXL* mutation of Rlv22 during nodule development.

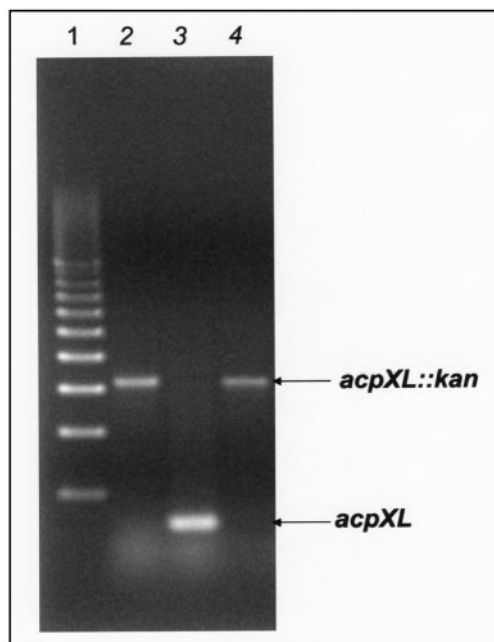

FIG. 1. PCR fragments amplified from Rlv3841 and Rlv22 bacteroids. Lane 1, 500-bp ladder; lane 2, PCR fragments from Rlv22 bacteroids; lane 3, PCR fragments from Rlv3841 bacteroids; lane 4, PCR fragments from a laboratory-grown culture of Rlv22. For the bacteroid preparations, isolated nodule bacteria were pooled from nodules of 10 pea plants. An approximately 300-bp PCR fragment indicates that the wild-type *acpXL* gene is present, while the mutated *acpXL*::*kan* PCR fragment is approximately 1,600 bp long.

Analysis of the lipid A from Rlv22 bacteroids. The lipid As from LPS preparations of parent Rlv3841 and mutant Rlv22 laboratory-grown bacteria and from bacteroids isolated from root nodules were analyzed to determine their fatty acid compositions (Table 2) and by MALDI-TOF MS (Fig. 2).

The composition analysis revealed that the lipid A from Rlv3841 laboratory-grown cells contained the same fatty acids at the same ratio that was observed for the lipid A from Rlv3841 bacteroids. In contrast, the Rlv22 laboratory-grown cells produced lipid A in which no long-chain $27OHC_{28:0}$ could be detected. However, the lipid A from Rlv22 bacteroids contained $27OHC_{28:0}$ at a level that was about 50% of the level observed in the Rlv3841 parent lipid A. In addition, the Rlv22 lipid A preparations contained noticeably increased levels of palmitate ($C_{16:0}$) and stearate ($C_{18:0}$) compared to the levels in the Rlv3841 lipid A samples.

The mass spectrum of the lipid A from laboratory-grown Rlv3841 (Fig. 2A) shows that there were two clusters of ions. The first cluster of ions ranged from *m*/*z* 1887.6 to 2058.0, with

TABLE 2. Fatty acid compositions of the lipid A purified from Rlv3841 and *acpXL* mutant Rlv22 laboratory-grown cells and bacteroids isolated from pea nodules[a]

Fatty acid	Mass % in:			
	Rlv3841 laboratory-grown cells	Rlv3841 bacteroids	Rlv22 laboratory-grown cells	Rlv22 bacteroids
$3OHC_{14:0}$	44	43	48	47
$3OHC_{15:0}$	3	3	2	2
$3OHC_{16:0}$	13	12	19	15
$3OHC_{18:0}$	24	23	31	24
$27OHC_{28:0}$	17	19	ND	11
$C_{16:0}$	Tr	Tr	+	+
$C_{18:0}$	Tr	Tr	+	+

[a] The values are relative mass percentages of total fatty acids. The levels of $C_{16:0}$ and $C_{18:0}$ in the lipid A samples could not be accurately quantified due to partial contamination of the samples with phospholipids; however, the Rlv22 samples contained higher levels (+) of these two saturated fatty acids than the Rlv3841 samples contained (trace amounts, Tr). ND, not detected. Bacteroids were isolated from pea nodules.

