The Lipopolysaccharide Lipid A Long-Chain Fatty Acid Is Important for *Rhizobium leguminosarum* Growth and Stress Adaptation in Free-Living and Nodule Environments

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Rhizobium bacteria live in soil and plant environments, are capable of inducing symbiotic nodules on legumes, invade these nodules, and develop into bacteroids that fix atmospheric nitrogen into ammonia. Rhizobial lipopolysaccharide (LPS) is anchored in the bacterial outer membrane through a specialized lipid A containing a very long-chain fatty acid (VLCFA). VLCFA function for rhizobial growth in soil and plant environments is not well understood. Two genes, *acpXL* and *lpxXL*, encoding acyl carrier protein and acyltransferase, are among the six genes required for biosynthesis and transfer of VLCFA to lipid A. *Rhizobium leguminosarum* mutant strains *acpXL*, *acpXL* *lpxXL*[−], and *lpxXL*[−] were examined for LPS structure, viability, and symbiosis. Mutations in *acpXL* and *lpxXL* abolished VLCFA attachment to lipid A. The *acpXL* mutant transferred a shorter acyl chain instead of VLCFA. Strains without *lpxXL* neither added VLCFA nor a shorter acyl chain. In all strains isolated from nodule bacteria, lipid A had longer acyl chains compared with laboratory-cultured bacteria, whereas mutant strains displayed altered membrane properties, modified cationic peptide sensitivity, and diminished levels of cyclic β -glucans. In pea nodules, mutant bacteroids were atypically formed and nitrogen fixation and senescence were affected. The role of VLCFA for rhizobial environmental fitness is discussed.

Rhizobia are gram-negative bacteria that are capable of thriving in both soil and plant environments. Each of these environments presents different challenges for bacterial growth, reproduction, and persistence. For example, in the soil, the bacteria can encounter drastic and rapid variations in temperature and humidity, such as drought or flooding, and competition with other microorganisms for nutrients, e.g., for plant exudates. In planta environments are considered more stable but can vary considerably depending on the bacterial location, e.g., whether they are located extra- or intracellularly in plant hosts.

Legume plant environments are of particular interest. On legume roots, rhizobia can induce a new plant organ, the symbiotic root nodule. Rhizobia invade and form persistent infections in nodule tissues and within cells, ultimately differentiating into bacteroids that fix atmospheric nitrogen into ammonia provided to the host-specific plant (Gage 2004; Long 2001; Murray 2011). To survive in changing extra- and intracellular plant micro-environments, rhizobia must be able to adapt to the varying conditions in these environments and circumvent plant defense responses.

Both soil and plant environments are considered stressful for the bacteria (Vriezen et al. 2007), and adaptations in the rhizobial cell envelope and surface are of critical importance for rhizobial survival under varying environmental conditions (Ferguson et al. 2005; Jones et al. 2007). Despite this critical importance, the role of the various bacterial cell-envelope components for bacterial cell adaptations is poorly understood, e.g., the unknown role of *Rhizobium leguminosarum* lipopolysaccharide (LPS) for bacterial soil and in planta survival. The LPS is the major component of the outer leaflet of the rhizobial outer membrane and consists of three main moieties, *O*-chain polysaccharide (OPS), core (outer and inner), and the lipid A. In comparison with lipid A from other gram-negative bacteria, *R. leguminosarum* lipid A has several distinguishing features, including i) the absence of two phosphate groups typically found on the 1- and 4' positions of the glucosamine (GlcN) disaccharide lipid A backbone, ii) the addition of a galacturonic acid to the 4' position once the phosphate has been removed, iii) the oxidation of the proximal glucosaminosyl residue to 2-aminogluconic acid (GlcNonate), and iv) a very long-chain fatty acid (VLCFA), 27-hydroxyoctacosanoate, linked as an acyloxyacyl ester to the β -hydroxyl group of the β -hydroxyacyl chain at the 2' position and a β -hydroxybutyrate modification of the 27-hydroxyl group of the VLCFA (Raetz et al. 2007; Ramadas Bhat et al. 1994) (Fig. 1A).

The importance of the VLCFA for lipid A functioning is only partially understood. As the VLCFA attached to lipid A has been found in most *Rhizobiaceae* species and also in a number of intracellular pathogens such as members of genera *Brucella*, *Bartonella*, and *Legionella* (Corbel 1997; Ramadas Bhat et al. 1991; Zähringer et al. 1995), its presence suggests important functions in bacterial host infections, regardless of whether the interactions are mutualistic or pathogenic. VLCFA has been shown to mediate LPS and lipid A anchoring to the outer

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membrane (Brown et al. 2011), and it may also have functions in outer membrane stability, conferring stress tolerance and adaptation during infection processes. Located in the outer leaflet of the outer gram-negative cell membrane, the VLCFA attached to lipid A seems ideally suited to mediate these functions, as it is likely to span the entire outer membrane.

The biosynthesis and addition of the VLCFA to the LPS lipid A requires a gene region encoding an acyl carrier protein (ACP) (*acpXL*), fatty acid synthesis proteins (*fabF1XL* and *fabF2XL*), β -hydroxyacyl-ACP dehydratase (*fabZXL*), an alcohol dehydrogenase (*adhA2XL*), and an acyltransferase (*lpxXL*) (Fig. 1B). Homologs of this gene region are present in most VLCFA-containing bacteria, including members of the *Rhizobiaceae* as well as in the above-mentioned pathogens (Ardissone et al. 2011; Brown et al. 2011; Colonna-Romano et al. 1990; Haag et al. 2009; Manterola et al. 2005; Sharypova et al. 2003; Vedam et al. 2003). A number of mutants encoding defects in this gene region have been constructed in one or both *acpXL* or *lpxXL* both of *R. leguminosarum*, *Sinorhizobium meliloti*, and *Rhizobium* sp. strain NGR234 (Ardissone et al. 2011; Brown et al. 2011; Haag et al. 2009; Sharypova et al. 2003; Vedam et al. 2003); all were devoid of the lipid A VLCFA, showing that the biosynthesis of VLCFA-containing lipid A is dependent on *acpXL* and *lpxXL*.

Fig. 1. A, Structures of lipid A from *Escherichia coli* and *Rhizobium leguminosarum*, demonstrating structural differences. In particular, note the very long-chain fatty acid (VLCFA) of the *R. leguminosarum* lipid A. **B,** Schematic of the gene region present in *R. leguminosarum* required for the biosynthesis of VLCFA onto the acyl carrier protein and transfer to the lipid A during lipopolysaccharide biosynthesis.

However, the functional importance of the VLCFA for cell-envelope stability and bacterial infectivity is still elusive for a number of reasons. First, various rhizobial species encoding a mutated *acpXL* gene exhibit a variable range of phenotypic impairments that are host dependent (Ardissone et al. 2011; Ferguson et al. 2005; Haag et al. 2009; Vedam et al. 2006). This perhaps indicates differing or multiple functions in the various legume host plants. Second, mutants of *R. leguminosarum* and *S. meliloti* lacking AcpXL were devoid of the VLCFA in their lipid A and substitute the VLCFA with a palmitoyl residue in their lipid A (Sharypova et al. 2003; Vedam et al. 2003), obscuring the phenotype that lacks any acyl chain at this position. Third, previous work also demonstrated partial restoration of VLCFA to the lipid A from bacteroids derived from *acpXL* mutants of *R. leguminosarum* and *S. meliloti* (Haag et al. 2009; Sharypova et al. 2003; Vedam et al. 2006), a still unexplained observation. Finally, in most legume hosts, mutants devoid of AcpXL showed effective but developmentally delayed phenotypes, e.g., pea or alfalfa (Haag et al. 2009; Vedam et al. 2004). Comparing the mutant bacteria used to inoculate the host plants with isolates from the developed nodules revealed modified stress-resistant properties of the bacterial isolates after the plant passage, perhaps indicating that the symbiotic host environments select rhizobial variants capable of delayed but effective symbiosis (Vedam et al. 2004).

Taken together, the function of the lipid A VLCFA is, despite past research efforts, not well understood and warrants additional analysis. The scope of this work was, therefore, to assess, in *R. leguminosarum*-pea symbiosis, the function of the lipid A VLCFA in a number of *R. leguminosarum* mutants (i.e., an ACP *acpXL*[−] mutant, an acyl transferase *lpxXL*[−] mutant, an *acpXL*[−] *lpxXL*[−] double mutant, as well as in their respective ex-nodule [ExN] mutant derivatives, i.e., isolates of each mutant obtained from pea nodules). In addition, we aimed to determine the lipid A structures from all LPS isolated from free-living, symbiotic, and nodule isolates for these strains and correlate this with their symbiotic and stress-tolerance phenotypes (i.e., nodule formation and resistance to salt, detergent, and the antibiotic polymyxin B [PmxB]). Both *acpXL* and *lpxXL* mutants did not add VLCFA to lipid A. In the *acpXL* mutant, a shorter acyl chain was transferred in place of VLCFA. However, in strains without *lpxXL*, neither VLCFA nor a shorter acyl chain was added. For both parent and mutant strains, nodule bacteroids had LPS with longer acyl chains than laboratory-cultured bacteria. The *acpXL* and *lpxXL* mutations had altered membrane stability properties, altered sensitivity to cationic peptides, and diminished levels of the osmoprotective cyclic β -glucans. When the host plant (*Pisum sativum* L., pea) was inoculated with parent or mutant strains, mutant bacteroids formed atypical irregular branched structures with multiple bacteroids per symbiosome. Mutant bacteroids were not packed as tightly in nodule plant cells and were senescing earlier than the wild-type rhizobia. Nitrogen fixation was also detrimentally affected.

