A *Rhizobium leguminosarum* Lipopolysaccharide Lipid-A Mutant Induces Nitrogen-Fixing Nodules with Delayed and Defective Bacteroid Formation

Vinata Vedam,^{2,1} Janine G. Haynes,¹ Elmar L. Kannenberg,^{3,2} Russell W. Carlson,² and D. Janine Sherrier¹

¹Department of Plant and Soil Sciences and the Delaware Biotechnology Institute, University of Delaware, Newark, DE 19711, U.S.A.; ²Complex Carbohydrate Research Center, University of Georgia, 220 Riverbend Road, Athens, GA 30602, U.S.A.; ³Department of Microbiology and Biotechnology, University of Tübingen, D072076 Tübingen, Germany

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Lipopolysaccharides from pea-nodulating strain *Rhizobium leguminosarum* bv. *viciae* 3841, as all other members of the family Rhizobiaceae with the possible exception of *Azorhizobium caulinodans*, contains a very long chain fatty acid; 27-hydroxyoctacosanoic acid (27OHC28:0) in its lipid A region. The exact function and importance of this residue, however, is not known. In this work, a previously constructed mutant, *Rhizobium leguminosarum* bv. *viciae* 22, deficient in the fatty acid residue, was analyzed for its symbiotic phenotype. While the mutant was able to form nitrogen-fixing nodules, a detailed study of the timing and efficiency of nodulation using light and electron microscopy showed that there was a delay in the onset of nodulation and nodule tissue invasion. Further, microscopy showed that the mutant was unable to differentiate normally forming numerous irregularly shaped bacteroids, that the resultant mature bacteroids were unusually large, and that several bacteroids were frequently enclosed in a single symbiosome membrane, a feature not observed with parent bacteroids. In addition, the mutant nodules were delayed in the onset of nitrogenase production and showed reduced nitrogenase throughout the testing period. These results imply that the lack of 27OHC28:0 in the lipid A in mutant bacteroids results in altered membrane properties that are essential for the development of normal bacteroids.

Additional keywords: bacteroid membrane.

Under limiting nitrogen conditions in the soil, rhizobia induce the formation of nitrogen-fixing nodules, specialized organs on the roots of their plant host. This remarkable natural process begins with an exchange of signals in the rhizosphere, progresses to infection of the legume root by rhizobia, and involves cross talk between the cell surface components of both the plant and bacterium.

Lipopolysaccharides (LPS) are complex glycolipid molecules that are major constituents of the outer leaflet of the gram-negative bacterial outer membrane (Raetz and Whitfield 2002). It is well-known that LPS play an important role in determining the virulence of enteric bacterial pathogens (Raetz and Whitfield 2002). It has also been shown that the LPS plays a crucial role in the interaction between members of the *Rhizobiaceae* and legume plants (Kannenberg et al. 1998).

Rhizobial cells are surrounded by extracellular polysaccharides (EPS), a more tightly bound acidic, capsular polysaccharide, and LPS, which makes up the outer leaflet of the bacterial outer membrane. The LPS, in concert with divalent calcium ions, adds stability to the extracellular sheath. In addition to this structural role, there is mounting evidence that components of cell wall polysaccharides act as signaling molecules that either promote tissue invasion, suppress a host defense response, or both (Brewin 1998). Although the LPS is a necessary component for symbiotic infection, its role and function during the process is not yet completely understood.

Conceptually, LPS is comprises three regions: the lipid A, the core, and the O-antigen region (Raetz and Whitfield 2002). Complete lipid A structures have been resolved for *R. etli* CE3 and also *R. leguminosarum* (Bhat et al. 1994; Que et al. 2000a and b). It has been shown that *Escherichia coli* and *R. leguminosarum* bv. *viciae* utilize the same biochemical pathway to catalyze formation of the lipid A precursor molecule Kdo₂lipid-IV_A from UDP-GlcNAc (Price et al. 1994). In later steps of LPS synthesis, the *E. coli* and *Rhizobium* spp. pathways diverge, and members of the genus *Rhizobium* produce a lipid A that is structurally very different from that of *E. coli* (Bhat et al. 1994; Brozek et al. 1996a and b; Que et al. 2000a and b). It has been hypothesized that the lipid A structural differences between the two bacteria play an important role in the biological niche that each occupies, i.e., that the unique structural features present on the rhizobial lipid A are necessary for establishing a normal nitrogen-fixing symbiosis with its legume host (Vedam et al. 2003).