the most intense ion at *m/z* 1914.0, and the other cluster of ions ranged from *m/z* 1625.7 to 1738.9, with the most intense ion at *m/z* 1652.0. The structures corresponding to the lipid A ions for the laboratory-grown Rlv3841 (structures I and II) have been reported previously (4, 35) and are shown in Fig. 2. The ion at *m/z* 1914.0 is consistent with the previously published *R. leguminosarum* or *Rhizobium etli* lipid A structure (4, 28, 29, 35), in which the lipid A has a disaccharide backbone consisting of a distal glucosaminosyl residue that is β-1,6 linked to a proximal 2-aminogluconate (GlcNonate) residue. At the 4′ position of the distal glucosamine there is an α-galacturonosyl residue substitution, and the β-glucosaminosyl-(1→6)-GlcNonate disaccharide is acylated with β-hydroxy fatty acids at the 2, 3, 2′, and 3′ positions. The $27OHC_{28:0}$ lipid A moiety is present as a secondary acyloxyacyl residue and is ester linked to the hydroxy group of the 3′-β-hydroxy fatty acid residue. The ions at *m/z* 2001.4 and above are due to molecules in which the 27-OH group of the $27OHC_{28:0}$ molecule is esterified with β-hydroxy-

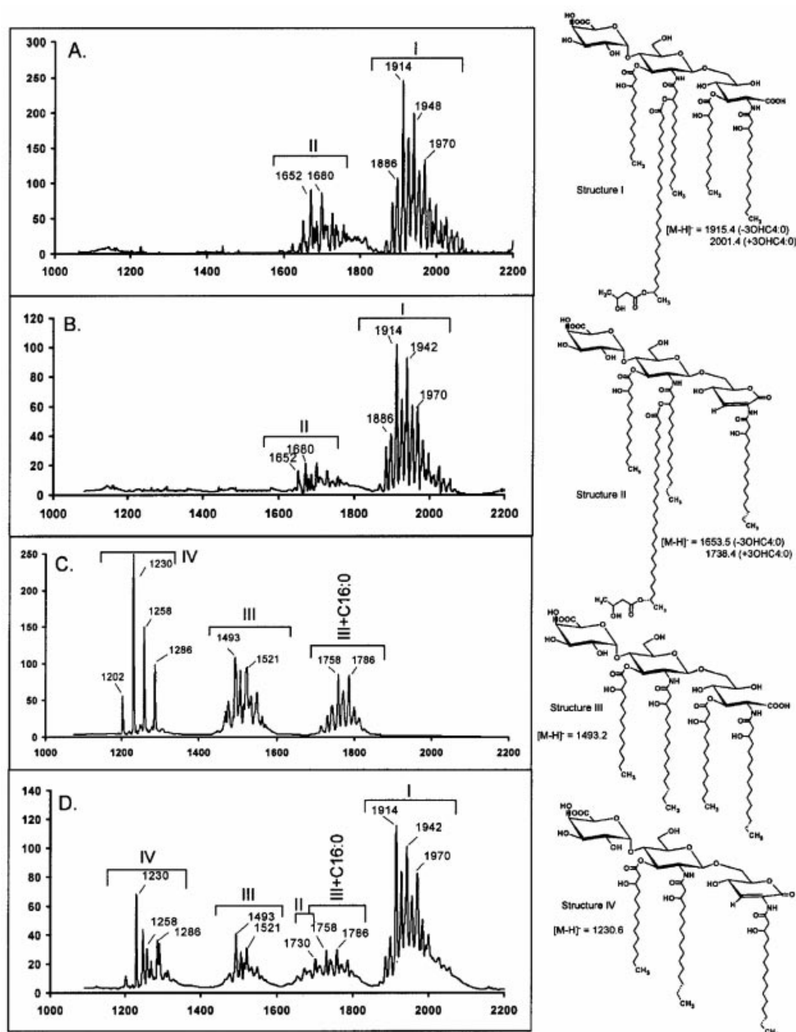

FIG. 2. MALDI-TOF MS spectra of lipid A from Rlv3841 and from its *acpXL* mutant Rlv22 isolated from laboratory-grown cultures and from bacteroids. (A) Lipid A from a laboratory-grown culture of parent strain Rlv3841; (B) lipid A from bacteroids of parent strain Rlv3841; (C) lipid A from a laboratory-grown culture of *acpXL*::*kan* mutant Rlv22; (D) lipid A from bacteroids of Rlv22. Structures I, II, III, and IV for the ions are also shown and are based on a composition analysis (described in the text) and on previously reported lipid A structures (28).