RESULTS

Construction of *R. leguminosarum* lipid A synthesis-impaired mutants.

Mutants impaired in lipid A biosynthesis were constructed by homologous recombination mutagenesis in strain *R. leguminosarum* bv. *viciae* 3841 (Rlv3841), replacing one or both of the functional wild-type genes *acpXL* and *lpxXL* with mutated inactive gene derivatives (i.e., an *acpXL::kan* [Vedam et al. 2003] and a *lpxXL::gent* derivative [Supplementary Fig. S1]). To our surprise, multiple attempts to create an *lpxXL*[−] mutant in the Rlv3841 background consistently failed. However, using the existing Rlv22 mutant

(a derivative of Rlv3841 with the *kan* cassette in *acpXL* [Vedam et al. 2003]) as recipient strain, several *acpXL*[−] *lpxXL*[−] double mutants were isolated and strain EL196 was chosen. The double mutant also allowed for the construction of an “effective *lpxXL*” mutant, namely, strain EL197, through partial complementation of strain EL196 with cloned parent *acpXL*. For the construction of the effective *lpxXL*[−] strain EL197, plasmid pVV5, harboring the parent *acpXL* (Vedam et al. 2003), was mobilized into EL196 to complement the chromosomal *acpXL::kan* gene mutation. The pVV5 plasmid was predicted to replicate at a low to moderate copy number of five to 30 copies (Buch et al. 2010; Khan et al. 2008; Kovach et al. 1995). The various constructs (Rlv22, EL196, EL197, EL198) were genetically verified through polymerase chain reaction (PCR) amplification of indicative fragments, using gene- and construct-specific primers (Supplementary Fig. S2), and *ertr* functionally verified with strain EL198 (i.e., EL196 pSSB-1). Plasmid pSSB-1 carries the cloned whole VLCFA-related gene region, including *acpXL* and *lpxXL*, complementing strain EL196 back to a wild-type phenotype.

LPS and lipid A isolation and characterization of rhizobia.

LPSs were isolated by phenol-water extraction from free-living bacteria, nodule bacteria and bacteroids, and ExN isolates of wild-type and mutant strains. After phenol-water extraction and phase separation, the bulk of the LPSs (>90% by weight) from laboratory-cultured bacteria and from nodule bacteria and bacteroid preparations was found in the water layer. The LPSs from laboratory-cultured parent strain Rlv3841 showed, after separation on sodium deoxycholate–polyacrylamide gel electrophoresis (DOC-PAGE) gels (Fig. 2), the well-known LPS banding pattern of LPS I and LPS II (Fig. 2, panel 1, lane 1) (Kannenberg and Carlson 2001). The comparison of this parent type LPS with the LPS from the laboratory-cultured *acpXL*[−] mutant

Rlv22 showed a similar banding pattern as observed for Rlv3841 but with all bands slightly shifted to a lower molecular weight. This small shift in the banding pattern has already been reported (Vedam et al. 2003) and is expected to be due to the loss of the VLCFA from the lipid A (Fig. 2, panel 1, lane 2). The LPS from strain EL196 (*acpXL*[−] *lpxXL*[−] double mutant) and its ExN isolate resembled each other and were very similar to the LPS banding pattern from laboratory-cultured Rlv22, including the slight shift to lower molecular weight LPS forms compared with Rlv3841 LPS. The LPS from strain EL197 (*lpxXL*[−]) also displayed a similar banding pattern to Rlv22 and EL196 LPS, including the shift to lower molecular weight LPSs compared with Rlv3841 LPS.

Since, for parent strain Rlv3841, nodule-dependent LPS modifications have been reported that involve a shift to higher molecular weight LPS forms (Kannenberg and Carlson 2001), it was of particular interest to compare the various wild and mutant-type LPSs from laboratory cultures with those isolated from nodule bacteria and bacteroids. The LPSs from nodule bacteria and bacteroids of strains Rlv22 and EL196 contained, instead of the usual LPS I ladder pattern, only two prominent higher molecular weight LPS I bands (Fig. 2, third lanes in panels 1 and 2). In addition, there were either no detectable or only minimally detectable levels of LPS II in these nodule bacteria and bacteroid-derived LPS fractions, which, together, is suggestive of conditional, nodule-dependent expression of LPS forms. Additionally, comparison of the LPSs from laboratory-cultured ExN Rlv22 and ExN EL196 with the LPSs from the respective laboratory-cultured bacteria showed largely the same banding pattern (Fig. 2, fourth lanes in panels 1 and 2), indicating that the modifications are transient and host dependent.

In contrast, the LPS from strain EL197 nodule bacteria and bacteroids had a banding pattern more similar to parent Rlv3841 nodule bacteria and bacteroid LPS in that it contained the various

Fig. 2. Alcian blue/silver-stained sodium deoxycholate-polyacrylamide electrophoresis gels. Separation of lyophilized lipopolysaccharide (LPS) fractions after hot phenol/water extraction and purification of the water-soluble LPSs. Section 1 shows separation of Rlv3841 free-living LPS compared with Rlv22 (i.e., Rlv3841 *acpXL::km*) free-living, bacteroid (Rlv22 Bac), and ex-nodule (ExN) (Rlv22 ExN) LPS. Section 2 shows separation of Rlv3841 free-living LPS compared with EL196 (i.e., Rlv3841 *acpXL::km*, *lpxXL::gm*) free-living, bacteroid, and ExN LPS. Section 3 shows separation of Rlv3841 free-living LPS compared with EL197 (i.e., Rlv3841 *acpXL::km*, *lpxXL::gm*, pVV5*acpXL*) free-living, bacteroid, and ExN LPS. Section 4 shows separation of Rlv3841 bacteroid LPS compared with EL198 (complemented double mutant) free-living LPS. In each sample well, 1 µg of dry weight of lyophilized LPS material from the water layer of phenol/water extractions in 1 µl of loading buffer was added.

LPS I bands to some degree but, also, a shift toward higher molecular weight LPS I bands and a LPS II than observed for Rlv3841 (Kannenberg and Carlson 2001). In mutants without the ACP AcpXL, i.e., Rlv22 and EL196, almost exclusively, two uniform, higher molecular weight forms of LPS I are formed; in contrast, mutant bacteria and bacteroids of EL197 (i.e., *lpxXL*⁻) express LPS forms with a variety of OPS chain lengths as well as LPS II without OPS (Fig. 2, third lane in panel 3 and first lane in panel 4). These LPS forms are very similar to the Rlv3841 parent bacteroid LPS. This also suggests that AcpXL can, to a degree, replace LpxXL with regard to the type of LPS observed on DOC-PAGE analysis but not the other way around.

Previous research showed that lipid A fatty acid composition can vary due to culture conditions (D'Haese et al. 2007; Kannenberg and Carlson 2001). To investigate this aspect further, the relative fatty acid compositions of the lipid A isolated from laboratory cultures, nodule bacteria and bacteroids, and laboratory cultures of ExN isolates of strain Rlv3841 and from the set of mutant derivatives involved in lipid A synthesis were investigated (Table 1). In agreement with previous findings, the main fatty acids identified in the different lipid As were C16:0, C18:0, five different 3OH fatty acids (i.e., 3OHC14:0, 3OHC15:0, 3OHC16:0, 3OHC18:0, and 3OHC18:1), and the VLCFA 27OHC28:0. Differing results observed in this study as compared with Vedam et al. (2006) for the same *R. leguminosarum* strains (Rlv3841 and Rlv22) may be attributed to differing LPS extraction methods.

The lipid As isolated from laboratory cultures from the mutant strains Rlv22, EL196, and EL197 were devoid of the VLCFA, leading in all three strains to an increase in the percentages of 3OH fatty acids (from 74% of the fatty acids in parent strain Rlv3841 to >95% in the derived *R. leguminosarum* mutant strains). Interestingly, under laboratory conditions, both C16:0 and C18:0 fatty acids are found only in small amounts (<2%) in extracts from all three mutants (Rlv22, EL196, EL197). This was in contrast to nodule conditions in which the relative percentage of C16:0 and C18:0 fatty acids of the lipid A greatly increased in strains Rlv22 Bac (*acpXL*⁻) and EL196 Bac (*acpXL*⁻/*lpxXL*⁻), concomitantly with a strong decrease in 3OH fatty acids, in particular in the 3OH C14:0. However, the strong increase of C16:0 and C18:0 was not found with lipid As derived from nodule bacteria from strain EL197 Bac (*lpxXL*⁻) and

the total amount of 3OH fatty acids stayed almost as high as found in laboratory culture (Table 1). A noticeable adaptation, however, also took place within the mix of 3OH fatty acids of this EL197 strain; the 3OHC14:0 was decreased, while 3OHC18:0 was increased. Taken together, these findings indicate that lipid A fatty acid composition is possibly fine-tuned for functioning under changing growth conditions and that LpxXL may be involved in C16:0 and C18:0 fatty acid transfer to lipid A, primarily under nodule conditions.

