UNDERSTANDING THE ROLE OF 27-HYDROXYOCTACOSANOIC ACID, A LIPID A COMPONENT OF RHIZOBIAL LPS IN THE RHIZOBIUM-PEA SYMBIOSIS.

by

VINATA VEDAM

(Under the Direction of Russell W. Carlson)

ABSTRACT

The initial stages of the *Rhizobium*-legume symbiosis involve several processes of finely tuned exchange of molecular signals, and ultimately result in the formation of root nodules on the host plant, where atmospheric nitrogen is fixed. Proper establishment of the symbiotic process is dependent on the structural uniqueness of surface molecules of the bacterium such as lipopolysaccharides. The gene encoding the specialized acyl carrier protein (acpXL) responsible for transferring 27-hydroxyoctacosanoic acid in *Rhizobium leguminosarum* was mutated in this study. The physiological and symbiotic phenotype of this mutant strain was characterized. Although the lab-grown mutant strain lacked this residue on the lipid A region, analysis of bacteroids from nodules infected with the mutant strain revealed reappearance of the fatty acid molecule. This is indicative that an *in planta* condition is capable of turning on an alternate pathway which is independent of the acpXL machinery for the addition of the 27-hydroxyoctacosanoic acid molecule. The effect of the mutation was perhaps most strongly observed at the ultrastructural level, which revealed misshaped bacteroids that were defective in their ability to divide synchronously with the symbiosome membrane hence resulting in multiple

bacteroids per membrane. Also, the inability of the mutant strain to grow on media with high osmolarity indicates defective/altered outer membrane properties.

INDEX WORDS: Lipopolysaccharides, LPS, *Rhizobium leguminosarum*, *Rhizobium etli*, lipid A, bacteroids, 27-hydroxyoctacosanoic acid, acyl carrier protein, acpXL

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DEDICATION

To my parents without whose blessings and support this thesis would not have been possible, for encouraging me, and for having been there in times when I needed them the most.

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CHAPTER 1

INTRODUCTION

Plant sources provide about 80% of the caloric and dietary protein needs of the people living in tropical countries, and the subtropical regions of Asia, Africa, and Latin America. Of the ever increasing world population, over 90% is estimated to reside in the tropics and the subtropics by the year 2035 (Waggoner, 1994). This will undoubtedly put a great pressure on the world's food resources. Nitrogen is the major limiting nutrient for most crop plants. Next to photosynthesis, acquisition of nitrogen is the second important function for plant growth and development (Vance, 1998). Production of high quality protein-rich food is entirely dependent on the availability of nitrogen. Application of nitrogen fertilizer is one method of providing plants the necessary nitrogen. The use of such fertilizers has increased tremendously in the developed countries, and has contributed to the increase in greenhouse gases, depletion of nonrenewable resources, imbalances in the atmospheric nitrogen cycle, and leaching of nitrites into the groundwater (Kinzig, 1994). In contrast, the high cost of nitrogen fertilizers, its production and transportation limit their use in developing countries. The use of nitrogen-fixing species in the fields reduces the use/requirement of fertilizers, thereby making farming practices both economically viable, and also environmentally friendly (Bohlool, 1992), (Vance, 1995). The primary source (80%) of biological nitrogen fixation in the agricultural environment is by the *Rhizobium*-legume symbiosis (Vance, 1996). The ecological niche for the *Rhizobium*-legume symbiosis is usually the root nodule. Root nodules are highly organized, hyperplastic tissue

masses derived from root cortical cells (Vance, 1996), (Hirsch, 1992). Nodules are grouped into two major categories depending on their meristematic activity, and fixed nitrogen transport products. Elongate nodules with indeterminate apical meristematic activity that transport fixed nitrogen as amides (alfalfa, pea and clover), and nodules that are spherical in shape with determinate internal meristematic activity that transport fixed nitrogen as ureides (soybean and bean) (Vance, 1998).

One of the important goals for agriculture is to enhance the use and improve the management of legume biologically fixed nitrogen, especially for economic reasons (Vance, 1998). Hence, the study of the *Rhizobium*-legume interaction is important not only for the basic understanding of the symbiosis, but also for agricultural and economic reasons.

In addition to the evident agronomic implication of this area of research, the *Rhizobium*-legume symbiosis is an exceptional model system to study the molecular basis in which a Gram-negative bacterium infects a eukaryotic cell. Since the *Rhizobium*-legume interaction involves a number of fundamentally significant cellular processes such as cell-cell recognition between a prokaryotic and eukaryotic cell, regulation/suppression of the plant defense response in order to facilitate symbiotic infection, and bacterial as well as plant cell differentiation processes (details are provided in the following sections). For Gram-negative bacterial pathogens, the lipopolysaccharide layer (LPS), which comprises the outer leaflet of the bacterial outer membrane, is crucial for establishing infection. In the case of enteric bacteria, the hydrophobic anchor of the LPS (lipid A/endotoxin) activates the host innate immune response (Raetz, 2002). The characteristic structural features of the *E. coli* lipid A, e.g. the two phosphate groups and the two fatty acyloxyacyl molecules are required for eliciting the endotoxic response in human cells (Raetz, 2002) (a detailed description of LPS structure is provided in the next chapter). There are

similarities between symbiotic and pathogenic interactions with regard to LPS acting as a virulence factor. Clearly, LPS plays a key role in determining virulence. An understanding of the structure-function of the LPS molecule will help us comprehend the role of LPS in invasion, and survival of Gram-negative bacteria in their host cells. This dissertation focuses on a single, unique component of the LPS from all members of the *Rhizobiaciae* which current data suggest is important in the infection of the host legume cell; a very long chained ω-1 hydroxy fatty acyl component known as 27-hydroxyoctacosanoic acid (27OHC28:0).