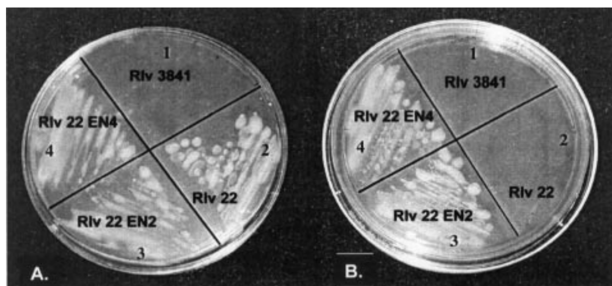

FIG. 3. Salt tolerance of the parent, *acpXL*::*kan* mutant Rlv22, and *acpXL*::*kan* mutant nodule isolates EN2 and EN4. (A) Growth on normal laboratory medium (see Materials and Methods) with kanamycin. All strains grew equally well on medium without kanamycin (data not shown). (B) Growth on normal laboratory medium with kanamycin and 0.5% NaCl.

butyrate. Other ions in this cluster are due to structural variants resulting from different fatty acyl chain lengths. In the second ion cluster, the most intense ion is the ion at *m/z* 1652.0 and is likely due to a structure (structure II) caused by elimination of the β-hydroxy fatty acyl residue from position 3 of the GlcNonate residue forming a 2,3-unsaturated 2-aminoglucono-1,5-lactone residue; this is a reaction which may be an artifact of the lipid A isolation procedure (20, 35).

The mass spectrum of the lipid A preparation from laboratory-grown mutant Rlv22 (Fig. 2C) also shows that there were two main ion clusters, one centered around the ion at *m/z* 1493.0 and the other centered around the ion at *m/z* 1230.0. A third minor cluster is centered around the ion at *m/z* 1758.0. The two main ion clusters represent structures that are devoid of $27OHC_{28:0}$ or 27O(β-hydroxybutyryl)$C_{28:0}$ (structures III and IV). These structures are identical to those previously reported for the mutant lipid A (35). We also reported previously (35) that the minor cluster of ions centered around *m/z* 1758.0 was due to replacement of $27OHC_{28:0}$ with a palmitoyl ($C_{16:0}$) residue. These ions were observed again in the current study, and this result, together with the composition data (Table 2), could have been due to structures in which $27OHC_{28:0}$ was replaced with a $C_{16:0}$ or stearoyl ($C_{18:0}$) residue.

Figures 2B and 2D show the mass spectra of the lipid A preparations from Rlv3841 and Rlv22 bacteroids, respectively. The mass spectrum of the Rlv3841 bacteroid lipid A preparation (Fig. 2B) is identical to that of the lipid A from the laboratory-grown culture (Fig. 2A) described above. The spectrum of Rlv22 bacteroid lipid A has four major ion clusters (Fig. 2D). Three of these ion clusters are identical to those observed for the lipid A from laboratory-grown Rlv22 (Fig. 2C). The fourth ion cluster is centered around the *m/z* 1914.0 ion and is identical to the second ion cluster for the lipid As from both the Rlv3841 bacteroids and laboratory-cultured cells. This result, together with the composition data described above, shows that the $27OHC_{28:0}$ moiety was present in some of the structures in the mutant bacteroid lipid A preparation. Also present in the Rlv22 bacteroid lipid A preparation were ions that were consistent with structures that lack $27OHC_{28:0}$ but contain an added $C_{16:0}$ or $C_{18:0}$ residue. In summary, the lipid A preparation from the Rlv3841 bacteroids appeared to have the same structures as the preparation from the Rlv3841 laboratory-grown cells. In contrast, Rlv22 laboratory-grown cells lacked $27OHC_{28:0}$ in their lipid A (Fig. 3C), but bacteroids from Rlv22-induced nodules produced some lipid A molecules that contained $27OHC_{28:0}$ (Fig. 3D).