Among the differences in the lipid A structures between *E. coli* and *R. leguminosarum* is the nature of the secondary fatty acids, i.e., the acyloxyacyl residues. *E. coli* adds two acyloxyacyl fatty acids, namely lauryl and myristyl residues, to the Kdo₂lipid-IV_A (Raetz and Whitfield 2002), whereas *R. leguminosarum* puts on a single, extra-long fatty acid residue, 27-hydroxyoctacosanoic acid (27OHC28:0) (Bhat et al. 1994; Que et al. 2000a and b). To accomplish this, *R. leguminosarum* requires a specialized acyl carrier protein, the AcpXL (Brozek et al. 1996a), and a minimum of one specific transferase, LpxXL (Basu et al. 2002). Due to the importance of LPS in rhizobia-legume symbiosis, we hypothesize that expression of the rhizobial LPS biosynthetic genes required for the structural features unique to the rhizobial lipid A, e.g., the synthesis and addition of 27OHC28:0, are critical for the formation of normal nitrogen-fixing root nodules.

Nodule formation is a multistep process, in which rhizobia respond to plant root exudates and move toward localized

regions or sites of the root where they subsequently attach themselves (Bhuvaneswari and Solheim 1985; van Rhijn and Vanderlayen 1995). Rhizobia promote curling, branching, and deformation of root hairs. Concomitant with root hair deformation, cortical cells divide to form a nodule primordium. In all rhizobial-plant interactions, fatty acylated chitin oligosaccharides, called Nod factors, are responsible for root hair deformation and cortical cell division (Carlson et al. 1994; D'Haese and Holsters 2002; Dénarié et al. 1992; Long 1996, 2001; Schultze and Kondorosi 1998; Spaik 2000). Curled root hairs entrap bacteria in a pocket of host cell wall. A lesion in the root hair cell wall occurs, followed by penetration of rhizobia through the cell wall into an invagination of the plasma membrane. The host plant responds by depositing cell wall material around the lesion (Callaham and Torrey 1981; Turgeon and Bauer 1982), forming a growing infection conduit termed an infection thread, which contains proliferating bacteria and matrix material (Rae et al. 1992; VandenBosch et al. 1989). The infection threads grow toward the nodule primordium (Libbenga and Harkes 1973; Newcomb 1981; Vasse and Truchet 1983; Wood and Newcomb 1989) and eventually unite with postmeristematic cells and release their bacterial contents into the host cell cytoplasm via an endocytosis-like infection process.

Peas form elongated indeterminate nodules in which the apical meristem is persistent, so there is a reiteration of cell division, cell invasion, and release of bacteria into the nodule cells. These processes occur in distinct developmental regions in an indeterminate nodule; each nodule includes a meristem, a prefixation zone, an interzone, a nitrogen fixation zone, and later in development, a senescent zone (Mylona et al. 1995). All of these developmental zones can be viewed in a medial, longitudinal section of the nodule.

In indeterminate nodules, nitrogen-fixation capability is always preceded by the release of bacteria via an endocytosis-like process into the plant cell cytoplasm. The result is an intracellular organelle known as the symbiosome, in which the bacterium is surrounded by a plant-derived membrane known as the symbiosome membrane (SM). This important developmental process occurs via unwallled infection droplets formed at the distal ends of infection threads. Droplets are delimited by a single membrane that is formed from the plasma membrane. Once the bacteria are engulfed by the plant cell, the bacterial cells within the symbiosome differentiate into their fully endosymbiotic, nitrogen-fixing form, known as bacteroids (Brewin 1998). Intactness of the SM seems to be a precondition for bacteroid function (Brewin 1998). In pea nodules, division of the bacteroids is followed immediately by division of the encompassing SM, so that each bacteroid is enclosed singly by a membrane. In nodules induced by some rhizobial LPS mutants, multiple bacteroids occupy a single symbiosome, indicating that the modification in the LPS may alter the bacterial cell surface interactions with the SM (Kannenberg et al. 1998). All members of the family *Rhizobiaceae* that have been examined, with the possible exception of *Azorhizobium caulinodans*, have LPS that contain the 27OHC28:0 fatty acid (Bhat et al. 1991a). In addition, long chain ω -1 hydroxy or oxo fatty acids are found in the lipid-A from a variety of gram-negative bacterial pathogens. These pathogens are facultative intracellular organisms that contain these lipid-A fatty acyl residues or homologs to *acpXL* or *lpxXL* (the long chain fatty acyl transferase), e.g., *Brucella abortus* (Bhat et al. 1991b), *Brucella melitensis* (Basu et al. 2002), *Legionella pneumophila* (Zahringer et al. 1995, 1999), *Bartonella henselae* (Bhat et al. 1991b), and *Rickettsia prowazekii* (Basu et al. 2000). This long chain fatty acid may provide extra stability to the bacterial outer membrane in that its alkyl

chain is of sufficient length to span the entire outer membrane bilayer. In addition, it has been observed that the level of 27OHC28:0 doubles in amount during *R. leguminosarum*-pea symbiosis (Kannenberg and Carlson 2001). This increase in 27OHC28:0 might be an adaptation to the physiological stresses of the endophytic environment.