CHAPTER 2

LITERATURE REVIEW

An overview of the role of lipopolysaccharides (LPSs) in the *Rhizobium*-legume symbiosis:

Members of the family *Rhizobeaceae* specifically five genera; *Rhizobium, Bradyrhizobium, Azorhizobium, Sinorhizobium* and *Mesorhizobium* and *Allorhizobium* (Rivas, 2002), (D'Haeze, 2002) are Gram-negative bacteria often found in association with plants and are capable of forming nitrogen-fixing symbioses with members of the *Fabeaceae* (the legume family) (Sprent, 1990), (VanBerkum, 1998). The *Rhizobium*-legume symbiosis has attracted study ever since Beijerinck (1887) demonstrated that bacteria were responsible for nodule formation (Long, 2001). Furthermore, since the identification of rhizobia as the source of fixed nitrogen in root nodules of legumes by Hellriegel and Wilfarth (1888), it has been speculated whether plants outside the family *Fabeaceae* could be manipulated to associate with rhizobia (Hirsch, 2001).

The *Rhizobium*-legume interaction is very specific, wherein certain rhizobial strains nodulate only specific legumes. This interaction begins with two free-living organisms and ends with their intimate co-existence. Bacteria are able to recognize certain plant signals, flavanoids, and induce the formation of a root nodule. Rhizobia invade the plant root tissue, and after establishing themselves in the root host cell, a nutrient exchange occurs in which the rhizobia deliver fixed nitrogen to the plant and receive carbon compounds from the plant (Long, 2001).

This molecular signaling between the plant and bacterium is quite complex, is dependent on several factors, and will be discussed in the following sections.

Prior to, and during the early stages of symbiosis, there is an intimate association of the bacterial outer surface with the roots of the host plant (Denny, 1995), (Schletter, 1995), (Leigh, 1992). In order to better understand the molecular basis of this interaction, and how the bacterium responds to different environments (rhizoshpere and *in planta*), it is essential to characterize the structures of the bacterial outer surface molecules. The outer surface of gram-negative bacteria consists of a number of different molecules. The carbohydrate molecules include the lipopolysaccharides (LPSs), capsular polysaccharides (CPSs), cyclic glucans, and extracellular polysaccharides (EPSs). Also present are porins, fimbriae and flagella. Additionally, rhizobia produce lipo chito oligosaccharides (LCOs) in response to the plant flavanoid signal molecules (Kannenberg, 1998). LPSs are complex glycoplipids that make up the outer leaflet of the bacterial outer membrane. The acidic CPSs are called K-antigens. These polysaccharides are believed to protect bacteria from host defense responses and are essential for pathogenic virulence (Kannenberg, 1998). The rhizobial K-antigens are distinguishable from other surface polysaccharides. The CPS of a rhizobial strain is different from its EPS as it is not excreted into the medium, is antigenic, and has a different structure (Kim, 1996), (Petrovics, 1993), (Reuhs and and Kim, 1993). The CPSs are also structurally distinct from LPSs, as they contain a higher ratio of Kdo (or a Kdo variant), and are not attached to a lipid-A anchor (Kannenberg, 1998). Cyclic ß- (1,2) glucans are cell-associated molecules that are known to provide functions for the free-living forms of the rhizobia during osmotic adaptations, and during the process of plant infection (Breedveld, 1998). EPSs are bacterial polysaccharide molecules that are excreted from the cell (Becker, 1998). They offer protection to the bacterial cell from environmental influences.

They also contribute to surface attachment, nutrient gathering, antigenicity, and, in many cases, symbiotic infection (Becker, 1998).

In general, an LPS can be divided into three structural regions: O-chain polysaccharide, core oligosaccharide, and lipid-A. The O-chain polysaccharide is a polymerized, repeating oligosaccharide, which is attached to the core region. The core oligosaccharide in turn is attached to a glucosamine residue of the lipid-A. The residue involved in this oligosaccharide-lipid linkage is 3-deoxy-D-*manno*-2-octulosonosyl (Kdo). The lipid region commonly consists of a disaccharide sugar backbone with glucosamine residues, which are both O- and N-fatty acylated. The lipid-A region serves as an anchor for the entire molecule in the bacterial outer membrane (Kannenberg, 1998). A detailed discussion of the LPS structure of *Rhizobium leguminosarum* will be provided in the next section.

The research reported in this thesis focuses on the LPS of *Rhizobium leguminosarum* 3841, a pea nodulating strain. More specifically, it focuses on determining the biosynthetic and symbiotic function of a single unique residue in its LPS; a very long chain fatty acid component, 27-hydroxyoctacosanoic acid (27OH-C28:0), of the lipid-A portion of the LPS.