Interestingly, 3% of the total fatty acids of EL197 bacteroid lipid A were VLCFA. Although VLCFA was found in the EL197 lipid A preparation, there was no lipid A containing VLCFA detected, using matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis (described further below), i.e., all ions observed were consistent with lipid A species that lacked the VLCFA. It is possible that VLCFA AcpXL may have been isolated during the phenol-water extraction and, therefore, the observed VLCFA may not be part of the lipid A. The larger amounts of VLCFA AcpXL may be present since this strain contains multiple copies of plasmid pVV5, which carries the *acpXL* gene.

Chain-length shifts in lipid-A structures from Rlv3841 laboratory-cultured bacteria and from nodule bacteria and bacteroids.

To gain further insight into lipid A structure, preparations isolated from laboratory-cultured bacteria and nodule bacteria and bacteroids were also analyzed by MALDI-TOF MS (Table 2; Figs. 3 and 4, Supplementary Figs. S3, S4, and S5). Figure 4 shows the structures I.A, I.B, II, III.A, III.B, IV, V.A, and V.B, predicted from the MALDI-TOF MS spectra and described in Supplementary Table S1. Each lipid A structure is heterogeneous with regard to one or both the fatty acid chain length or proximal GlcN structure, creating a cluster of ions this way.

As shown in Figure 3, lipid A from the laboratory-cultured parent strain Rlv3841 has four major clusters of ions consistent with structures I.A, I.B, II, III.A, III.B, and IV. The VLCFA is attached to structures I.A and I.B with either GlcN (I.A) or GlcNonate (I.B) as proximal sugar and to structure II, which is

Table 1. Relative fatty acid compositions (major constituents only) of lipid A from *Rhizobium leguminosarum* Rlv3841, Rlv22, EL196, and EL197 free-living bacteria, bacteroids (Bac), and ex-nodule (ExN) bacteria

Strain	Fatty acid percentages ^w							
	C16:0 ^x	C18:0	3OH					27OH
			C14:0	C15:0	C16:0	C18:0	C18:1	C28:0
Rlv3841	4	5	46	2	9	13	4	16
Rlv3841 Bac	8	9	20	4	4	8	7	41
Rlv3841 ExN	6	12	37	2	7	9	2	25
Rlv22	2	ND ^y	57	7	13	18	1	ND
Rlv22 Bac	14	11	36	12	6	9	11	ND
Rlv22 ExN	4	4	43	4	13	29	4	ND
EL196	1	1	57	5	12	22	3	ND
EL196 Bac	21	26	26	11	5	5	6	ND
EL196 ExN	2	14	32	10	11	29	2	ND
EL197	1	1	53	5	12	24	4	ND
EL197 Bac	1	3	30	4	12	41	6	3 ^z
EL197 ExN	3	17	46	9	9	15	2	ND

^w Fatty acid percentages are calculated based on the ratios of gas chromatography area values.

^x Small amounts of C16:0 and C18:0, in mutants that lack *acpXL* but not *lpxXL* can be due to the fact that *lpxXL* can add these fatty acids, to a small degree, from other *acp*-fatty acid substrates. However, a small portion of these two fatty acids could also be due to slight contamination by phospholipids. However, the large increases in these fatty acids in the EL196 and Rlv22 bacteroid lipopolysaccharide preps are unlikely to be explained completely by phospholipid contamination.

^y ND = none detected.

^z Very long-chain fatty acid (VLCFA) was present in EL197 bacteroid lipid A preparations; however, this long-chain fatty acid was not attached to the lipid A component. It is a possibility that VLCFA was attached to AcpXL and extracted from the cells during the phenol/water extraction procedure.

missing 3OHC14:0 on the three-position of the proximal modified GlcNonate. The loss of this fatty acid is believed, however, to be an artifact of the mild acid hydrolysis procedure used to isolate lipid A (Price et al. 1995); due to elimination of the fatty acyl residue and production of a proximal 2-ene-GlcNonono-lactone residue on the lipid A. Thus, structures I and II are due to pentaacylated lipid A species (II being an artifact of I). Structures III.A, III.B, and IV were observed to a lesser extent and are tetraacylated structures that lack the VLCFA. The ions observed for structure III were of the shorter fatty acyl chains (only 1448.1, 1464.0, 1476.1, and 1492.1 m/z) in comparison with ions observed from the mutants (1447.9, 1463.9, 1475.9, 1491.9, 1513.9, 1520.0, 1548.0, and 1577.9 m/z). Structures III and IV are similar to I and II but lack the VLCFA.

The lipid A from Rlv3841 nodule bacteria and bacteroids also displayed structures I.A, I.B, II, and IV (Fig. 4). However, many of these lipid A structures had a shift toward longer chain fatty acids in each ion cluster. For example, ion 1914.6 m/z is a longer fatty acid chain variant of 1886.5 m/z , and ions 2012.7 and 1984.6 m/z are longer fatty acid chain variants of ion 1956.5 m/z . The structures I.A and I.B with the shortest chain lengths (ions 1870.5 and 1886.5 m/z , respectively) were only observed in the laboratory-cultured parent and not the nodule bacteria or bacteroids. Both the laboratory cultures and nodule bacteria and bacteroid Rlv3841 had the same ions for structure II. Structure III, the tetraacylated lipid A, was observed in laboratory-cultured Rlv3841 only and not in the nodule bacteria or bacteroids. For structure IV (the artifactual elimination product of structure III), Rlv3841 laboratory-cultured lipid A lacked two ions, m/z 1259.9 and 1301.9, that were observed in the bacteroid lipid A. These ions are 16 mass units greater than corresponding structure IV ions, and structures for these ions remain unassigned. However, they could be oxygen adducts of structure IV molecules. The nodule bacteria and bacteroids contained all of the observed ions for variations of structure IV (except for the three with the shortest acyl chain lengths 1173.8, 1201.8, and 1229.9 m/z).

Additional structures and chain-length shifts in lipid A from mutants in laboratory culture versus lipid A from mutant nodule bacteria.

Laboratory-cultured Rlv22 (*acpXL*[−]) had structures III.A, III.B, IV, and V.B., all of which lack the VLCFA (Table 2; Fig. 3). Structure V is similar to structure I, except that a C16:0 or C18:0 is added instead of the VLCFA. Based on peak intensity, structure V was less abundant than structures III and IV in the laboratory-cultured bacteria and structure V.A may be present at insufficient levels for detection. The Rlv22 nodule bacteria and bacteroid lipid A showed ion clusters tending toward having longer chain fatty acids. Structure V with the additional C16:0 or C18:0 was also more abundant, i.e., the relative intensity of its ion was greater in the nodule bacteria and bacteroid-derived lipid A sample, as compared with the lipid A from laboratory culture. The proximal-GlcN form of structure III was not observed in Rlv22 nodule bacteria and bacteroid lipid A as was observed for Rlv3841 nodule bacteria. Only the shorter chain ions (1448.1 and 1476.1 m/z) were observed for laboratory-cultured Rlv22 structure III.A. Rlv22 structure III.B. had two observed ions thought to be sodiated structures that were not present in any other strains, one (1513.9 m/z) present only in the laboratory culture and one (1577.9 m/z) observed in both laboratory culture and nodule bacteria and bacteroid Rlv22. For structure IV, the laboratory-cultured Rlv22 lacked the ions that are unassigned and may be oxygen adducts of structure IV, while the ions 1273.9 m/z and above were observed in the nodule bacteria and bacteroids. The proximal-GlcN form of structure V was not observed in Rlv22 laboratory culture, and only the ions corresponding to the proximal-GlcNonate form with shorter chain-length structures were observed (1786.2 m/z and below).