Throughout nodule development, rhizobia must modulate plant development, prevent elicitation of plant defense responses, and maintain cell vitality. Due to the intimate contact of the rhizobial LPS molecule with the plant during the process of infection and invasion, it can be envisioned that the LPS plays a role, maybe even a crucial one, in the initiation of infection, root tissue invasion, bacterial release, bacteroid development, and bacteroid senescence (Kannenberg et al. 1998). Although there have been reports of close, physical contact between the SM and the bacteroid itself (Bradley et al. 1986), there has been no conclusive molecular evidence of this interaction, and the involvement of the LPS in the adhesion process has not yet been resolved (Brewin 1991).

The focus of the work presented here is the role of bacterial LPS, specifically the 27OHC28:0 long chain fatty acyl component of the lipid A in nodule development. Previously, we constructed a *R. leguminosarum* strain mutated in *acpXL*, the gene that encodes for the acyl carrier protein unique to 27OHC28:0 (Vedam et al. 2003). The *R. leguminosarum* strain deficient in *AcpXL* was unable to add the 27OHC28:0 fatty acid residue to the lipid A region of its LPS, resulting in a tetraacylated lipid A. This mutant was unable to grow at pH 5.0 and in 0.5 M NaCl. In spite of this sensitivity to acidic conditions, the mutant was able to form nitrogen-fixing nodules on pea even though acidic conditions occur within the nodule, raising the possibility that the bacterium may have some mechanism of partially compensating for the loss of this fatty acid within the nodule. However, nodulation by the mutant was significantly delayed in comparison to that by the parent strain. Therefore in this study, a detailed examination of the symbiotic phenotype of the *acpXL* mutant was performed. Peas were inoculated with the mutant strain or the corresponding parent strain, were grown under controlled environmental conditions, and were observed for any macroscopic differences in nodule formation, such as size and nodule numbers. Further, nodules were analyzed microscopically to discern ultrastructural differences. In this study, we discuss the symbiotic phenotype of the *R. leguminosarum acpXL* mutant with its host plant *Pisum sativum* and propose a possible role for this long chain fatty acid in the pea symbiosis.

RESULTS

Nodule formation.

To test the effect of the *acpXL* mutation on nodule formation, plants were inoculated with the *acpXL* mutant, and nodule development was monitored. Control plants were inoculated with the parent bacterium, *R. leguminosarum* bv. *viciae* 3841. Nodules formed on the plants inoculated with either the parent or the mutant (Fig. 1). Light microscopic analysis of these nodules showed that the nodule tissue was invaded by bacteria (Fig. 1). However, in comparison to nodules induced by the parent bacterium, the progression of nodule occupancy by the mutant was delayed. In the parent nodule, the meristem, invasion zone, and fixation zone typical of a mature nitrogen-fixing nodule were present 7 days postinoculation (dpi) (Fig. 1A). The infection and the nitrogen fixing zones were prominent, containing a large number of cells invaded by infection threads in the invasion zone, and cells were packed densely with nitrogen-fixing bacteria in the mature zone. In contrast, the nodules formed by the mutant contained a meristem typi-

cal of an indeterminate nodule, but the infection and nitrogen-fixing zones were sparsely occupied by the invading bacteria (Fig. 1B). In addition, the cells in the nitrogen-fixation zone contained large vacuoles and a limited number of intercellular bacteria, an appearance that is similar to an earlier stage of development in parent nodules. In the prefixation zone, the mutant and parent nodules contained numerous infection structures, including infection droplets and infection threads. In general, the infection structures in the mutant nodules were similar to those in the parent nodule; however, aberrant infection threads containing a higher proportion of matrix material were occasionally identified in the prefixation zone of mutant nodules (Fig. 2).