Properties of EN *acpXL* mutant isolates from Rlv22-infected pea plants. In order to determine if passage through the plant had selected for an Rlv22 variant that constitutively expressed an alternative mechanism for synthesis of $27OHC_{28:0}$, we isolated and examined a number of ex nodule mutant isolates. We showed previously that the Rlv22 *acpXL*::*kan* mutant was unable to grow in the presence of 0.5% NaCl or at pH 5.0 (35). Sixteen Rlv22 EN isolates were grown under the same test conditions as the original mutant. We observed that all of the Rlv22 EN isolates were able to grow in the presence of 0.5% NaCl (Fig. 3). On plates with acidic growth conditions (pH 5), the Rlv22 EN isolates showed stronger growth than the original Rlv22 mutant, although the growth was not as good as the growth of wild-type strain Rlv3841 in the same experiment. PCR analysis to detect either *acpXL* or *acpXL*::*kan* was performed with the genomic DNA from two Rlv22 EN isolates, EN2 and EN4, using primers specific for *acpXL*. The results were the same as those obtained for the bacteroid preparations shown in Fig. 1 and showed that the PCR products obtained from the Rlv22 EN isolates were each 1.56 kb long, which is consistent with the size of *acpXL*::*kan*, and were the same size as the PCR product from the original Rlv22 strain. A PCR product indicating that there was an intact *acpXL* gene was not detected in either the EN2 or EN4 isolate.

In order to determine the presence of $27OHC_{28:0}$ in the lipid A from the two Rlv22 EN mutant isolates, lipid A was prepared from these two strains grown under normal laboratory conditions or in the presence of 0.5% NaCl. The lipid A samples were then analyzed by MALDI-TOF MS to determine their fatty acyl compositions. The original Rlv22 mutant was also included in this study as a control. The results are shown in Fig. 4. The mass spectrum of the lipid A from the original Rlv22 mutant (Fig. 4A) was the same as the mass spectrum described above and shown in Fig. 2C. The mass spectra of the lipid As from EN2 and EN4 grown under normal laboratory conditions (Fig. 4B) or in the presence of 0.5% NaCl were identical to one another and very similar to the mass spectrum of the lipid A from the original Rlv22 mutant in that all of the mass spectra lacked the $27OHC_{28:0}$ residue. The minor difference between the spectra of EN2 and EN4 lipid A preparations and the spectrum of the Rlv22 lipid A was that the third minor ion cluster, likely due to replacement of $27OHC_{28:0}$ with a palmitoyl or stearoyl residue, was significantly less intense in the EN2 and EN4 lipid A preparations.

DISCUSSION

We previously showed that *R. leguminosarum acpXL* mutant Rlv22 is unable to add $27OHC_{28:0}$ to its lipid A, exhibits delayed nodulation and nitrogen fixation, forms many aberrantly shaped bacteroids, exhibits disrupted synchrony of bacterium and symbiosome membrane division, and, under laboratory culture conditions, is unable to grow at a low pH (pH 5.0) or in the presence of 0.5% NaCl (35). Sharypova et al. (31) showed that an *acpXL* mutant of *S. meliloti* also lacked $27OHC_{28:0}$ in its lipid A, was sensitive to deoxycholate, exhibited delayed nodulation of *Medicago sativa*, and had reduced competitive

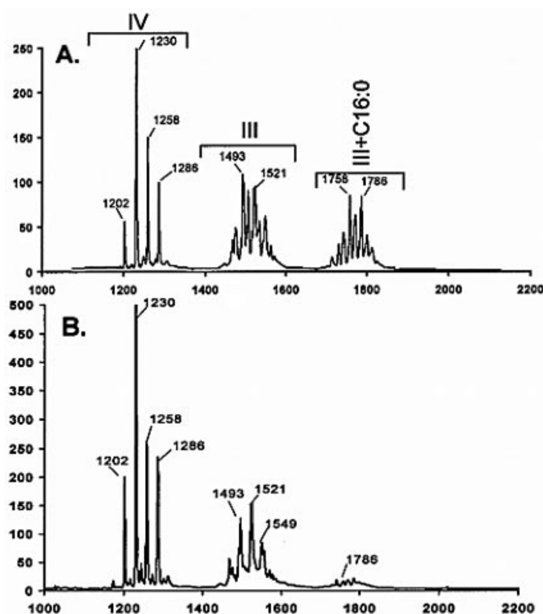

FIG. 4. MALDI-TOF MS spectra of lipid A from laboratory-grown cultures of Rlv22 (A) and ex nodule isolate EN2 (B). The mass spectrum of the lipid A from ex nodule isolate EN4 was identical to that shown for EN2, and the mass spectra of the lipid A preparations from the same cultures grown under laboratory conditions in the presence of 0.5% NaCl were identical to the mass spectra shown.