Rhizobium **LPS mutants in nodule development:**

In order to assess the possible functions of LPS in nodule development, the symbiotic phenotypes of several mutants with defective LPSs have been analyzed. A majority of *Rhizobium* LPS mutants do indeed form nodules (Noel, 2000). Many instances have been reported however, wherein nodule development is severely impaired by LPS mutants, often resulting in Fix^- or strongly reduced nitrogen-fixing abilities (Kannenberg, 1998). The phenotype of LPS defective mutants depends to some extent on whether the host legume forms determinate or indeterminate

nodules. The most severe phenotypes have been reported for LPS mutants that are symbionts of hosts that form determinate nodules. An example is a mutant of a *B. elkanii* strain 62A101c that lacks the O-chain moiety of the LPS, and has several symbiotic phenotypes, depending on which variety of soybean is chosen as the host plant. On a number of soybean varieties, the mutant does not induce any nodules, and on others it causes white empty bumps with severe abnormalities (Kannenberg, 1998). *Rhizobium etli* mutants lacking the O-chain polysaccharide are symbiotically defective in that they induce small white nodules on bean hosts, which are devoid of leghemoglobin (Noel, 1986). In these nodules, a nodule meristem is formed, and development seems normal for the first few days, but becomes aberrant later on, with central, instead of normal peripheral vascular bundles. The infection is aborted in the root hair cells in bloated infection threads, and the bacteria are embedded in a matrix structure. The molecular basis for this deterioration is not known (Noel, 1986). On hosts forming indeterminate nodules, *R. leguminosarum* bv. *viciae* LPS mutants completely lacking the O-chain polysaccharide develop nodules with abnormal infection threads, and only sporadically release the bacteria into the nodule cells (Perotto, 1994), (deMaagd, 1989), (Priefer, 1989). The resulting bacteroids senesce early, and fix little to no nitrogen. Cytological analysis in pea nodules show that the walls of infection threads have secondary modifications, and probably callose depositions, accumulation of intercellular matrix composed of plant derived glycoprotein, and sporadic cell death. These responses have been thought to be mild plant host defense reactions (Perotto, 1994). The LPS-defective mutants that were released into host pea root cells differentiated into abnormally swollen, and elongated bacteroids. In these cases, rhizobial cell division is impaired, and synchronous growth and division of the bacterial and peribacteroid membranes (which normally occurs during development of indeterminate nodules in peas) is disrupted (Perotto, 1994). In pea

root nodules, the colonization of plant cells by rhizobia relies in active growth and branching of transcellular infection threads, which deliver the bacteria to newly formed cells. If plasticity of the infection thread wall is decreased by secondary metabolites like lignins, both growth and branching are affected, and the number of nodule cells reached by the infection threads will be much less than normal. Hence, abnormal infection threads and impaired tissue invasion by these LPS-defective bacteria may be the consequence of a plant defense response triggered by mutants defective in LPS macromolecules (Perotto, 1994). The involvement of LPS mutations in eliciting host defense responses can be explained variously. One likely explanation is that the region of the LPS that is modified/absent in the mutant may contain a positive signal required for recognition by the plant to promote development of a symbiotic rather than a pathogenic interaction (Kannenberg, 1998). Alternatively, the O-chain polysaccharide maybe needed to shield the core region, which may otherwise be a determinant for pathogenicity and elicit a defense response when it comes in contact with the plant cell surface (Kannenberg, 1998). Yet another plausible theory is that the altered LPS structure of the LPS-defective mutants could act indirectly to release/expose a metabolite that elicits the host defense response.

It is difficult to make strong conclusions regarding the specific roles of rhizobial LPSs based on these, and other examples of mutant LPS phenotypes on host plants. Mutants such as those discussed above with major defects in their LPS structures could be pleiotropic with regard to their entire surface. The removal of the O-chain polysaccharide moiety exposes the other outer membrane components of the bacterium to the plant cell, and the interaction of these components (LPS core, outer membrane proteins, phospholipids, etc) may be the cause for the damaging effect on symbiosis. These mutants also probably have weak and leaky membranes, allowing for the entry and exchange of molecules that may be potentially harmful for the bacterium, and

therefore to the symbiosis. Despite these issues, results obtained by the study of the mutants such as described above have provided a platform for a better understanding of the requirement for LPS in symbiosis. Thus, although rhizobial LPSs have been shown to be crucial for symbiotic infection, their precise function(s) during this process of symbiosis is not clearly understood (Stacey, 1991), (Priefer, 1989), (deMaagd, 1989), (Perotto, 1994), (Cava, 1989), (Kannenberg, 1998), (Noel, 1986). However, it has been concluded that for proper function in symbiosis, complete LPS molecules composed of a sizeable O-chain, core oligosaccharide, and lipid A are needed (Noel, 1986) , (Kannenberg, 1992).

The structure of rhizobial LPSs:

In addition to the requirement of a structurally complete LPS for symbiosis, the literature also shows that a *Rhizobium* has the ability to alter its LPS in response to the environment of the host. This literature is described in section C below; however, before reviewing these data, it is necessary to discuss the structural details of the rhizobial LPS.

The techniques used to elucidate rhizobial LPS structures are similar to those employed for the enteric bacteria. LPSs are in general complex molecules and are structurally very heterogeneous, and hence present several difficulties for structural analyses. The analysis is complicated further, due to the fact that rhizobial LPSs do not have some of the common structural features found in the enteric LPSs (Kannenberg, 1998). Nevertheless, all rhizobial LPSs that have been examined so far seem to share the same overall structural motif as the enteric bacteria.

The main aim of this discussion is to delineate the structure of the *Rhizobium leguminosarum* LPS, since this is the organism used in the research presented in this thesis.