The above results suggest that formation and development of nodules are affected by the *acpXL* mutation and the resulting lack of 27OHC28:0 in the bacterial LPS. To examine this possibility further, roots were harvested from plants inoculated with either parent or mutant bacteria at 3, 5, 7, 9, and 11 dpi to

compare the progression of nodule development. Nodules were dissected from inoculated root systems, counted, and measured. Because nodules were harvested from the plants under a dissecting microscope, young nodules unemerged from the root were also harvested, counted, and measured. Emerging nodules were easily distinguished from emerging lateral roots by shape and vascular anatomy (Fig. 3). In this study, unemerged nodules were defined as nodules that had not yet broken through the epidermis. Analysis of the nodules induced by the parent compared with those by the mutant demonstrated statistical differences between the numbers of nodules (Fig. 4A). Initially, at day 3, plants inoculated with either parent or mutant produced the same number, 35, of nodules. However, by 5 dpi, the average number of nodules induced by the mutant was significantly less than by the parent bacteria, 40 vs. 100 nodules per plant, respectively. After five days, nodule numbers continued to increase in the mutant-inoculated plants, while in the parent-inoculated plants, the nodule numbers remained steady. At 7 dpi, plants inoculated with the mutant bacteria still had significantly fewer nodules (65 nodules per plant) than in the parent case (110 nodules per plant). By 9 dpi, the nodule counts equalized between the parent- and mutant-infected plants, and this trend continued into the last sampling date 11 dpi.

In addition to differences in nodule numbers, the size and shape of nodules induced by mutant and wild-type bacteria during the developmental timecourse were also dissimilar. The length of each nodule and the number of unemerged nodules formed on inoculated plants were analyzed in detail. The number of unemerged nodules formed by the parent and the mutant were counted and were expressed as a percentage of total nodules (Fig. 4B). At 3 dpi, 100% of the nodules produced in plants inoculated with either bacterium were unemerged. Two days later, 45% of the nodules on plants inoculated with the mutant were unemerged nodules, whereas only 20% of the nodules on plants inoculated with the parent were unemerged. At 7 dpi, the nodules showed more growth but the mutant still exhibited statistically fewer emerged nodules (22% remained unemerged) than the parent (10% of total nodules remained unemerged). By 9 dpi, as observed with nodule numbers, no

Fig. 1. Light micrographs of nodules at 7 days postinoculation. **A**, Nodule induced by the parental (3841) and **B**, the mutant (22) strain of *Rhizobium leguminosarum* bv. *viciae*. Regions of the mature nodule include nodular meristem (1), prefixation zone (2), interzone (3), nitrogen fixation zone (4). The mature nitrogen fixation zone is not present in most mutant nodules at this developmental stage. Scale bar = 100 μ m.

Fig. 2. Light micrograph of aberrant infection structure in the prefixation zone of a mutant nodule at 7 days postinoculation. The infection thread (indicated by arrows) contains numerous undifferentiated bacteria and leads to cells (arrowhead) with a swollen thread and enlarged invasion structures. Scale bar = 10 μ m.

difference was detected in the number of unemerged nodules on plants inoculated with parent and mutant bacteria.

The average length of nodules formed by the two bacterial strains were the same in parent and mutant nodules, with the exception of the developmental timepoint 7 dpi (Fig. 4C). On this sampling date, nodules formed by the mutant measured about 0.6 mm (mean nodule length) compared with nodules formed by the parent, which measured approximately 0.75 mm (mean nodule length). These data demonstrate that the progression of nodule development by the mutant deviates from that of the parent. Interestingly, the differences occur early in the course of nodule development, around and before the time when nitrogen fixation is initiated.

Nitrogen fixation.

To evaluate whether the measured differences in the nodule formation and growth could be related to the onset of nodule maturation, nitrogen fixation was monitored as a function of nodule development. During the course of this study, it was noted that nodules induced by the parent bacteria turned pink earlier than nodules induced by the mutant. At 7 dpi, nodules induced by parent bacteria were beginning to turn pink, while on the same date all mutant-induced nodules remained white. By 9 dpi, nodules on both sets of plants were pink. To confirm this visual indication that mutant-induced nodules were fixing nitrogen, nitrogenase activity was characterized by acetylene reduction assays. Small amounts of ethylene, indicative of nitrogenase activity, could be detected in both the parent and mutant nodules at 5 dpi. However, the mutant nodules consistently had lower levels of activity compared with that of the parent nodules at all timepoints (Fig. 5). The comparison of nitrogenase content was also analyzed by monitoring the amount of nitrogenase protein in the nodule during development induced by the parent and mutant strains. Total proteins were extracted from nodules at the different timepoints, 100 µg of protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was probed with antinitrogenase antibodies (Fig. 6). Nitrogenase was not detected in parent or mutant nodules 5 dpi. The cross-reactive protein was first detected in the parental but not the mutant nodule extract 7 dpi. In contrast, a very faint band was detected in mutant nodule extract 9 dpi, and the protein increased in the mutant nodule extract 11 dpi but was still significantly less than that observed in the parent-induced nodule.

Comparison of parent and mutant nodulation by electron microscopy.