ability. However, nodules elicited by this mutant on roots of *M. sativa* and *Medicago truncatula* had normal morphology and fixed nitrogen. Sharypova et al. did not describe an effect of the *acpXL* mutation on *S. meliloti* bacteroid development. The aberrant bacteroids that we observed for the Rlv22 mutant (34) support the idea that the $27OHC_{28:0}$ moiety of lipid A plays an important role in bacteroid development and in the synchronous division of bacteria and the symbiosome membrane. However, the ability of the mutant to eventually form nitrogen-fixing nodules in a process in which it is likely to encounter acidic pH conditions and changes in osmolarity raised the question of whether Rlv22 adapts in some manner to the loss of $27OHC_{28:0}$ within the host plant. In this study, we addressed this question by examining the lipid A isolated from Rlv22 bacteroids and from ex nodule *acpXL* mutant isolates EN2 and EN4 grown under laboratory conditions at normal NaCl concentrations and in the presence of 0.5% NaCl. We found that, unlike laboratory cultures, Rlv22 bacteroids are able to add $27OHC_{28:0}$ to their lipid A. Furthermore, we observed that the *acpXL*::*kan* mutation was maintained in Rlv22 bacteroids and in the EN isolates of Rlv22 and that, when grown under laboratory conditions, the EN isolates were still unable to add $27OHC_{28:0}$ to their lipid A. These results indicate that in Rlv3841 an alternative mechanism is induced within the nodule that can functionally replace the inactivated AcpXL in the *acpXL*::*kan* mutant. The presence of an alternative to *acpXL* for the synthesis of $27OHC_{28:0}$ that is induced within the host suggests that this fatty acyl residue in lipid A may be essential for the symbiotic interaction between *R. leguminosarum* and peas.

As mentioned above and consistent with our results with Rlv3841, recent work with *S. meliloti* also showed that an *acpXL* mutant lacked the $27OHC_{28:0}$ residue in its lipid A (31). A subsequent study (15) demonstrated that the *S. meliloti acpXL* mutant contained another lipid A species, in which a $C_{18:0}$ residue replaced the $27OHC_{28:0}$, a result that is consistent with our results (21; this study), which shows that the Rlv22 mutant can produce a lipid A molecule in which the $27OHC_{28:0}$ moiety is replaced by a $C_{16:0}$ or $C_{18:0}$ residue. Since replacement of $27OHC_{28:0}$ by $C_{18:0}$ in the *S. meliloti acpXL* mutant was prevented by a second mutation in the gene encoding the $27OHC_{28:0}$ acyltransferase (*lpxXL*), it was concluded that LpxXL is able to transfer either $C_{18:0}$ or $27OHC_{28:0}$ to the acyloxyacyl position of *S. meliloti* lipid A (15). This report (15) also showed that the *S. meliloti acpXL* mutant, an *lpxXL* mutant, and an *lpxXL acpXL* double mutant were able to form, in a delayed manner, nitrogen-fixing nodules. The latter two mutants were particularly sensitive to stressful conditions, such as detergents or increased salt concentrations. While these results also suggest that in *S. meliloti* there may be alternative mechanisms that are activated within the host that can functionally replace the mutated *acpXL*, as well as *lpxXL*, there has been no analysis of the lipid A from *S. meliloti acpXL* or *lpxXL* mutant bacteroids.