Since LPSs are complex structures, a single preparation usually contains a heterogeneous mixture of molecules due to various lengths of the O-chain polysaccharide, and variation in the glycosyl and fatty acyl residues of the lipid-A region. Molecules with the O-polysaccharide are called smooth LPS, or LPS I. Apart from these components, there are molecules which lack the O-polysaccharide called rough LPS, or LPS II. Molecules that have truncated O-chains (specially LPS mutants) of various lengths are called semi-rough LPSs, or LPS III, IV, V etc. The complete structure of the lipid-A core region of *R. etli* has been elucidated and is identical in the LPSs from strains of *R. leguminosarum* biovars viciae, trifolii, phaseoli, and *R. etli* (Bhat, 1994b), (Raetz, 1993), (Price, 1995).

The best-characterized rhizobial LPSs are those of *R. etli* and *R. leguminosarum* strains. The structure of the lipid A region is shown in Fig. 1. The lipid A from was isolated after mild acid hydrolysis of the LPS and its structure determined. This lipid A has a very unusual structure when compared to that from many other bacteria, specifically those from the closely related enteric bacteria such as *E. coli* (Bhat, 1994b), (Que, 2000b;Que, 2000a), (Que, 2000b). It is devoid of phosphate, contains only hydroxy fatty acids, and contains 27OH-C28:0 as the single acyloxyacyl residue. It has a GalA residue at the 4'-position instead of phosphate, and a 2-aminogluconate instead of GlcN-1-phosphate. Both the GlcN and the GlcN-onate residues are N-acylated at the 2-position, and O-acylated at the 3-position with β-hydroxy fatty acids of various lengths. Fatty acid analysis of the lipid A of *R. etli* revealed the presence of five fatty acids: 3-OH-C14:0, 3-OH-C15:0, 3-OH-C16:0, 3-OH-C18:0, and 27-OH-C28:0.

The organization of the core oligosaccharide and the point of attachment of the O-chain in the LPS (Fig. 2) was established by the analysis of LPSs from mutants completely lacking in the O-chain, or having severely truncated forms of the O-chain (Kannenberg, 1998). The core

(this links the lipid A to the O-chain polysaccharide) is a complex, branching octasaccharide that contains three 3-deoxy-D-*manno*-2-octulosonoysl (Kdo), three GalA, one mannose and one galactose residues (Forsberg, 1998) (Fig. 2). In the case of *R. etli* CE3, there is an outer core at the reducing end of the O-chain polysaccharide that is attached to the external core Kdo residue (see Fig.2). This outer core region of the *R. etli* CE3 LPS consists of an N-acetylquinovosamine (QuiNAc), Man, and fucose (Fuc) trisaccharide (Forsberg, 1998). The core structure of *R. etli* CE3 and *R. leguminsoarum* 3841 are identical based on High Performance Anion-Exchange Chromatography HPAEC results, NMR analysis, and their reaction with monoclonal antibodies (Forsberg, 1998), (Kannenberg, 1996), (Carlson, 1995).

The O-chains of *Rhizobium leguminosarum* vary from strain to strain, and consist of a polymerized repeating oligosaccharide composed of deoxy and methylated deoxyglycosyl residues (Kannenberg, 1998). Additionally, O-polysaccharides are often highly acetylated, making them very hydrophobic. A unique feature of the *R. leguminosarum* O-chains isolated after mild acid hydrolysis of the LPS is that they have a Kdo residue at their reducing ends, a feature not found in the enteric LPSs. This is the core external Kdo residue, Kdo III, and thus far is uniquely found as part of the inner core of *R. leguminosarum* and *R. etli* (see Fig. 2). Only a few rhizobial O-chain polysaccharide structures have been reported. As an example, the O-chain of *R. etli* CE3 (LPS I) consists of five repeating trisaccharide units made from a Fuc-GlcA disaccharide backbone with a terminal 3-methyl-6-deoxytalose (3Me-6deoxyTal) attached to each Fuc residue (Forsberg, 1998). The O-chain polysaccharide terminates with a 2,3,4-tri-O-methyl fucose (Forsberg, 1998). In some strains, the number of O-chain repeats varies greatly, and results in a “ladder” pattern on PAGE gels (Carlson, 1984). For other strains, the number of repeats is not as variable, and hence PAGE analysis results in only a few major bands. Gram-

negative bacteria can vary their O-chain polysaccharide structures in response to environmental conditions (Whitfield and and Valvano, 1993).

Rhizobium **alters its LPS structure in response to the plant host cell environment:**

LPS epitope changes in rhizobia during symbiosis:

Investigations into LPS changes of animal pathogens have suggested that they respond to different environments by structurally modifying their LPSs (Kannenberg, 1998). The plant host also provides *Rhizobium* with different micro-environmental conditions, and these environmental challenges result in several surface changes in the *Rhizobium* LPS (Kannenberg, 1998). Detailed biochemical studies using monoclonal antibodies (Mabs) have been performed in nodule and free-living bacteria to investigate any subtle structural changes in the LPS during symbiosis, and under culture conditions of low pH and oxygen, which are thought to mimic the nodule environment. The Mabs were developed to have specificity for the O-chain (Brewin, 1986), (Kannenberg, 1992), or the core region of the LPS (Lucas, 1996). The results of these studies have indicated that there are indeed changes in LPS epitope expression during symbiosis (Kannenberg, 1998), and these changes in LPS epitope expression occur mainly in the O-chain region. It was shown that different types of LPSs can be expressed during symbiosis, and this depends on the type of environment that the bacteria are exposed to (Kannenberg, 1998), (Kannenberg, 1989), (Brewin, 1986), (Kannenberg, 1992), (VandenBosch, 1989). LPS epitope expression has been most extensively investigated in *R. etli* and *R. leguminosarum* strains, and a few important conclusions have been drawn. LPS epitopes are expressed either constitutively, or