To determine whether the LPS modification altered the cell-cell interaction between the host and bacterium, pea root nod-

Fig. 3. Light micrographs of representative root and nodules collected for nodule measurement studies. **A**, Nascent lateral root, distinguished easily from nodule primordium by the width of the meristem. **B**, Unemerged nodule that has not broken through the root epidermis and appears as a slight root bulge with a wide meristem. **C**, Emerged nodule. Scale bars = 100 µm.

Fig. 4. Summary of nodule measurement data. Nodules were counted and measured 3, 5, 7, 9, 11 days postinoculation (dpi). **A**, Mean number of nodules per plant ($n = 5$), showing statistically fewer nodules in mutant-inoculated plants at 5 and 7 dpi. **B**, Percentage of the total number of nodules per plant that were unemerged from the root surface ($n = 5$), demonstrating differences in nodule numbers between mutant- and parent-induced nodules at 5 and 7 dpi. **C**, Mean length of all nodules from five plants, showing a difference between parental and mutant nodules at 7 dpi. Means were separated by Tukey's honest significant difference test, $p \leq 0.05$. Significant differences are indicated by an asterisk (*).

ules infected with the parent were compared with those infected with the mutant by transmission electron microscopy. Plants were sampled during a developmental timecourse at 5, 7, 9, and 11 dpi. At 5 dpi, nodules infected with parent or mutant strains contained the typical tissue zones with cell invasion, infection, and bacteroid development. In both cases, bacteria could be observed within the host cells, but they were not yet showing the characteristic branching of mature bacteroids, and the infected cells still contained large vacuoles (data not shown). Consistent with the results described above and shown in Figure 1, at day 7 postinoculation, infected cells in mutant nodules had low bacterial occupancy, with the greatest portion of the cellular content consisting of vacuoles. Individual infected cells usually contained one large vacuole. In contrast, the parent-infected counterparts showed a greater level of cellular occupancy by the microbial symbiont. By 9 dpi (Fig. 7), both mutant- and parent-induced nodules showed high levels of cellular occupancy. However, significant differences were seen in the bacteroid shapes of the mature, nitrogen-fixing region of the mutant-induced nodules. Mature bacteroids in the parent nodules were frequently Y-shaped, a characteristic feature of *R. leguminosarum* bv. *viciae* bacteroids (Fig. 7A and E). The mutant nodules, in contrast, contained unbranched and abnormally branched, irregularly shaped bacteroids (Fig. 7B, C, D, and F). There are numerous bulges on the surface of the mutant bacteroids (Fig. 7C), unusual branching profiles, and large size (Fig. 7F). These features were first detected in nodules at 7 dpi, occurred more frequently at 9 dpi, and were very common at 11 dpi. Despite the severe differences in morphology between the mutant and parental bacteroids, no differences could be detected in the shape of the bacterial strains when grown in culture (data not shown).

In addition to the differences in bacteroid shape, the mutant nodules exhibited additional cellular defects in the mature nitrogen fixation zone. Cells contained membranous proliferations in the vacuole or cytoplasm (Fig. 7C). These may represent regions of bacteroid degradation or sites of mistargeting of plant cellular membranes to the vacuole or empty inclusions

containing no bacterial cells within the plant cytoplasm. Such membranous inclusions were not observed in corresponding parent-nodule sections. Further, a dramatic difference noted between the mutant and parent nodules was that the SM frequently did not enclose a single bacteroid but, instead, was contiguous, enclosing several adjacent bacteroids.

DISCUSSION

During symbiosis, two significant changes have been observed to occur to the *R. leguminosarum* cell: i) the LPS becomes hydrophobic and ii) the entire bacterial cell becomes hydrophobic (Kannenberg and Carlson 2001). The increase in LPS hydrophobicity is most likely due to the fact that there is a doubling of the 27OHC28:0 residue on the lipid A and that there is an increase in methyl and, possibly, in acetyl groups on the O-chain polysaccharide (Kannenberg and Carlson 2001). The increase in hydrophobicity of the entire cell surface is likely due to the increase in O-chain polysaccharide methyl and acetyl groups as well as to a decrease in acidic EPS as infection proceeds. These observations suggested that both cell surface hydrophobicity as well as the increase in 27OHC28:0 are essential for the establishment of a normal symbiosis.