EL196 (*acpXL*[−]/*lpxXL*[−]) and EL197 (effective *lpxXL*[−]) both had structures III.A, III.B, and IV in both free-living and bacteroid lipid A (Table 2, Fig. 3). EL196 nodule bacteria and bacteroids had structure III.A lipid A with longer chain-length fatty acyl residues (ion 1560.2) than that from the laboratory-cultured strain and did not have the ions thought to be sodiated that were observed for Rlv22. The EL196 nodule bacteria and bacteroid lipid A did not have the ions for the predicted shortest chain-length structures that were observed for the laboratory culture (1173.8 and 1201.8 m/z). Neither the laboratory strain nor the bacteroids had the ions for the unassigned structure IV molecules (which could be oxygen adducts). Both laboratory-cultured and nodule bacteria and bacteroid EL197 lipid A had all of the observed ions for structure III.A, but, for structure III.B, the nodule bacteria and bacteroid lipid A had an additional ion predicted to have longer acyl chains. EL197 nodule bacteria and bacteroids had ions predicted to be hydroxylated or sodiated in addition to the ions observed for the laboratory culture. Both the EL196 and EL197 bacteroid lipid A were shifted toward longer chain fatty acid structures. Mutation of the *lpxXL* gene prevented the addition of the VLCFA as well as substitution of the VLCFA with shorter chain fatty acid. The shift toward longer chain fatty acids from bacteroids in comparison with laboratory-cultured strains is also shown by the fatty acid compositions in which C16:0 and C18:0 percentages increase between laboratory-cultured bacteria and bacteroids while the fatty acid percentages of C14:0 decreased (Table 1).

Increased mutant sensitivities to stressors.

The sensitivity and resistance of the mutants to stress in vitro was tested by growing the strains in the presence of increased levels of NaCl (increased osmolarity), in the presence of the detergents sodium dodecyl sulfate (SDS) or DOC (Table 3), and with the cationic peptide antibiotic PmxB (Fig. 5), known to affect gram-negative cell-membrane architecture, thereby inhibiting growth (Teuber and Bader 1976). The mutant strains Rlv22, EL196, and EL197 were all sensitive to NaCl at 0.5% in the growth medium, while parent strain Rlv3841 and the fully complemented double mutant EL198 were able to grow at this salt concentration. A related observation was made in attempts to grow the bacteria on media plates supplemented with detergents SDS or DOC. On media containing 0.35 mM SDS, the wild-type strain Rlv3841 grew, while all three mutants Rlv22, EL196, and EL197 were unable to grow at this concentration. Similarly, while the parent Rlv3841 strain was able to grow on DOC plates with up to 3 mM DOC in the medium, mutants Rlv22 and EL196 were considerably more growth-sensitive and stopped growing at 0.76 mM DOC. Mutant EL197 was even more susceptible to DOC than Rlv22 and EL196 and was unable to grow at a concentration of 0.64 mM DOC. An uneven picture emerged in attempts to grow the mutants on PmxB gradient plates; while

Table 2. Lipid A structures of *Rhizobium leguminosarum* under free-living and nodule growth conditions

Strain	Lipid A structure ^z							
	I.A	I.B	II	III.A	III.B	IV	V.A	V.B
Rlv3841	+	+	+	+	+	+	−	−
Rlv3841 Bac	+	+	+	−	−	+	−	−
Rlv22	−	−	−	+	+	+	+	+
Rlv22 Bac	−	−	−	−	+	+	−	+
EL196	−	−	−	+	+	+	−	−
EL196 Bac	−	−	−	+	+	+	−	−
EL197	−	−	−	+	+	+	−	−
EL197 Bac	−	−	−	+	+	+	−	−

^z + and − indicate the presence or absence of each lipid A structure by strain.

Rlv22 and EL196 were more sensitive and displayed diminished growth in comparison with the parent Rlv 3841 and the complemented double mutant EL198 (Fig. 5), mutant EL197 showed the wild-type level of resistance toward PmxB.

Following passage through the plant, it was previously shown that the Rlv22 ExN isolate gained parental levels of tolerance to NaCl, despite being unmodified with regard to *acpXL* during the plant passage (Vedam et al. 2003). This surprising but unexplained result was confirmed for Rlv22 ExN and was also found to be true for the ExN isolate of mutant EL196. Similarly to NaCl salt sensitivity, the Rlv22 ExN and EL196 ExN isolates both showed increased tolerance to SDS and DOC.

However, none of these mutant ExN isolates regained resistance to DOC to the same level as the parent strain or to the complemented double mutant. In contrast to Rlv22 ExN and EL196 ExN, the EL197 ExN isolate did not gain tolerance to NaCl; the EL197 ExN isolate displayed the same level of sensitivity toward salt, SDS, and DOC that was observed with its parent EL197 before plant passage. With regard to PmxB, all three ExN mutant isolates did not exhibit increased tolerance to PmxB, as was observed for SDS, DOC, and NaCl. Moreover, EL197 ExN was found to be more susceptible to PmxB as compared with the parent EL197 strain before the plant passage.

Fig. 3. Matrix-assisted laser desorption-ionization time of flight mass spectrometry (MALDI-TOF MS) spectrum of lipid A as isolated from laboratory cultures of strains Rlv3841 (parent), Rlv22 (*acpXL*), EL196 (*acpXL/lpxXL*), and EL197 (*lpxXL*) and their proposed lipid A structures deduced from MALDI-TOF MS analysis. Structures I.A, I.B, II, III.A, III.B, and IV were observed in Rlv3841 lipid A. Structures III.A, III.B, IV, V.A, and V.B were observed in Rlv22 lipid A. Structures III.A, III.B, and IV were observed in EL196 and EL197 lipid A. Very long-chain fatty acid (VLCFA) was only observed in the parent strain; Rlv22 was able to substitute a shorter chain fatty acid for the VLCFA; and neither EL196 nor EL197 was able to add VLCFA or the shorter chain fatty acid.

Decreasing ratios of cyclic β -glucans from mutants and ExN parent strains.

R. leguminosarum is known to use cyclic β -glucans for hypo-osmotic adaptation and their synthesis is osmoregulated (Breedveld et al. 1990; Griffiths et al. 2008). Thus, the ability of Rlv22 and EL196 to regain resistance to NaCl and detergents after passage through the plant may have been due to a change in the amount of cyclic β -glucans found in the periplasm of ExN strain isolates. Since the cyclic β -glucan is composed of glucose connected by 1,2-glycosidic linkages, 2-linked glucose (2-Glc) levels were determined, to estimate the relative amounts of cyclic β -glucan present in the parent, the mutants, and their ExN isolates, as described below. The results are shown in Figure 6. Rlv3841 and EL198 laboratory culture bacteria had greater amounts of 2-Glc (2-Glc/Ino ratios of 30.9 and 18.9, respectively) compared with mutants Rlv22 and EL197 (3.4 and 2.1 ratios, respectively). EL196 had a barely detectable amount of 2-Glc (a 0.5 ratio). Only the ExN isolate of Rlv22 showed a modest increase in the relative amount of 2-Glc from laboratory to ExN culture. The EL196 and EL197 ExN isolates were unchanged in their levels of 2-Glc. Interestingly, Rlv3841 and EL198 both displayed a dramatic decrease in the 2-Glc/Ino ratio, i.e., 13- and fourfold decreases, from amounts found in laboratory cultures to ExN derivative cultures, possibly indicating that cyclic β -glucans may be downregulated in these bacteria during

nodule passage. These results imply that the restoration of salt and detergent tolerance in the ExN isolates is unlikely to be caused by an increased production of cyclic β -glucan. However, the lesser amounts of cyclic β -glucans observed in Rlv22, EL196, and EL197 could possibly have impacted the ability of those mutants to infect the host. The reason for the decrease in

Table 3. Osmotic tolerance and detergent sensitivity of laboratory-cultured and ex-nodule (ExN) *Rhizobium leguminosarum* strains^z

Strain	NaCl%	SDS (mM)		DOC (mM)			
	0.50	0.35	0.52	0.64	0.76	1.00	2.00
Rlv3841	+	+	+	+	+	+	+
Rlv3841 ExN	+	+	+	+	+	+	+
EL198	+	+	+	+	+	+	+
Rlv22	-	-	-	+	-	-	-
Rlv22 ExN	+	+	-	+	+	-	-
EL196	-	-	-	+	-	-	-
EL196 ExN	+	+	-	+	+	-	-
EL197	-	-	-	-	-	-	-
EL197 ExN	-	-	-	-	-	-	-

^z Strains were grown on tryptone-yeast extract plus calcium media for 3 days at 30°C with the indicated concentrations of NaCl, sodium dodecyl sulfate (SDS), or sodium deoxycholate (DOC). + and - indicate whether that strain of *Rhizobium leguminosarum* was able to grow on the media.

Rlv3841 Laboratory Cultured

Rlv3841 Bacteroid

Fig. 4. Matrix-assisted laser desorption-ionization time of flight mass spectrum of the lipid A from strain Rlv3841 laboratory culture (top) and nodule bacteria or bacteroids (bottom). The m/z mass ions of the bacteroid-derived lipid A spectrum, generally, have longer fatty acyl chains than in those observed for lipid A from Rlv3841 laboratory culture. Ions between 1173.3 and 1323.9 m/z represent Structure IV. Ions between 1448.1 and 1492.1 m/z represent Structure III. Ions between 1624.3 and 1822.4 m/z represent Structure II. Ions between 1870.5 and 1068.7 m/z represent Structure IV.

the levels of cyclic β -glucans for Rlv3841 and EL198 ExN isolates is currently unknown.