The nature of this alternative host-induced mechanism for synthesizing $27OHC_{28:0}$ is unknown. However, it has been reported that *S. meliloti* possesses multiple ACPs. Apart from the four known major ACPs in rhizobia, genomics has predicted the existence of additional ACPs (19). The complete sequence of *S. meliloti* indicates that there are at least two novel ACPs. One of the ACP genes is located on the Sym plasmid (it is located in a cluster of four genes, all closely linked, perhaps belonging to one operon), and the other is located on the chromosome. The *Mesorhizobium loti* genome also contains an operon similar to the operon identified in *S. meliloti* (19). Since rhizobia possess multiple ACPs, it is possible that *R. leguminosarum* contains an additional ACP that could be activated in planta, thereby compensating for the disrupted *acpXL* in Rlv22 during symbiosis. In this regard we have located in the Rlv3841 genome sequence (www.sanger.ac.uk), which has not been fully annotated yet, two such possible *acp* gene candidates. One *acp* candidate is located on the chromosome, and the second is located on the symbiotic plasmid, pRL10. These two putative *acp* genes encode ACPs that are very similar to one another and to ACPs from *Agrobacterium* and a number of *Burkholderia* strains, particularly strains of the pathogens *Burkholderia mallei* and *Burkholderia pseudomallei*. The Rlv3841 ACP gene region in pRL10 is shown in Fig. 5. The *acp* gene is next to a gene encoding an acyl-ACP dehydrogenase. It is preceded by DNA sequence binding motifs for NifA and for alternative sigma factor 54 in a DNA region that exhibits sequence similarity to a region that precedes the *fixWABC* genes in *R. leguminosarum* UPM791 (26). This location suggests that expression of the *acp* gene may be regulated by the O_2 status of the bacterial cell. The possibility that the other ACPs may be involved in the in planta ability of the *acpXL*::*kan* mutant to synthesize $27OHC_{28:0}$ is under investigation.

$27OHC_{28:0}$ in the lipid A or orthologs of *acpXL* or *lpxXL* have been found in a number of bacterial species, including several pathogens that cause chronic intracellular infections. In all cases in which the gene sequences can be compared, there

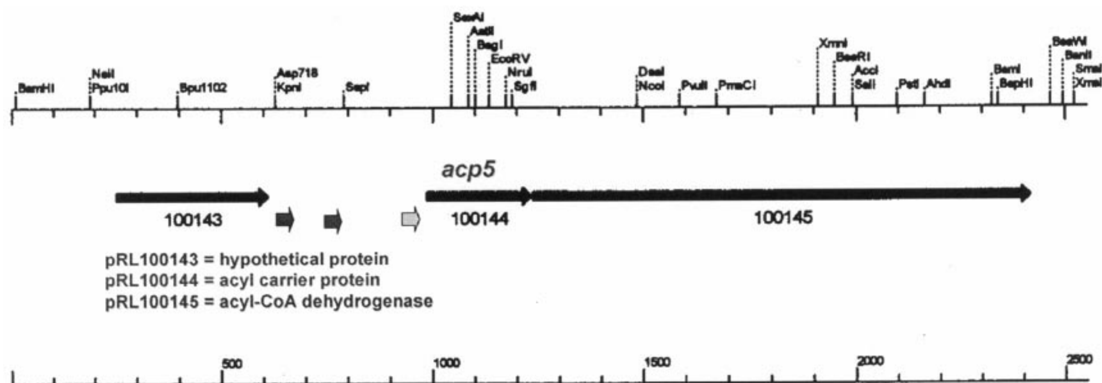

FIG. 5. Gene region for the putative *acp* gene located on symbiotic plasmid pRL10 of Rlv3841. The sequence between pRL100143 and pRL100144 (*acp*) contains sequences similar to the *nifA* upstream activating sequence (left small arrow) and to sigma 54 (middle small arrow) and ribosomal (right small arrow) binding sequences. acyl CoA, acyl coenzyme A.

is a very similar arrangement of genes encoding fatty acyl-ACP synthase orthologs (three or four genes) flanked by *acpXL* on one side and by *lpxXL* on the other side. The arrangement of this gene region is shown for several bacterial species in Fig. 6. There is some variability in this gene region; some species do not contain the putative fatty acid ACP dehydratase (Orf1) gene, while others do not contain the dehydrogenase (Orf4) gene. In all cases, genes encoding proteins with high levels of similarity to AcpXL, LpxXL, Orf2 (a putative 3-oxoacyl ACP synthase), and Orf3 (a putative second 3-oxoacyl ACP synthase) are present. The only exception to the high level of sequence similarity in this gene region is the region from *Legionella pneumophila*. However, the arrangement of the genes in *L. pneumophila* is identical to the arrangement of this region in the other bacterial species shown. Therefore, it is highly likely that this gene region in all of these bacterial species consists of a cassette of five to six genes that are specifically required for the synthesis of very-long-chain ω-hydroxy or -oxo fatty acids and their transfer to lipid A.