In a recent report from our laboratories, it was suggested (Vedam et al. 2003) that this long fatty acid may function by increasing the stability of the bacterial membrane. Both increased membrane stability and cell surface hydrophobicity are conditions that could be necessary for the endocytotic invasion of, and survival within, the host cell. In addition with regard to pea nodules, these conditions may be essential for the synchronous division of the bacterial cell and SM. To test these suggested functions of 27OHC28:0, the gene encoding for its unique acyl carrier protein, *acpXL*, was mutated. In our previous paper (Vedam et al. 2003), it was shown that the lipid A from this mutant lacked 27OHC28:0 as expected, was unable to grow under acidic conditions (pH 5.0) or at increased salt concentration (0.5 M NaCl), had an increase lag phase of growth, and was delayed in its ability to nodulate pea. However, in spite of these physiological defects, the mutant was able to form nitrogen-fixing nodules on pea. A delayed nodulation phenotype that still formed nitrogen-fixing nodules was also reported for an *acpXL* mutant of *Sinorhizobium meliloti*, the symbiont of *Medicago sativa* (Sharypova et al. 2003).

In this paper, we present more detailed characterization of the symbiotic phenotype of the *R. leguminosarum acpXL* mutant. The following observations were made of the mutant in

Fig. 5. Relative nitrogenase activity in mutant *Rhizobium leguminosarum* bv. *viciae* 22 nodules versus parental *Rhizobium leguminosarum* bv. *viciae* 3841 nodules. Ethylene production was measured from tissues 1, 3, 5, 7, 9, and 11 days post inoculation. Values depicted are the mean of three independent measurements and are expressed as a percentage of the parental strain activity. Asterisks (*) indicate timepoints when no activity was detected in either the mutant or the parent strain.

Fig. 6. Relative levels of nitrogenase present in nodules of the parental (3841) or mutant (22) strain of *Rhizobium leguminosarum* detected by Western blot analysis. Proteins were extracted 5, 7, 9, and 11 days postinoculation (dpi). Protein extracts (100 ug in each lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were transferred to nitrocellulose, were probed with antinitrogenase antibodies, and were visualized using chemiluminescence. A single major reactive band was observed in the range of 31 kDa.

Fig. 7. Transmission electron micrographs of mutant- and parent-infected nodules. **A**, *Rhizobium leguminosarum* bv. *viciae* 3841 bacteroid 9 days postinoculation (dpi), showing characteristic wild-type branching. **B**, An abnormally branched mutant bacteroid, 9 dpi. **C**, Misshapen mutant bacteroid 9 dpi, showing numerous bulges. The double arrowhead indicates unusual membranous profiles in the vacuole, a feature often viewed in cells undergoing symbiosome degradation. Single arrowheads point to the abnormally continuous symbiosome membrane surrounding multiple bacteroids. **D**, An unusually large and abnormally branched mutant bacteroid 9 dpi. **E**, Low magnification view of parental infected cells in the fixation zone, containing characteristic branched bacteroids. **F**, Low magnification view of mutant infected cells in the fixation zone illustrating aberrant bacteroid features, including large size, misshapen form, and multiple bacteroids contained within one symbiosome unit (indicated by barbed arrowheads). The vacuole is unusually large for this stage of nodule development. Scale bars = 1 μ m.

comparison with the parent. i) The mutant was delayed in nodulation, as previously reported (Vedam et al. 2003), and produced a reduced number of nodules at early postinoculation timepoints but caught up with the parent at later timepoints. ii) The mutant produced nodules with a delayed onset of nitrogenase production and with reduced nitrogenase at later timepoints. iii) The mutant formed very irregularly shaped and enlarged bacteroids, in contrast to the parent, which formed the normal elongated or Y-shaped bacteroids. iv) The mutant frequently formed symbiosomes containing multiple numbers of the irregularly shaped bacteroids while, with the parent, each symbiosome contained a single bacteroid.

The delay in nodulation and reduced nodule number at early timepoints may be due to the fact that the mutant has an increased lag period of growth (Vedam et al. 2003). It is also known that the mutant does not grow under acidic conditions (e.g., pH 5.0) or at 0.5 M NaCl (Vedam et al. 2003). Since the nodule environment could be either acidic, of higher osmolarity, or both, it is possible that the growth rate of the mutant within the nodule is reduced due to stress caused by these conditions, resulting in the delay in nodulation and reduced nodule number at the earlier timepoints. The delay in the expression of nitrogenase could also be explained in a similar manner. The sensitivity of the mutant to both acidic conditions and increased salt concentration may be due to a decrease in outer membrane stability caused by the lack of 27OHC28:0 on the lipid A.