in a regulated fashion, and have been grouped together in various classes. The types of LPS modifications depend on whether or not the host forms determinate or indeterminate nodules. In pea nodules induced by *R. leguminosarum* 3841, VandenBosch et al., showed that an epitope on the O-chain (recognized by MAC 203) is expressed in bacteroids after release into the plant cytoplasm, and before the expression of nitrogenase (VandenBosch, 1989). Kannenberg et al., showed that constitutive epitopes were expressed through all the invaded zones of the nodule. Further, regulated LPS epitopes followed either a radial pattern of expression, or a linear axis of symmetry along the axis of nodule development (Kannenberg, 1994). Most of the changes in LPS epitope expression occurred after bacterial release into the symbiosome during bacterial multiplication, suggesting that LPSs expressed by bacteria within the infection threads (i.e. prior to release into the symbiosome) resembled those of free-living bacteria (VandenBosch, 1989). The mechanism by which the LPS epitope changes are regulated is not yet understood, and the structures of the different epitopes have not been established. It can be concluded that there are a number of factors involved in regulating and producing the different LPS structural variations (Kannenberg, 1998), and that LPS expression and adaptation is generated via changes by both plant-specific physiological conditions and factors .

Changes in LPS hydrophobicity in Rhizobium leguminosarum during bacteroid development:

In a study by Kannenberg et al. (Kannenberg, 2001), modifications to the LPS structure caused by varying growth conditions (namely standard, acidic, and reduced oxygen) of *R. leguminosarum* 3841 were investigated by biochemical methods. The LPSs were extracted by the hot-phenol water method (Westphal, 1965), and chemically analyzed. Results showed that the LPS of *R. leguminosarum* 3841 grown under standard conditions was predominantly hydrophilic, and was found in the water phase after the extraction procedure. In contrast, the LPS

of *R. leguminosarum* 3841 grown in low oxygen conditions was mainly hydrophobic, and extracted into the phenol phase (Kannenberg, 2001). The core region of the LPS from these bacteria grown under different conditions was structurally unaltered, and this suggested that the structural modifications should occur in the O-chain and/or the lipid A regions. Analysis of the lipid A region revealed a dramatic increase (two to three-fold) in the amounts of the long chained fatty acid (27OHC28:0) (Kannenberg, 2001).

The hydrophobic LPSs from cells grown under low-oxygen conditions resembled those from nodule bacteria, implying that the structural modifications in the LPSs from the low-oxygen grown cells occur during cell differentiation from free-living rhizobia to bacteroid forms. The expression of hydrophobic forms of LPSs during symbiosis is perhaps an indication of the need for LPS structural changes, as well as overall surface properties, and may support the possibility that the rhizobial LPS is part of a required, non-specific hydrophobic surface interaction (Kannenberg, 2001). In the study by Kannenberg et al, it was observed that nodule bacteria adhered to hydrocarbons significantly more than the bacteria cultured under standard conditions. This indicated that there was either an overall increase in cell surface hydrophobicity, or that there were regions/areas in the cell surface that have increased hydrophobicity and adhesion properties (Kannenberg, 2001). Such hydrophobic areas on the bacteroid surface could play an important role in the plant-bacterium attachment through proximity, and adhesion at the plant-bacterial interface (Kannenberg, 2001). In such a case, the interaction is likely to affect a number of symbiotic functions. These include the endocytotic invasion step in which the rhizobia enter the plant cells, synchronous division in the symbiosomes, and transfer of important compounds such as nitrogen and carbon dioxide (Kannenberg, 2001). The chemical modifications that are

involved in the formation of the hydrophobic LPSs, and their actual role in symbiosis are still unclear.

Biosynthesis of rhizobial lipid A (*R. etli* and *R. leguminosarum*):

It is clear from the above discussion that LPS plays crucial, if yet unknown, roles in *Rhizobium*-legume symbioses. Changes occur to both the carbohydrate and the lipid-A portions of the LPS that may be essential for proper symbiotic infection. Since the topic of this thesis is in regard to the function of a unique rhizobial lipid-A component, 27OHC28:0, it is essential to briefly discuss the initial common biosynthetic pathway of the rhizobial and enteric (*E. coli*) lipid As, and the subsequent divergence of this pathway in *R. leguminosarum* that leads to its final unique lipid A.

In gram-negative bacteria, UDP-GlcNAc is a precursor of the disaccharide backbone of lipid A. The biosynthetic pathway has been well studied, and proceeds via 3-O-acylation of UDP-GlcNAc, followed by deacetylation, N-acylation, disaccharide formation, 4' phosphorylation, Kdo addition, and late acylation (Fig. 3). However, not much is known regarding lipid A biosynthesis in gram-negative bacteria with novel lipid A structures Biosynthesis of the lipid A region of the *E. coli* LPS is essential for its viability (Raetz, 1993). The lipid A portion from enteric bacteria is responsible for triggering the endotoxic response in human cells. Structural features of the lipid A that are essential for this toxicity include the presence of a glucosamine disaccharide backbone, phosphate groups, and certain fatty acyl residues (Kannenberg, 1998). As described in the previous section, the lipid A from *R. leguminosarum* is nonphosphorylated, and contains 2-deoxy-2 aminogluconic acid in place of the reducing glucosamine residue on the *E. coli* lipid A. Additionally, *R. leguminosarum* contains