Altered nitrogen fixation, nodule number, and bacteroid morphology.

To explore the symbiotic phenotype and also to obtain ExN isolates for analysis, each strain was used to inoculate host pea plants. Each of the mutants was able to induce effective nodules

on pea plants and formed pink root nodules. Variability was observed between one or more of the *acpXL* and *lpxXL* mutant phenotypes with regard to the efficiency of the interaction (i.e., nitrogen fixation), onset of nodulation, nodule number and average nodule size, nodule internal organization, and bacteroid properties such as shape, occupation density, and size.

Nitrogen fixation was measured by exposing the plant root system from different days postinfection (dpi) to acetylene and measuring the percentage of ethylene evolved (Table 4). At 14 dpi, pea root nodules induced by parent strain Rlv3841 produced a significantly higher percentage of ethylene as compared with EL196 (*acpXL*/*lpxXL*) nodules and EL197 (*lpxXL*) nodules. Rlv22 (*acpXL*) and strain EL198 (complemented double mutant) had intermediate percentages of ethylene production that were not significantly different from nodules from Rlv3841. At 21 dpi, a similar trend was seen. The highest percentage of ethylene production was observed for Rlv3841 nodules; it was significantly higher than EL196 and EL197. The nodules induced by Rlv22 and EL198 had, again, intermediate levels of acetylene production but were not significantly different than Rlv3841 nodules. At 28 dpi, host plants and nodules induced by strain Rlv3841 and the fully complemented EL198 strain were developmentally ahead of the plants from mutant-induced nodules to the extent that they exhausted their medium humidity and dried out, preventing another reading to be taken for these strains. At this time point, the nodules of Rlv22 had a significantly higher percentage of acetylene production than either EL196 or EL197. Thus, at all time points, plants infected with mutant strains impaired in LPS lipid A biosynthesis were less effective in nitrogen fixation compared with plants infected with the parent wild-type strain.

The ability of legume hosts to autoregulate nodule number has been shown to lead to an increased number of nodule initiations when individual nodules are not fully functional (Vasse et al. 1993). Since the *acpXL* and *lpxXL* mutations both lead to impaired VLCFA insertion into lipid A and, presumably, to impaired nitrogen-fixation efficiency (discussed above), we checked whether pea plants infected with these mutants are also affected in nodule number and size (Table 5). The state of nodule development was scored visually as immature (small, white) or mature (large, pink). We found that a common feature of host plants that were infected with mutants impaired in lipid A VLCFA biosynthesis was a delay in the formation of mature nodules and a tendency to have more small nodules. At 14 dpi, plants infected with parent and mutant strains had more immature than mature nodules but the number of nodules did not significantly differ between strains. At 21 dpi, plants infected with EL196 had significantly more immature nodules than those infected with any other strain. EL198 had the most mature nodules, Rlv3841 and Rlv22 had an intermediate number, and EL196 and EL197 had the fewest mature nodules at 21 dpi.

Fig. 5. Growth of laboratory-cultured and ex-nodule isolates of Rlv3841, Rlv22 (*acpXL*), EL196 (*acpXL*/*lpxXL*), EL197 (effective *lpxXL*), and EL198 (double mutant fully complemented with pSSB1) isolates on tryptone-yeast extract gradient plates ranging from 0 to 50 μ g/ml of polymyxin B. Solid black lines indicate the approximate distance of growth of each isolate.

Fig. 6. Comparison of the levels of 2-linked glucose (2-Glc) measured by partially methylated alditol acetate analysis. Rlv3841, Rlv22 (*acpXL*), EL196 (*acpXL*/*lpxXL*), EL197 (effective *lpxXL*), and EL198 (complemented double mutant) from laboratory-cultured (black bars) and ex-nodule (white bars) isolates were measured.

Table 4. Ethylene production (as percentage of used acetylene volume) of nodule pea root systems induced by *Rhizobium leguminosarum* strains

Strain	Percentage of ethylene				
	14 dpi (<i>n</i>) ^y	% Wild type (WT)	21 dpi (<i>n</i>)	% WT	28 dpi (<i>n</i>)
Rlv3841	9.93 $\pm$ 2.12 a (7)	100	5.81 $\pm$ 2.04 a (10)	100	ND ^z
Rlv22	7.24 $\pm$ 3.26 a (6)	72.9	4.66 $\pm$ 2.26 a (14)	80.2	12.32 $\pm$ 0.90 a (5)
EL196	4.63 $\pm$ 0.86 b (6)	46.6	2.73 $\pm$ 1.24 b (23)	47.0	9.67 $\pm$ 1.49 b (5)
EL197	2.85 $\pm$ 0.68 b (6)	28.7	3.28 $\pm$ 1.47 b (20)	56.5	7.96 $\pm$ 1.79 b (5)
EL198	7.64 $\pm$ 1.30 a (5)	76.9	4.55 $\pm$ 1.84 a (22)	78.3	ND

^y Percentages are calculated from the ratio of the area under the peak for acetylene to the area under the peak for ethylene. *n* is the number of plant root systems analyzed for acetylene reduction. Values shown $\pm$ standard deviation. Different letters indicate significance differences in percentage ethylene when compared with the parent strain Rlv3841 at 14 and 21 dpi ($P < 0.05$). At 28 dpi, differing superscripts within the column indicate significance differences in percentage ethylene between strains ($P < 0.05$).

^z ND = not determined.

Overall, plants infected with double mutant EL196 had significantly more total nodules than those infected with Rlv3841, Rlv22, or EL198, while EL197 had an intermediate total nodule number.

We also investigated nodule tissue organization and bacteroid morphology of the mutants. While nodules of Rlv3841, Rlv22, and EL198 at 21 dpi had largely comparable nodule organization (not taking into account smaller developmental stage differences of the various nodules investigated), the nodules induced by EL196 and EL197 showed differences with diminished numbers of infected plant nodule cells and aberrations in nodule cell organization. Infected nodule cells were more loosely spaced and most of the cells lacked a visible vacuole (Supplementary Fig S6). Bacteroid shape was also affected. Examples of bacteroids from 14, 21, and 28 dpi of

strains Rlv3841, Rlv22, EL196, EL197, and EL198 are shown in Figure 7. Strains Rlv3841 and EL198 both formed mostly normally shaped elongated Y-structure bacteroids, while Rlv22, EL196, and EL197 formed abnormally shaped bacteroids with increased branching, and nodule cells containing symbiosomes with multiple bacteroids (Figure 7 shows an example in the 28-dpi panel). Some bacteroids of Rlv22, EL196, and EL197 also showed regions of dark spots or granules, which have been suggested to be a sign of bacteroid senescence (Fig. 8) (Melino et al. 2012). Comparable structures have not been discovered in respective nodules induced by the wild-type parent Rlv3841 strain or the complemented mutant EL198.

DISCUSSION

To thrive in soil and plant environments, rhizobia must be able to adapt to the stressors in these environments (Vriezen et al. 2007), e.g., low water activity and antimicrobial compounds, and results of this and other work support that bacterial LPS plays an important role in providing tolerance to these stressors. It is likely to be of importance for both survival within the rhizosphere and the establishment of symbiosis with host plants (Araujo et al. 1994; Frayse et al. 2003). In particular, lipid A with its VLCFA is expected to be crucial for rhizobial survival in both soil and host-plant environments, as it anchors the LPS to the outer membrane of the rhizobial cell envelope.

R. leguminosarum LPS and lipid A are environment-dependently modified.

Overall, the relative fatty acid composition comparison of the lipid A from laboratory culture, nodule bacteria and bacteroids, and ExN bacteria suggests three adjustments in the lipid A fatty acid composition from free-living bacteria to nodule bacteria and bacteroids: i) a shift toward higher molecular weight LPS in all cases but particularly in mutants defective in *acpXL*,

Table 5. Number of immature (small, white) and mature (large, pink) nodules for Rlv3841, Rlv22, EL196, EL197, and EL198 at 14 and 21 days postinfection (dpi)^y

Strain	<i>n</i>	Immature ^z	Mature	Total ^z
14 dpi				
Rlv3841	7	122	25	147
Rlv22	6	93	22	114
EL196	6	99	12	111
EL197	6	90	0	90
EL198	5	92	14	105
21 dpi				
Rlv3841	14	63 b	43	106 b
Rlv22	15	70 b	44	114 b
EL196	13	151 a	26	178 a
EL197	12	91 b	30	121 b
EL198	14	32 b	54	85 b

^y *n* is the number of plant root systems analyzed.

^z Different letters indicate significance differences in the number of nodules when compared with the parent strain Rlv3841 ($P < 0.05$).