The delay in nodulation in the mutant is overcome by day 9 postinoculation. However, at that time and at later times, the level of nitrogenase in the mutant nodules remains lower than in parent nodules, and the appearance of the mutant bacteroids is dramatically different from those of the parent. These two effects suggest that the mutant has a defect in bacteroid maturation. This defect in bacteroid maturation is further indicated by the observation that the mutant is disrupted in the synchronous division of the bacterial cell and SM resulting in multiple, irregularly shaped bacteroids within a single symbiosome. Under normal circumstances, and as observed with the parent strain, the synchronized division of the bacterial cell and the SM leads to each symbiosome containing a single bacterium. The mechanism of this synchronous division process is not known. However, it is likely to involve intimate contact between the bacterial membrane and the SM. In fact, an earlier report provides evidence of physical contact between the LPS of the bacteroid membrane and the SM (Bradley et al. 1986). A possible mechanism for the synchronous division process is that the bacterial-SM membrane contact guides, i.e., pulls, the SM around the dividing bacterial cell, resulting in each daughter cell being surrounded by an SM. This contact may create tension on the bacterial outer membrane, which would be countered by the stabilizing force provided by the lipid A portion of the LPS. The 27OHC28:0 fatty acyl component, which increases the hydrophobic character of the lipid A and could span the entire bilayer of the outer membrane, would likely be a crucial factor in this stabilization. In the absence of 27OHC28:0, the force anchoring the lipid A to the bacterial outer membrane would be reduced, causing disruptions at numerous points in the bacterial membrane during the bacterial-SM division process. Thus, the result of this defective division process may be the multiple, irregularly shaped, enlarged bacteroids within a single symbiosome that are observed for this *acpXL* mutant.

While the *acpXL* mutant has the distinct phenotype just described, it was somewhat surprising that it was not even more symbiotically defective. As we previously reported (Vedam et al. 2003), the LPS from a laboratory-grown culture has a tetraacylated lipid A that lacks the 27OHC28:0. The

outer membrane comprised of such a lipid A is essentially missing one-third of this fatty acyl content and, therefore, was expected to be quite unstable. In addition, the *acpXL* mutant, as mentioned above, was shown to be sensitive to acidic conditions and to increased salt concentration (Vedam et al. 2003), even though it may face such conditions (of low pH) within the host nodule. Some possible explanations for the ability of the *acpXL* mutant to survive within the host cell environment are: i) the host environment may not be as severe as the laboratory conditions tested or ii) the mutant can respond to the host environment or to host signals and adapt in some unknown manner that partially compensates for the weakened outer membrane caused by the lack of 27OHC28:0. While it has been shown that *acpXL* mutant bacteria recovered from the nodule were genetically stable, i.e., the *kan* cassette was still retained within the *acpXL* gene (Vedam et al. 2003), it is necessary to examine in more detail the properties of the mutant as isolated from the nodule, as well as the LPS from mutant bacteroids. This work is currently in progress.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

The *R. leguminosarum* bv. *viciae* 3841 (Wood et al. 1989) and *R. leguminosarum* bv. *viciae* 22 (Vedam et al. 2003) were grown on solid or liquid tryptone yeast extract medium at 28°C, as previously described (Vedam et al. 2003). Cultures were supplemented with kanamycin (50 µg ml⁻¹), and streptomycin (5 µg ml⁻¹), as appropriate.

Plant nodulation assays.

Pisum sativum 'Early Alaska' seeds were surface-sterilized for 30 s in ethanol, were rinsed four times in sterile water, were soaked 3 min in 6% sodium hypochlorite (Clorox), and were rinsed 10 times with sterile water. Seeds were sown in 250-ml flasks on 150 ml of sterile Fahraeus N-free medium (Nutman 1970) solidified with 1% Bacto-agar (Becton Dickinson, Sparks, MD, U.S.A.). The flasks were capped with sterile foam stoppers and were placed in a dark cabinet at room temperature. After seven days, the etiolated pea hypocotyls were gently pulled out of the mouth of the flask, and the foam plug was replaced to support the shoot. Flasks were wrapped in black plastic, and the plants were grown at 21°C, 23% relative humidity, and 78 µmol · s⁻¹ · m⁻² light for 14 h per day.

For inoculation of the plants, parent and mutant bacteria were grown to an optical density of 1.1 at 600 nm, were collected by centrifugation, and were resuspended in water. Plants (14 days after sowing) were inoculated with either the parent or the mutant at a concentration of 5 × 10⁸ CFU/plant.

Nodule counts and measurements.

Plants inoculated with parental (*R. leguminosarum* bv. *viciae* 3841) or mutant (*R. leguminosarum* bv. *viciae* 22) bacteria were harvested 3, 5, 7, 9, and 11 dpi. On each sample date, five plants from each treatment were randomly selected for nodule measurements. The root system of each plant was harvested into 100 ml of 2.25% sodium hypochlorite (from Clorox) and was cleared by vacuum for 15 min and then by soaking at atmospheric pressure for an additional 25 min. Root systems were rinsed thoroughly under running water for 2 min and then allowed to soak in polished water for 20 min prior to dissection.