only one acyloxyacyl residue, 27OH-C28:0. Galacturonic acid is linked to the 4' position of the non-reducing glucosaminoglycosyl residue instead of the phosphate as in the *E. coli* lipid A. These structural differences lead to the following question: Is there a distinctly different biosynthetic pathway for the unique rhizobial lipid A structure? The details of the lipid A biosynthetic pathway in *E. coli* have been extensively worked out by Raetz and co-workers (Raetz, 1993). In *E. coli*, the steps leading up to the synthesis of Kdo_2-lipid-IV_A are essential for cell viability, and the presence of the 4' phosphate is required for the transfer of the two Kdo residues from CMP-Kdo to lipid IV_A. After the synthesis of Kdo_2-lipid-IV_A, further processing occurs and the acyloxyacyl fatty acids are added to form the mature *E. coli* lipid A (Kannenberg, 1998). In spite of the unique rhizobial lipid-A structure compared to that of *E. coli*, it was shown that *R. leguminosarum* contains the same enzymes as *E. coli* that can convert UDP-GlcNAc into Kdo_2-lipid-IV_A (Price, 1994). This result indicated that *R. leguminosarum* has unique enzymes, as shown in Fig.3, which are able to convert the Kdo_2-lipid-IV_A precursor into the mature rhizobial lipid A structure. Hence, *R. leguminosarum* should possess: phosphatases (both 4' and 1), an oxidation system which converts the reducing end glucosamine to 2-aminogluconate, a transferase which transfers the GalA residue to the 4' position, and a unique acyl transferase for the incorporation of the 27OHC28:0 residue. All of these enzymes (except the GalA transferase) that are involved in the synthesis of the rhizobial lipid A have been identified (Kannenberg, 1998), (Basu, 2002).

Biology of the *Rhizobium*-legume symbiosis:

During nodulation, the rhizobia and their host plants are in intimate contact with one another. This interaction ultimately results in the formation of the nodule; a structure where the

bacteria are enclosed in ‘infected cells’. The host root cells are packed densely with infecting bacteria however, there is no obvious defense response. This lack of defense response is most likely mediated through (controlled via) surface determinants of the rhizobia. The surface molecules that are likely candidates are LPSs and EPSs, as shown by mutant analysis (Hadri, 1998). The possible roles of LPS in the various stages of symbiosis are discussed in a later section.

Steps leading up to formation of a mature nodule -

Formation of infection threads and growth: Curling of root hairs precedes their invasion by rhizobia. The curled root hair is further distorted into a ‘Shepherd’s crook’ (Hadri, 1998). This final curling does not occur as a result of the presence of Nod factors alone, and seems to depend on the presence of live rhizobia on the root hair surface. There is a pocket formed by the curled root hair, and within this pocket bacterial intrusion occurs (Brewin, 1998a). Bacteria enter the root hair by an apparent weakening of its cell wall, and further, by invagination of the plant membrane (Brewin, 1998a). This is followed by the formation of an infection thread, which is formed by the deposition of plant cell wall materials (van Spronsen, 1994), (Newcomb, 1985) (see Fig.4). The wall of the infection thread prevents direct contact between the bacteria, and the plant cell membrane (Brewin, 1998a). In this way, a tube-like structure is formed by which the bacteria enter the plant. The growth of infection threads is hypothesized to be by vesicle incorporation at the tip, akin to pollen tube growth. Infection threads grow across cytoplasmic space, and then fuse with the mother cell wall, releasing contents of the lumen into the intercellular matrix (Brewin, 1991). The process of transcellular infection repeats itself into the post-meristematic cells. In mature indeterminate nodules such as peas, there is a sub-meristematic zone, and the invasion zone where branched infection threads pass through and into

recently divided cortical cells, thus, generating the central, invaded tissue zone where nitrogen fixation occurs. Transcellular infection threads are not observed in determinate nodules such as those formed on *Phaseolus vulgaris* (Brewin, 1998a).

Nodule initiation and formation: Nodule primordia are formed in the inner layers of the root, and hence the infection thread (IT) must pass through several layers of root cortical cells. In order to accommodate the polar growth of infection threads, the cytoskeleton of the root cortical cells has to modified suitably (Gualtieri , 2000). Briefly, before penetrating the cortical cells, the nucleus moves to the center of the cell and the cytoplasm and microtubules rearrange to form a 'cytoplasmic bridge'. These bridges are more or less radially aligned in the cortex, and guide the infection thread to the nodule primordium. The cytological changes indicate that the cortical cells that are preparing for infection thread penetration enter the cell cycle, although they do not undergo division (Gualtieri , 2000). Tissue and cell invasion is accompanied by the differentiation of host cells derived from the apical meristem.

Endocytosis and bacteroid formation: As the nodule develops, the nitrogen fixing capability of *Rhizobium* is always preceded by the endocytosis of bacteria (Brewin, 1998a). In indeterminate nodules, the endocytosis occurs from unwalled infection droplets that extrude from infection threads. These droplets are bound by a single membrane, which is continuous with the plasma membrane (Gualtieri , 2000). In pea, the symbiosomal membrane divides synchronously with the bacteria so that a symbiosome membrane singly encloses each bacterium (see Fig.5). When the bacteria eventually stop dividing, they differentiate into the fully endosymbiotic nitrogen-fixing form known as bacteroids. This process is associated with a change in bacterial cell morphology to the characteristic "Y" shape (Brewin, 1998a). During the synchronous division of bacteria and the symbiosome membrane, the two membranes are in close contact to one another, which

could be essential for the endocytotic process in pea and possibly other legume hosts, which form indeterminate nodules. The O-antigen of bacterial LPS seems to be involved in this process, since mutants defective in O-antigen production do not undergo normal endocytosis (Perotto, 1994). Intimate surface interactions between the two membranes could explain how the simultaneous division of the symbiosome membrane accompanies the division of bacteroids. In hosts that form determinate nodules, synchronous division between the bacteria and the symbiosome membrane does not occur and, therefore, it is common to observe multiple bacteroids surrounded by a single symbiosome membrane. Close membrane contact between the infecting bacterium and the determinate-nodule forming hosts first occurs within the infection thread and may be the reason why LPS rhizobial mutants of these hosts are defective that this earlier stage of symbiosis than with indeterminate nodule-forming hosts.