Fig. 7. Transmission electron microscopy images of individual Rlv3841, Rlv22, EL196, EL197, and EL198 bacteroids. Scale bar = 2 μm.

indicating that bacteroid LPS from all strains contained higher molecular weight OPS than the LPS of laboratory-grown cultures; ii) an increase in the percentage of C16:0 and C18:0 fatty acids; iii) a decrease in the percentage of 3OH fatty acids (in particular that of 3OHC14:0); and iv) a pronounced increase in the relative percentage of 27OHC28:0 in the LPS from the Rlv3841 parental strain (Table 1). These effects on lipid A composition could be the result of conditional LPS structure modification in nodule bacteria and bacteroids or the result of nodule selection pressure favoring the growth of bacterial variants with modified lipid A composition. If, in fact, the latter would be the case, one could expect the lipid A fatty acid adjustments observed during nodule development would be permanent and also would be found in the derived laboratory-cultured ExN isolates, but that was not the case. Taken together, the observed LPS and lipid A structural modifications indicate that genetic selection is not taking place but that a conditional tailoring of LPS and lipid A structure is occurring in the bacteroid due to nodule conditions.

With regard to the mutants, the *acpXL* mutant was able, in part, to replace the VLCFA with C16:0 or C18:0; however, none of the *lpxXL* mutants were able to do this. Similar results were also observed for *AcpXL* and *LpxXL* from *Sinorhizobium meliloti* (Ferguson et al. 2005; Haag et al. 2011), corroborating for this bacterium that *LpxXL* is required for the transfer of the VLCFA as well as the C16:0 or C18:0 to the lipid A to form a pentaacylated lipid A (Ferguson et al. 2005).

It has been previously reported that the *acpXL* mutants of *R. leguminosarum* and *S. meliloti* are able to partially restore the addition of VLCFA while in plant nodules (Haag et al. 2009; Vedam et al. 2006). This restoration of the lipid A VLCFA in the nodule environment was not observed in this work and awaits further investigation. The VLCFA was detected, however, in lipid A preparations from EL197 bacteroids; but MALDI analysis of the lipid A from EL197 did not show any detectable lipid A species containing the VLCFA. A likely explanation for the VLCFA in the EL197 lipid A preparations could be that it was present as the VLCFA *AcpXL* conjugate that was not further processed because of the nonfunctional acyltransferase. This VLCFA *AcpXL* may have been present in high levels due to the extra copies of *acpXL* in EL197 and isolated with lipid A.

Stress resistance of *R. leguminosarum acpXL*[−] and *lpxXL*[−] mutants.

A precondition for rhizobial survival in both soil and plant environments is the ability of the bacteria to adapt to local stressors, such as changes in osmolarity, humidity, and plant defense responses (e.g., antimicrobial peptides) (Brewin 1991; Erbs and Newman 2012; Kannenberg and Brewin 1989; Van de Velde et al. 2010). Each of the mutants, Rlv22 (*acpXL*[−]), EL196 (*acpXL*[−]/*lpxXL*[−]), and EL197 (effective *lpxXL*[−]), was affected in at least some of their stress-related properties, i.e., compared with the parental strain, they became slower growing (Supplementary

Fig. S7), sensitive to sodium chloride, detergents (SDS or DOC) (Table 3), the antimicrobial peptide PmxB (Fig. 5), or desiccation (Supplementary Fig. S8), and had lower levels of osmoprotective cyclic glucans (Fig. 6). These findings emphasize the importance of lipid A and the VLCFA for rhizobial stress adaptation and corroborate previous work that showed sensitivity to high osmolarity, detergents, and desiccation in other laboratory-cultured VLCFA mutants (Brown et al. 2011; Vanderlinde et al. 2010; Vedam et al. 2003). Slow growth and susceptibility to salt, detergents, desiccation, and a diminished amount of cyclic β -glucans are expected to be due to a loss of membrane stability caused by the lack of a VLCFA on the lipid A.

In this and previous work, it has been demonstrated that, after symbiotic passage through pea plants, the ExN isolates of *acpXL* and *lpxXL* mutants of *R. leguminosarum* and *S. meliloti* have restored tolerance to detergents and salt (Brown et al. 2011; Sharypova et al. 2003; Vedam et al. 2006). The mechanism for this restoration is still elusive. Vanderlinde and Yost (2012), studying detergent tolerance in the same *R. leguminosarum* strain, hypothesized that an inherited and not an adaptive mechanism restores detergent tolerance during the host-plant passage. Examination of our mutants showed that none of the ExN isolates from our set of mutants had lost the genetic mutation; a result that pointed toward an adaptive mechanism underlying our observations. To further examine possible mechanisms for restoration of detergent and osmotic tolerance, we investigated a known osmoprotectant in *R. leguminosarum*, the cyclic β -glucans from the parent strain, and mutant laboratory-cultured and ExN isolates. Compared with the parent strain, the amount measured of 2-Glc was low for the laboratory-cultured mutants that lack VLCFA and remained low for all strains, following passage through the plant. Thus, the amount of 2-Glc did correlate with the restoration of osmotic tolerance seen in the mutant ExN isolates. Therefore, the restoration to parental tolerance levels of stressors was not due to isolation of ExN isolates with increased levels of cyclic β -glucans. The mechanism for restoration of osmotic and detergent tolerance in mutant ExN isolates remains unknown.

Nodule development of *R. leguminosarum acpXL*[−] and *lpxXL*[−] mutants.

The lack of the VLCFA still allowed for effective pea nodules to develop, but nodule development was altered. Host plants that were infected with mutants that lack VLCFA were slower to form mature nodules, tended to have more small, often white nodules and had irregular infected-nodule cell organization, and symbiosomes containing multiple aberrantly shaped bacteroids that prematurely senesce. Since LPS has been suggested to be of importance in the later stages of nodule development (Kannenberg et al. 1998; Scheidle et al. 2005), it is tempting to speculate that the plant phenotypes observed in this report could be due to the defective LPS lipid As, which may lead primarily to destabilization of the bacterial membrane and,

Fig. 8. Transmission electron microscopy images of individual mature bacteroids exhibiting degradation. Arrows are pointing to areas of degradation.

also, possibly to some recognition by the host, causing a plant defense reaction and delayed nodule development. The sensitivity we found in some mutants that lack VLCFA toward the antimicrobial peptide PmxB seems to point in this direction. In addition to destabilization of the membrane, a defective interaction between nodule-specific cysteine-rich (NCR) peptides and the bacterial membrane of the mutants that lack VLCFA may be another mechanism leading to the alteration of nodule development, irregular nodule cell organization, and aberrantly shaped bacteroids. NCR peptides are produced in indeterminate nodules, are required for the prevention of bacterial cell division, and are necessary in forming an effective symbiosis (Mergaert et al. 2003; Van de Velde et al. 2010). The interaction of these mutants, as well as their LPSs, with these different classes of peptides that act upon bacterial LPS is a further area of research that should be explored.

Overall, the mutants have complex phenotypes and the mutant lipid As seem to have an impact on rhizobial uptake by nodule cells, on the interaction of the peribacteroid membrane and the bacterial surface necessary for bacteroid and symbiosome development. Bacteroid stability and, possibly, nutrient uptake may be leading to a delay in symbiotic developments or to premature senescence or both. Thus, the results of this report confirm and strongly support the importance of a pentaacylated lipid A with attached VLCFA for the stability and functioning of the outer membrane of the *R. leguminosarum* cell envelope in both symbiotic as well as soil environments. Many open questions about the functional details, however, remain unanswered, and much needs to be learned about the role of the VLCFA in rhizobial cell-envelope stability and stress adaptation.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

Bacteria and plasmids used in this study are listed in Table 6. *Escherichia coli* strains were grown on Luria-Bertani (LB) medium at 37°C with appropriate antibiotics (Vedam et al. 2003). *R. leguminosarum* strains were grown at 30°C on tryptone-yeast extract (TY) medium with added calcium (Noel et al. 1986) and appropriate antibiotics. Antibiotics were used at the following concentrations (µg/ml): tetracycline, 10; kanamycin,

50; gentamicin, 25; streptomycin, 200; and ampicillin, 50. For β-galactosidase blue and white colony screening, 100 µl of 40 mM isopropylthiogalactopyranoside (IPTG) and 100 µl of 2% 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) were spread onto each plate.

Plant growth and inoculation conditions.

For germination, 'Early Alaska' pea seeds were rinsed with 70% ethanol for 30 s, were washed 3× with sterile distilled (d) H₂O, surface sterilized with 6% sodium hypochlorite for 1 min, and were washed 10× with sterile dH₂O. The sterilized seeds were transferred to 300 ml of nitrogen-free Fahraeus medium (Nutman 1970) in 500-ml foam stoppered flasks and were allowed to germinate for 10 to 13 days at 22°C for 14 h of light, followed by 10 h of darkness at 18°C, and ambient relative humidity. Following germination, stems were manipulated out of the top of the flask and the foam stopper was gently reinserted, allowing the stem and leaves of the plant to grow outside the flask. Each plant was inoculated with 1 ml of an approximately 0.15 optical density at 600 nm (OD₆₀₀) log phase culture (approximately 10⁸ CFU/ml) by dripping onto the seed and emerged roots. Each flask was then covered with a brown paper bag and was taped closed, to minimize light exposure to the roots while leaving the foam stopper and stem and leaves of the plant exposed to light. The pea plants were allowed to grow each day as described above for up to 28 dpi.