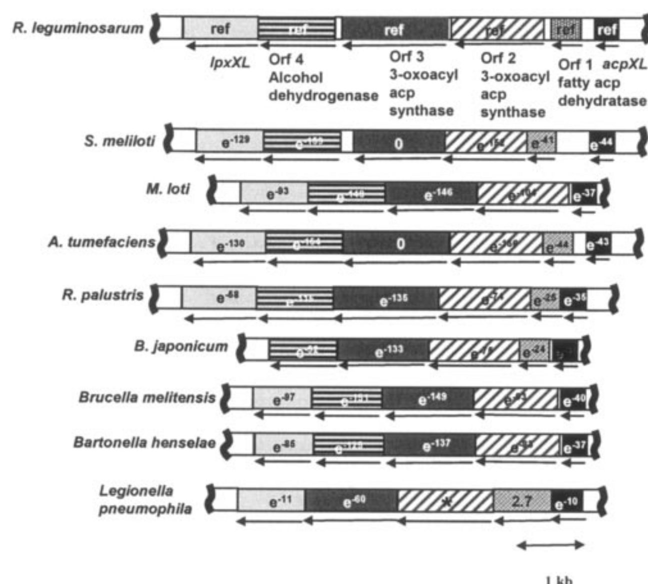

FIG. 6. Sequence comparison for the translation products of the gene region coding for the synthesis of $27OHC_{28:0}$ and its transfer to the lipid A from a number of gram-negative bacterial species. The E-values reflect the levels of sequence similarity of the protein products to the protein products from Rlv3841 (shown at the top). An asterisk indicates a translated *L. pneumophila* Orf2 protein sequence that did not exhibit similarity to Orf2 from the other species shown; however, it did exhibit similarity (e^{-11}) to Orf3 of *L. pneumophila*. *R. palustris*, *Rhodopseudomonas palustris*; *B. japonicum*, *Bradyrhizobium japonicum*.

In the case of the brucellae, their commonality with rhizobia is that they are both endocytosed into their host cells and are able to persist and function within host-derived membrane compartments (symbiosomes and phagosomes, respectively) (7, 8, 30). Thus, it is possible that the $27OHC_{28:0}$ lipid A component in both of these taxa plays an important role in their virulence and persistence within their hosts. Recently, another connection between *Brucella abortus* and rhizobia has been reported with regard to a protein called BacA and its importance for producing lipid A with $27OHC_{28:0}$ (16). The BacA protein was found to be necessary for infection of alfalfa by *S. meliloti* (17) and was also shown to be present in *B. abortus* and necessary for this pathogen to maintain a chronic infection in its host (24). The BacA protein exhibits sequence similarity with a peroxisomal membrane protein family (16) that is thought to play a role in the transport of long-chain fatty acids (6, 18, 36, 41). BacA mutants of *S. meliloti* and *B. abortus* both produce lipid A preparations in which the level of $27OHC_{28:0}$ is reduced to about 50% of the normal level (16). Unlike AcpXL, which is specifically required for the synthesis of 27-$OHC_{28:0}$, it is likely that the BacA protein has multiple functions that affect symbiotic infection, including a function which affects the level of 27-$OHC_{28:0}$ in lipid A and another function which is essential for infection.

In summary, we showed that the *acpXL* mutation is partially suppressed by a possible host-activated alternative mechanism for the synthesis of $27OHC_{28:0}$. This result suggests that the presence of $27OHC_{28:0}$ in rhizobial lipid A is essential for symbiosis. Further investigation is in progress to (i) identify the alternative mechanism for $27OHC_{28:0}$ synthesis that occurs in planta and (ii) prepare and characterize the symbiotic phenotypes of mutants (e.g., deletion of the entire *acpXL-lpxXL* region) that are unable to synthesize $27OHC_{28:0}$ both ex planta

and in planta. In addition to increasing our understanding of the molecular basis of the *Rhizobium*-legume symbiosis, determining the role of $27OHC_{28:0}$ in the symbiotic process should also provide information regarding the virulence mechanism of the brucellae and possibly several other pathogen species that cause chronic intracellular infections.

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