The molecular players of Rhizobium-legume symbiosis

LPS does not seem to be absolutely necessary for initiating infection of indeterminate nodules, a conclusion based primarily on the fact that most LPS mutants are able to invade plant host tissue to some degree (Kannenberg, 1998). Bacterial release is a process closely resembling endocytosis. In the infection threads, rhizobia lose their ‘capsule’ (constituted by surface carbohydrates such as EPS or CPS) before or after the process of internalization (Brewin, 1998b). The fact that the number of invaded pea or vetch plant cells is significantly reduced in case of LPS mutant rhizobia is suggestive that LPSs may be involved in cell internalization; however, the mechanisms of LPS involvement remain unclear (Perotto, 1994). Within the symbiosome compartment, rhizobia divide and differentiate into mature nitrogen-fixing bacteroids. Bacteroids that are formed by rhizobia with LPS mutations have been studied in pea nodules (Kannenberg, 1998), and impairments observed have indicated that a complete LPS-I

(i.e. LPS which contains the O-chain polysaccharide) is essential for normal bacteroid development (Perotto, 1994), (Kannenberg, 1992). It is possible that adhesion between the bacterial and the peribacteroid membranes is involved in maintaining the synchronous division process since bacteroids derived from rhizobial LPS mutants show a disruption in this synchrony (Brewin, 1998b). The molecular basis for bacteroid/peribacteroid membrane interaction is not known and requires further investigation (Brewin, 1998b).

Nod factors induce nodule formation. The highly specific exchange of signals between plant host and bacteria result in nodule formation involving "nodulation" genes, which are responsible for the biosynthesis and secretion of these 'Nod factors' (Downie, 1998). The type of infection, and the structural and developmental characteristics of the nodule are specified by the plant, rather than by the rhizobial strain. This is indicative that the host has genetic information for symbiotic infection and nodulation, and that the role of the bacterium is to turn on this program with highly specific signals (J Denarie, 1996). Nodulation genes (*nod, nol,* and *noe*) are required for infection, nodule formation, and control of host specificity (J Denarie, 1996). Regulatory as well as structural nodulation genes are required for signal exchange, which occurs in early symbiosis. All rhizobia studied so far have at least one copy of *nodD*, whose gene-product is a transcriptional activator of the LysR family. In the presence of plant signals such as flavonoids in root exudates, NodD activates the transcription of structural *nod* genes. Activation of regulatory Nod proteins by diverse plant signals constitutes the first level of control of host specificity (J Denarie, 1996), (van Rhijn, 1995). Structural *nod* genes are classified into two types: the 'common' genes such as *nodABC* genes, and 'species specific' genes. The common genes are so called because they are found in all rhizobial species, and play a very important role (their inactivation results in a complete loss of ability to elicit any detectable plant response). The

species-specific genes are present in various combinations in different species or biovars. Mutations in these genes result in an alteration of the host range of nodulation (J Denarie, 1996). Nod factors were found to be amphiphilic, and when analyzed by MS and NMR, were found to be lipo-chitooligosaccharides (LCOs). These molecules have a backbone of β-1,4-linked N-acetyl glucosamine residues in which the terminal glucosamine residue is N-fatty acylated. This chitin oligomer backbone is diversely substituted in various species on the two terminal glucosamine residues (J Denarie, 1996). The *nodABC* genes are required in all rhizobial species for the synthesis of this N-acylated chitin oligosaccharide, and different rhizobial species produce nod factors with either four or five glucosamine residues (J Denarie, 1996). The GlcN N-fatty acyl substitutions differ among species, and seem to contribute to host specificity. In most species, the non-reducing terminal GlcN is N-acylated with a common fatty acid as well as N-methylated. Nod factors cause root hair deformation and nodule formation. However, viable bacteria are required for root hair curling (Hadri, 1998). Root hair deformation starts with a swelling of the tip, and it is likely that nod factor-induced swelling involves growth. Nod factors also induce rapid changes in the root epidermis such as alkalinization and membrane depolarization, followed by calcium spiking (Ehrhardt, 1996), (Ehrhardt, 1992).
In addition, nod factors can also induce preinfection thread formation, thick short root formation, and stimulate cortical cell division, with accompanying starch accumulation, resulting in a nodule.

Biosynthesis of rhizobial fatty acids and the role of acyl carrier proteins:

The cell envelope of Gram-negative bacteria has three distinct layers; the inner/cytoplasmic membrane, peptidoglycan, and the outer membrane. The membranes contain phospholipids, and proteins. The LPS component is found in the outer membrane layer. The

phospholipid composition of the *Rhizobiaceae* is more complex than in *E. coli*. The rhizobial LPSs, as already discussed earlier, are also significantly different than the structures found in *E. coli*. The rhizobia also contain granules of polyhydroxybutyrate (PHB), and these can account for as much as 98% of lipid extracts (Geiger, 1998).