Root systems were scanned systematically under a dissecting microscope, and all nodules were excised and reserved in polished water. Length and width of each emerged and unemerged nodule were measured using Zeiss Axiovision software, and the number of nodules and nodule primordia on each root

system was determined. A total of 3,613 nodules were measured for this study. Nodule measurement data were subjected to analysis of variance, and the means were separated using Tukey's honest significant difference test ($p \leq 0.05$).

Acetylene reduction assay.

Nitrogen fixation was measured with acetylene reduction assays, as previously described (Wacek et al. 1976). Briefly, detached roots with nodules of the parent or the mutant from each timepoint were placed in glass bottles with screw caps and fitted rubber membranes. Acetylene (1 ml) was added to the bottles, and after an hour of incubation at room temperature, the amount of ethylene generated from the reduction of acetylene via nitrogenase was measured by GC analysis (isotherm at 70°C, with a 50 m $\times$ 0.53 mm, 10- μ m, Al₂O₃-KCl column (Chrompak; Varian, Inc., Palo Alto, CA, U.S.A.). The experiment was performed in triplicate.

Light and electron microscopy.

Nodules were collected 3, 5, 7, 9, and 11 dpi for microscopy. Tissues were harvested directly into fixative containing 1% glutaraldehyde (Sigma, St. Louis), 4% formaldehyde in 100 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] buffer, pH 7.0. Larger nodules were cut in half or thirds to aid fixation and infiltration. Tissues were fixed overnight at 4°C with rotation, were rinsed three times with room temperature water, were postfixed with 1% OsO₄ for 3 h, and were dehydrated in a graded dilution series of acetone. Tissues were infiltrated in Epon 812-Araldite resin and were heat cured at 70°C for 48 h (Mollenhauer 1964).

For light microscopic evaluation, 0.5- μ m longitudinal nodule sections were cut with glass or diamond knives (Delaware Diamond Knife, Wilmington, DE, U.S.A.) on a Reichert Ultracut E ultramicrotome. Sections were collected onto gelatin-coated glass slides, were counter stained with 0.1% (wt/vol) basic fuchsin or 0.1% (wt/vol) basic fuchsin and 1% toluidine blue O in 1% NaBO₂·4H₂O, and were observed and documented with a Zeiss Axioskop 2 microscope and a Zeiss AxioCam digital camera.

For electron microscopy, 90-nm-thick sections were collected onto hexagonal gold grids and were counterstained with alkaline lead citrate (Reynolds 1963) for 5 to 7 min and with 0.5% uranyl acetate for 12 min. Samples were visualized and documented on a Zeiss CEM 902 TEM (Carl Zeiss, Inc., Oberkochen, Germany).

Protein extraction and Western analysis.

Nodules were harvested 5, 7, 9, and 11 dpi from peas inoculated with the parent or the mutant and were homogenized at 4°C in extraction buffer containing 100 mM TRIS, 1 mM EDTA, and 1% (vol/vol) protease inhibitors (Sigma). Samples were centrifuged at 16,100 $\times$ g, and supernatant was retained. Proteins were precipitated from the supernatant overnight in 80% acetone at -20°C and were recovered by centrifugation at 2,000 $\times$ g for 10 min.

Protein content was quantified using the Bio-Rad DC protein assay (Bio-Rad, Hercules, CA, U.S.A.). Samples were resuspended in Laemmli buffer (Laemmli 1970), and 100 μ g of each sample was separated on 12% SDS-PAGE gels, using Bio-Rad mini protean 3 gel equipment. Separated proteins were transferred to nitrocellulose membrane (Osmonics, Inc., Minnetonka, MN, U.S.A.) as described (Towbin et al. 1979). Blots were stained with Ponceau S stain (Salinovich and Monterlaro 1986). Protein blots were destained, blocked for 1 h in 1% skim milk and TBST (Tris-buffered saline, 0.05 [wt/vol] Tween-20), and probed with a universal antibody mixture

against dinitrogenase reductase (a gift from P. Ludden, University of California, Berkeley, U.S.A.) diluted 1:1000 in 0.1% skim milk and TBST. Blots were rinsed in Tris-buffered saline and were incubated with goat anti-rabbit antibody conjugated to horseradish peroxidase (Sigma). Immunoreactive proteins were detected with enhanced chemiluminescence and documented with X-ray film (Kodak, Rochester, NY, U.S.A.).

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