Bacteroid isolation.

For chemical analysis, plant nodules with bacteroids were collected at 21 dpi. Nodules from approximately 50 plants per strain were removed from the roots with forceps and were collected in a 0.5 M sucrose buffer (50 mM Tris, 10 mM dithiothreitol [DTT], 0.5 M sucrose, pH 7.4).

Subsequent to their harvest, the nodules were removed from the buffer and were ground with a mortar and pestle in a small amount of a freshly prepared buffer with 0.5 M sucrose 5% (wt/vol) polyvinylpyrrolidone and (5 mM) p-aminobenzamidine. The slurry of plant material and nodule bacteria (i.e., predominantly bacteroids) was filtered through a syringe packed with 1 cm of glass wool. The bacteroids from the filtrate were further isolated by sucrose gradient ultracentrifugation. The sucrose gradients consisted

Table 6. Bacterial strains and plasmids used in this study

Strain/plasmid	Characteristics ^z	Source
<i>Escherichia coli</i>		
XL1 Blue	<i>endA1, recA1, gyrA96, thi, hsdR17</i> (rk ⁻ , mk ⁺), <i>relA1, supE44, lac</i> , (F ⁺ , <i>proAB</i> ⁺ , <i>lacIqZ</i> ΔM15::Tn10[Tet ^r])	Stratagene Corp.
DH5α	Φ80 <i>dlacZ</i> ΔM15, <i>recA1, endA1, gyrA96, thi-1, hsdR17</i> (rk ⁻ , mk ⁺), <i>supE44, relA1, deoR, Δ(lacZYAargF)</i> U169	Stratagene Corp.
<i>Rhizobium leguminosarum</i> bv. <i>viciae</i>		
Rlv3841	Strain 300 Str ^r , Fix ⁺	Wang et al. 1982
Rlv22	Strain 3841 <i>acpXL::km</i> Str ^r , Kan ^r , Fix ⁺	Vedam et al. 2003
EL196	Strain Rlv22 <i>lpxXL::gm</i> Str ^r , Kan ^r , Gen ^r , Fix ⁺	This study
EL197	Strain EL196 pVV5 with <i>acpXL</i> , Str ^r , Kan ^r , Gen ^r , Tet ^r , Fix ⁺	This study, Vedam et al. 2003
EL198	Strain EL196 pSSB-1 with <i>acpXL,lpxXL</i> , Str ^r , Kan ^r , Gen ^r , Tet ^r , Fix ⁺	This study, Basu et al. 2002
Plasmids		
pSSB-1	pLAFR-1 with 22-kb <i>Rhizobium leguminosarum</i> genomic DNA (RL2810 to RL2832)	Basu et al. 2002
pBS II SK+	2.96-kb phagemid derived from pUC19, <i>lacZ</i> , Amp ^r	Stratagene Corp.
pVV5	pVV1 derivative cloned into pBBR1MCS-3 containing <i>acpXL</i>	Vedam et al. 2003
pDVB1	pBS SK derivative with 2.6-kb insert from pSSB-1 containing <i>lpxXL</i>	This study
pDVB2	pDVB1 containing <i>aacC1</i> cassette from pMS255	This study
pDVB3	pDVB2 derivative cloned into pEx18Tc	This study
pMS255	Gen ^r cassette in pSVB30	Becker et al. 1995
pEX18Tc	Broad-host range suicide vector, Tet ^r <i>sacB</i> ⁺	Hoang et al. 1998
pRK600	Mobilizing plasmid	Finan et al. 1986
pRK2013	Mobilizing plasmid	Ditta et al. 1980

^z Tet^r, Str^r, Kan^r, Gen^r, and Amp^r = resistance to tetracycline, streptomycin, kanamycin, gentamycin, and ampicillin.

of 2 ml of each of the following: 70, 52, 35, 25, and 17.5 sucrose in Tris buffer (50 mM Tris, 10 mM DTT, pH 7.4). The bacteroid filtrate was layered on top of the sucrose gradient and was ultracentrifuged at $100,000 \times g$ for 4 h. The bacteroid layer, a dark pink section between the 70 and 52% sucrose layers, was removed from the gradient, was diluted in 30 ml of buffer (50 mM Tris, 10 mM DTT, pH 7.4), and was centrifuged at $4,000 \times g$ for 30 min. The pellet was resuspended in extraction buffer (0.05 M $\text{Na}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 M EDTA, 0.05% [wt/vol] NaN_3 , pH 7), and was stored at -20°C .

ExN bacterial isolation.

To allow for comparison of strain properties before and after passage through the nodules, for each mutant, isolates were obtained from mature, fully developed root nodules. This isolate is referred to as the ExN isolate. Several nodules were picked from a root system and each nodule was individually washed and surface sterilized with 6% sodium hypochlorite for approximately 30 s and was then washed four times with sterile dH_2O . The individual nodules were crushed with a pair of sterile forceps onto a TY plate with the appropriate antibiotics and were streaked with a sterile loop for individual colonies. The resulting colonies were restreaked for purity and were stored in glycerol stocks. Each ExN isolate was confirmed through correct antibiotic resistance and retaining the antibiotic resistance cassettes within the correct gene through PCR.

Mutant construction.

Plasmid pSSB-1, containing the *R. leguminosarum* chromosomal gene region with the cluster of genes required for VLCFA biosynthesis and attachment to the lipid A (Basu et al. 2002), was used for isolation of the *lpxXL* gene RL2812. For that, the plasmid was digested with restriction enzyme *Pst*I, creating a 2.6-kb fragment harboring *lpxXL* with flanking regions stretching 1,271 bp upstream (including the alcohol dehydrogenase RL2813 and a small fragment of *fabF1XL*) and 423-bp region downstream of *lpxXL* (encoding a small fragment of RL2811, a hypothetical protein). This fragment containing *lpxXL* was cloned into pBlue-script pBSII SK⁺ creating pDVB1. The pDVB1 plasmid was used to transform *E. coli* XL1-B cells and was grown on LB plates with ampicillin, X-Gal, and IPTG. White colonies were selected and restreaked, and the derived plasmid preparations were restriction-digested and viewed on an agarose gel to confirm the presence of the construct.

For *lpxXL* gene interruption, the gentamicin-resistance cassette *aacC1* (Becker et al. 1995) was inserted into *lpxXL*. For that, plasmid pDVB1 was restriction-digested with *Acc*III and *aacC1*-containing plasmid pMS255 with *Xma*I and the fragments were ligated to form pDVB2. *E. coli* DH5 α was transformed with pDVB2 and was selected for growth on LB plates with gentamicin. Transformants were restreaked and the constructs were confirmed through restriction digestion and gel electrophoresis of the isolated plasmid. Both forward and reverse orientations of the gentamicin cassette were obtained and an orientation opposite to the *lpxXL* gene orientation was used.

For *lpxXL* inactivation, *lpxXL* carrying the *aacC1* insertion was cloned into the broad host range suicide vector pEX18Tc (Hoang et al. 1998). The pDVB2 fragment containing *lpxXL::gm* was *Sac*I-digested, gel-extracted, and ligated with the *Eco*R1-digested pEX18Tc to form pDVB3, which was transformed into *E. coli* DH5 α and was selected for antibiotic resistance on LB plates with gentamicin and tetracycline. The correct transformants were confirmed by restriction digestion and gel electrophoresis.

Plasmid pDVB3 was introduced into *R. leguminosarum* bv. *viciae* 3841 and 22 through triparental mating with *E. coli* DH5 α , pDVB3 as the donor, Rlv3841 or Rlv22 as the recipient,

and *E. coli* pRK2013 (Ditta et al. 1980) as the helper. Single recombinants were selected on TY with gentamicin and streptomycin and were then replica-plated onto TY with gentamicin, streptomycin, and 10% sucrose for selection of double recombinants. Three double *acpXL::km lpxXL::gm* mutants were isolated (EL196, DM13, DM15). However, no *lpxXL::gm* single mutants could be constructed. Mutations were confirmed by PCR with primers for *lpxXL* (AATGAGGGTGGACGTTCTTG and GTTCGACTACGATCCCGAAA) and *acpXL* (ATTGCAGAA ACCAGCGAGAT and AGAAACCTTGCTTCGTTGA).

Because we failed to isolate a *lpxXL::gm* mutant, the *acpXL::km*, *lpxXL::gm* double mutant EL196 was partially complemented with the parental *acpXL* (cloned in plasmid pVV5) to create effective *R. leguminosarum lpxXL⁻* mutant EL197, i.e., *acpXL⁻lpxXL⁻acpXL⁺*. The pVV5 plasmid (Vedam et al. 2003) with the *acpXL* gene was introduced into EL196 through triparental mating of *E. coli* pVV5 donor strain, with *R. leguminosarum* EL196 as the recipient, and *E. coli* pRK600 as helper strain. Transformants were selected on TY with gentamicin, kanamycin, and tetracycline. Loss of complementing plasmid pVV5 in lab culture of EL197 and in planta was not observed. Full complementation of the *R. leguminosarum* EL196 mutant was accomplished by the triparental mating of *E. coli* pSSB1 as donor, *R. leguminosarum* EL196 as acceptor, and pRK2013 as helper creating *R. leguminosarum* strain EL198. Transformants were selected on TY agar with tetracycline and streptomycin and were confirmed with PCR.

