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 (27OHC28:0) in their lipid A. A *Rhizobium leguminosarum* 3841 acpXL mutant (named here Rlv22) lacking a functional specialized acyl carrier lacked 27OHC28:0 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 27OHC28:0 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 27OHC28:0 to the lipid A of Rlv22 lipopolysaccharides is partially restored in Rlv22 acpXL mutant bacteroids. We hypothesize that *R. leguminosarum* bv. *viciea* 3841 contains an alternate mechanism (e.g., another acp gene) for the synthesis of 27OHC28:0, which is activated when the bacteria are in the nodule environment, and that this is an alternative mechanism which functionally replaces acpXL and is responsible for the synthesis of 27OHC28:0-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. *viciea* 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 (27OHC28:0). 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...
fatty acyl component 27OHC_{28:0} in lipid A. This fatty acyl component is present in the LPS of members of the *Rhizobium* family (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 a 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 (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 Sharpyova 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 50% 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 aerochic 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, ground for preparation of bacteroids.

**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 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 (GAGGGGTTTTAAATGATCA and AGGCTTGCCCGTTTGA), 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

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<th>Strain</th>
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<tr>
<td><strong>R. leguminosarum bv. viciae 3841</strong></td>
<td>Strr' Fix' NaCl'</td>
<td>37</td>
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<tr>
<td><strong>R. leguminosarum bv. viciae 22</strong></td>
<td>Strr' Km' Fix' NaCl'</td>
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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 out onto TY agar plates. The plates were developed, and three single colonies 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 0.25 M EDTA/5% phenol, titrated to pH 8.9 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 ultrasonic 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 H2O 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 previously determined (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 genetical 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.

**Analysis of the lipid A from Rlv22 bacteroids.** The lipid A from LPS preparations of parent Rlv3841 and mutant Rlv22 laboratory-grown bacteria and from bacteroids isolated from root nodules was 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 27OHC28:0 could be detected. However, the lipid A from Rlv22 bacteroids contained 27OHC28:0, 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 (C16:0) and stearate (C18:0) 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...
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 a β-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-
butyrate. Other ions in this cluster are due to structural vari-
ants 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 elimi-
nation of the β-hydroxy fatty acyl residue from position 3 of the GlcNonate residue forming a 2,3-unsaturated 2-aminoglu-
cono-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 labora-
tory-grown mutant Rlv22 (Fig. 2C) also shows that there were

two major 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 major ion clusters represent structures that are devoid of 27OHC28:0 or 27O(β-hydroxybutyryl)C28:0 (structures III and IV). These structures are identical to those previously reported for the mutant lipid A (35). We also reported pre-
viously (35) that the minor cluster of ions centered around m/z 1758.0 was due to replacement of 27OHC28:0 with a palmitoyl (C16:0) 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 27OHC28:0 was replaced with a C16:0 or stearoyl (C18:0) 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 prepara-
tion (Fig. 2B) is identical to that of the lipid A from the labora-
tory-grown culture (Fig. 2A) described above. The spec-
trum 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).

DISCUSSION

We previously showed that R. leguminosarum acpXL mutant Rlv22 is unable to add 27OHC28:0 to its lipid A, exhibits de-
layed 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 27OHC28:0 in its lipid A, was sensitive to deoxycholate, exhibited delayed nodulation of Medicago sativa, and had reduced competitive

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 kan-
amycin. All strains grew equally well on medium without kanamycin (data not shown). (B) Growth on normal laboratory medium with kanamycin and 0.5% NaCl.
R. leguminosarum lipid A may be essential for the symbiotic interaction between the host and the bacterium. Reduced within the host suggests that this fatty acyl residue in lipid A is necessary for the function of the bacteroid. However, nodules elicited by this mutant on roots of peas and white clover grown under laboratory conditions, the EN isolates were still able to add 27OHC28:0 to their lipid A. Furthermore, we observed that the Rlv22 mutant had normal morphology and 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 27OHC28:0 moiety of lipid A plays an important role in bacteroid development and in the symbiotic 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 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 *poxL*, there has been no analysis of the lipid A from *S. meliloti* acpXL or *poxL* mutant bacteroids.

The nature of this alternative host-induced mechanism for synthesizing 27OHC28:0 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 isolated 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 O2 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 27OHC28:0 is under investigation.
is a very similar arrangement of genes encoding fatty acyl-ACP synthase orthologs (three or four genes) flanked by \textit{acpXL} on one side and by \textit{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 \textit{Legionella pneumophila}. However, the arrangement of the genes in \textit{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 \(\omega\)-hydroxy or -oxo fatty acids and their transfer to lipid A.

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 270HC\textsubscript{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 \textit{Brucella abortus} and rhizobia has been reported with regard to a protein called BacA and its importance for producing lipid A with 270HC\textsubscript{28:0} (16). The BacA protein was found to be necessary for infection of alfalfa by \textit{S. meliloti} (17) and was also shown to be present in \textit{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 \textit{S. meliloti} and \textit{B. abortus} both produce lipid A preparations in which the level of 270HC\textsubscript{28:0} is reduced to about 50% of the normal level (16). Unlike AcpXL, which is specifically required for the synthesis of 27-OHC\textsubscript{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\textsubscript{28:0} in lipid A and another function which is essential for infection.

In summary, we showed that the \textit{acpXL} mutation is partially suppressed by a possible host-activated alternative mechanism for the synthesis of 270HC\textsubscript{28:0}. This result suggests that the presence of 270HC\textsubscript{28:0} in rhizobial lipid A is essential for symbiosis. Further investigation is in progress to (i) identify the alternative mechanism for 270HC\textsubscript{28:0} synthesis that occurs in planta and (ii) prepare and characterize the symbiotic phenotypes of mutants (e.g., deletion of the entire \textit{acpXL-lpxXL} region) that are unable to synthesize 270HC\textsubscript{28:0} both ex planta.

\textbf{FIG. 5.} Gene region for the putative \textit{acp} gene located on symbiotic plasmid pRL10 of Rh3841. The sequence between pRL100143 and pRL100144 (\textit{acp}) contains sequences similar to the \textit{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.

\textbf{FIG. 6.} Sequence comparison for the translation products of the gene region coding for the synthesis of 270HC\textsubscript{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 Rh3841 (shown at the top). An asterisk indicates a translated \textit{L. pneumophila} Orf2 protein sequence that did not exhibit similarity to Orf2 from the other species shown; however, it did exhibit similarity (e\textsuperscript{-11}) to Orf3 of \textit{L. pneumophila}. \textit{R. palustris}, \textit{Rhodopseudomonas palustris}; \textit{B. japonicum}, \textit{Bradyrhizobium japonicum}. 2132 VEDAM ET AL. J. BACTERIOL.
and in planta. In addition to increasing our understanding of the molecular basis of the Rhizobium-legume symbiosis, determining the role of 27OHCA 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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