LPS extraction and purification.

Each mutant and, in addition, a derived ExN isolate from each mutant obtained from the mutant-induced host root nodules (here termed ExN isolates) were grown at 30°C overnight in TY liquid media with added calcium (Noel et al. 1986) and appropriate antibiotics, were collected by centrifugation from 4-liter cultures (approximate $\text{OD}_{600} = 1.0$), were washed with 0.9% NaCl, and were washed $3\times$ with sterile dH_2O , and the pellet was suspended in 20 ml of sterile dH_2O . LPS was isolated by hot phenol-water extraction (Westphal and Jann 1965). Briefly, the cell pellet in 20 ml of sterile dH_2O and 20 ml of hot phenol was heated at 65°C for 45 min, was cooled for 5 min in ice water, was centrifuged (7,000 to 10,000 rpm for 25 min, using a JA-20 fixed-angle rotor) to separate the water and phenol layers, and the water layer was collected. Then, 18 ml of dH_2O was added to the phenol layer and the extraction procedure was repeated twice more, after which pooled water layers and the phenol layers were dialyzed (molecular weight cut-off [MWCO] = 2,000). Following dialysis against dH_2O , LPS was lyophilized and was further purified with RNase, DNase, and proteinase treatments.

For bacteroid LPS isolation, the bacteroid pellet was extracted by micro hot phenol-water extraction (Westphal and Jann 1965). Micro hot phenol-water extractions were completed as described for free-living and ExN bacteria, except volumes of 1 ml were used instead of 20 ml. Water layer LPS was used for all analyses.

LPS characterization.

Isolated LPSs were separated on a DOC-PAGE gel and were visualized using Alcian blue/silver staining as described (Reuhs et al. 1993). One microgram of LPS in loading buffer was loaded into each well of the DOC-PAGE gel and was separated by gel electrophoresis.

To isolate the lipid A component of the LPS, 1 mg of LPS was subjected to mild acid hydrolysis by incubation in 1 ml of 1% acetic acid at 100°C for 2 h. (Caroff et al. 1988). Following hydrolysis, the lipid A was isolated by an initial extraction with 2 ml of MeOH/chloroform (1:1) and two subsequent extractions with 1 ml of chloroform. The chloroform layers containing the lipid A were dried and weighed.

Lipid A preparations were analyzed by GC-MS (combined gas chromatography and mass spectrometry) of trimethylsilyl glycosides and MALDI-TOF MS. Samples were analyzed in a 30-m DB-5 column following methanolysis in methanolic 1 M HCl and trimethylsilylation (Ramadas Bhat et al. 1991; York et al. 1985). The lipid A was also analyzed by MALDI (MS) using an AB SCIEX TOF/TOF 5800 high-resolution mass spectrometer in the negative-ion reflective mode. Lipid A preparations were dissolved in 3:1 chloroform/methanol, and 2 μ l were mixed with 2 μ l of THAP (2,4,6 trihydroxyacetophenone monohydrate) and were spotted on a plate for mass analysis.

Mutant sensitivity phenotype analysis.

To assess osmotic tolerance of the bacterial strains, rhizobial cells were streaked onto TY plates containing 0.25, 0.50, and 0.75% NaCl. Sensitivity to detergents was determined by streaking strains on TY agar plates containing SDS at 0.17, 0.35, 0.52, and 0.69 mM or DOC at 0.51, 0.64, 0.76, 1, 2, and 3 mM. Following 3 days of incubation at 30°C, the relative amounts of growth were assessed visually and were assigned a + or – value. Resistance to PmxB was analyzed by streaking strains (10 μ l of a liquid culture at OD₆₀₀ = 0.2) onto TY gradient plates containing 0 to 50 μ g of PmxB per milliliter.

Cyclic β -glucan analysis.

Cyclic β -glucans coextract with LPS during phenol-water extraction, thus, the relative amount of cyclic β -glucans can be estimated using a preparation of crude LPS. LPS for each mutant and the parent strain and their ExN isolates was extracted as described above from 1-liter cultures (OD₆₀₀ of approximately 1.0) and was dialyzed with MWCO = 1,000. The amount of total carbohydrate in the water layer was analyzed by a phenol sulfuric acid test. For each strain, 100 μ g of lyophilized material extracted into the water layer was diluted in 100 μ l of dH₂O. Then, 100 μ l of 5% phenol in water and 500 μ l of concentrated H₂SO₄ was added to each sample. Samples were heated at 99°C for 5 min and were then cooled to room temperature. Absorbance was measured at 460 nm and total carbohydrate for each sample was calculated, based on a glucose standard curve, from 5 to 60 μ g of glucose. Based on the results from the total carbohydrate analysis, the weight of lyophilized LPS equivalent to 200 μ g of carbohydrates was utilized for linkage analysis.

Linkage analysis of neutral sugars (particularly 2-Glc) was determined using the Ciucanu permethylation method (Ciucanu and Kerek 1984), and conversion to partially methylated alditol acetates was determined as described previously (Darvill et al. 1985). Analysis was performed using combined GC-MS. It is expected that the 2-Glc analyzed was present within the periplasm and was not secreted 2-Glc, due to washing of the cells removing secreted 2-Glc prior to LPS isolation. A known inositol standard was used for quantification of 2-Glc.

Acetylene reduction assay.

To assess nitrogen fixation, the amount of acetylene reduced to ethylene was determined. For each plant, the root system was removed at the seed coat and was placed inside a 100-ml glass bottle with a septum stopper. Acetylene gas (1 ml) was injected into each bottle. After 1 h, 0.3 ml of gas was removed from the bottle through the diaphragm and was injected into a gas chromatograph (isothermal at 70°C, with a 25 m by 0.53 mm, 10 μ m Al₂O₃/KCl column). The ratio of ethylene to acetylene (area under the peak) was calculated for determination of acetylene reduction.

Nodule preparation for microscopy.

Plant roots were removed from Fahraeus medium and smaller sections of roots with nodules were harvested. Nodules

to be used for confocal microscopy were placed into a 4% formaldehyde, 100 mM PIPES fixative solution, and nodules to be used for transmission electron microscopy (TEM) were placed into a 4% formaldehyde, 1% glutaraldehyde, 100 mM PIPES fixative solution. Under a stereo microscope, nodules were dissected longitudinally perpendicular to the outgrowth at the root, with a double-edged razor blade, while submerged in the fixative solution, and a section containing the middle of the nodule attached to the root was collected. Sections were transferred to 8 ml of fixative in a scintillation vial and were fixed overnight at 4°C with gentle agitation.

TEM.

Nodule sections fixed in 4% formaldehyde, 1% glutaraldehyde, and 100 mM PIPES were rinsed 3 $\times$ for 15 min with distilled water and were postfixed in 1% osmium tetroxide for 2 h, with rotation. Postfixed samples were rinsed 3 $\times$ for 15 min with distilled water with rotation. Samples were then dehydrated stepwise in solutions of acetone in water (10, 30, 50, 70, 90, and 100% with 100% repeated a second time) for 30 min each, while rotating at room temperature and were subsequently infiltrated stepwise with graded solutions of Epon Araldite (25, 50, 75%, in acetone and 100% with 100% repeated a second time) for 1 h each, with rotation at room temperature. The final 100% Epon Araldite infiltration resin was held overnight, with rotation, at room temperature. The next day, the resin was replaced with fresh 100% Epon Araldite for infiltration with rotation, for 8 h. Resin was, again, replaced with 100% Epon Araldite and was transferred with nodule sections into an aluminum weigh boat and was polymerized at 65°C for 72 h. Embedded nodule samples were cut out and were mounted onto acrylic mounting stubs for trimming on a microtome. Once trimmed, 70-nm parallel sections were cut with a diamond knife and were collected onto gold hex mesh grids. Grids were submerged into 2% aqueous uranyl acetate (Catalano et al. 2004) for 15 min and were rinsed eight times with distilled water. Sections were stained with lead citrate for 10 min and were washed eight times with distilled water (Reynolds 1963). Once dry, grids with sections were carbon coated. Sections were imaged with a Zeiss Libra 120 TEM at 80 kV.

Nodule and bacteroid measurements.

Nodule size was determined from five to 15 pea plants per bacterial strain. All nodules on each plant root system were visually categorized as immature (small, white) or mature (large, pink). TEM images were analyzed with ImageJ. Statistical significance was determined using the GLM procedure of SAS. Mean separation was conducted by analysis of variance and Tukey's honestly significant difference test. Significance was determined at $P < 0.05$.

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