The predominant fatty acids in the *Rhizobiaceae* family include palmitic (16:0), *cis*-palmitoleic (16:1(9*c*)), and *cis*-vaccenic (18:1(11*c*)). This is similar to *E. coli*, however the relative amounts of *cis*-vaccenic acid and the fatty acids derived from it exceed 50% of the total fatty acids in most members of the *Rhizobiaceae*, and hence are much greater than found in *E. coli* (Geiger, 1998). In the course of normal fatty acid biosynthesis, 3-hydroxy fatty acids are also formed. In the *Enterobacteriaceae* 3-hydroxymyristic acid is a common substituent in the lipid-A. The *Rhizobiaceae*, in addition to 3-hydroxy fatty acids, also contain very long chain (ω-1)-hydroxy fatty acids (Geiger, 1998), as previously described. The unusually long 27OH-C28:0 was originally identified as a major structural fatty acyl component in the LPS of *R. leguminosarum* bv. *trifolii* (Hollingsworth, 1989).

The biosynthesis and transfer of fatty acids is very important during the formation of membranes, storage lipids, distinct lipoproteins, or certain amphiphilic signal molecules in Gram-negative bacteria. In higher animals, fatty acid biosynthesis occurs on a multi-enzyme complex. In most bacteria, fatty acids are formed by the catalytic activity of a number of monofunctional enzymes. A small (molecular mass <10 000) protein carries the growing fatty acyl chains via a thioester linkage of a 4P-phosphopantetheine prosthetic group which itself is attached to a conserved serine residue. This protein is called the acyl carrier protein (Acp). Acps are also involved in the transfer of fatty acyl chains during phospholipid, lipid-A, and hemolysin biosynthesis (Geiger, 2002). In *E. coli*, a single, essential protein AcpP, which is encoded by the

constitutive *acpP* gene, performs all of these functions. The biosynthesis of the complex surface molecules of the rhizobia involves several Acps. Besides the constitutively expressed AcpP, three other specialized Acps are reported in rhizobia: (1) NodF-is a flavonoid inducible protein required for nod factor synthesis. (2) AcpXL is required for the synthesis and transfer of 27OH-C28:0 to the sugar backbone of the lipid-A. (3) RkpF is an Acp homologue that is involved in the biosynthesis of capsular polysaccharides (Geiger, 2002).

NodF, found in *Sinorhizobium meliloti*, is an Acp homolog and carries a 4P-phosphopantetheine prosthetic group characteristic of Acps. All rhizobial species that make α, β multiply unsaturated fatty acids contain *nodFE* genes. In addition to *Sinorhizobium* species, *Rhizobium leguminosarum* bvs. viciae, trifolii, *R. galegae*, and *Mesorhizobium huakuaii* all produce fatty acyl residues with unusual α, β unsaturation (Lerouge, 1990), (Schultze, 1992), (Spaink, 1991), (van der Drift, 1996), (Yang, 1999), (Poinsot, 2001). In addition to NodF, the NodE protein is required for the synthesis of the α, β multiply unsaturated fatty acids are essential for the formation of nodules on legume roots of plants belonging to the galegoid family (Geiger, 2002).

As discussed in the previous section, the lipid-A of all examined members of the family *Rhizobiaceae*, with the possible exception of *Azorhizobium caulinodans*, contain 27OHC28:0 (Bhat U.R., 1991). A specialized Acp, AcpXL, is required for the synthesis and transfer of this fatty acid residue onto the lipid-A precursor, Kdo_2-$lipid_{IV}A$ (Brozek, 1996). A unique transferase, LpxXL, transfers 27OH-C28:0 from AcpXL onto the hydroxy group of the β-hydroxy fatty acid attached to the 2' position of the Kdo_2-$lipidIV_A$ (Basu, 2002).

The RkpF Acp homolog was found in the *fix-23* locus of *S. meliloti* strain 41 that is involved in the production of Kdo-rich CPS that can functionally compensate for the missing

EPS *exo* minus mutants (Geiger, 2002). One of the four *fix-23* complementation units was shown to have six ORFs that have high similarity and similar organization to the rat fatty acid synthase multi-functional enzyme domains. The sixth ORF was proposed to encode an ACP (Petrovics, 1993), and was named RkpF. The overall amino acid similarity between all the Acps (AcpP, AcpXL, NodF and RkpF) is between 26 and 32% (Brozek, 1996). Further, a well-conserved amino acid region is present around the phosphopantetheine-binding site. Thus, the *Rhizobiaceae* have multiple Acps that are required for the synthesis of the various cell surface glycolipids, polysaccharides, and Nod factor signal molecules.

Phylogenetic distribution of 27OHC28:0, and Gene organization around *acpXL* in *R. leguminosarum*:

27-Hydroxyoctacosanoic acid is an unusual fatty acid and differs from other hydroxylated fatty acids of LPSs in that its hydrocarbon chain is approximately twice their usual length. In addition, its functional hydroxy group is present at the penultimate position of the hydrocarbon chain, rather than at the normal C-3 position (Bhat U.R., 1991). The family *Rhizobiaceae* belongs to the α-2-subgroup of the class *Proteobacteria*. *Protebacteria* comprises of the nitrogen fixing rhizobia, as well as intracellular pathogenic bacteria, and nitrifying bacteria (Bhat U.R., 1991). In an investigation of the distribution of 27OH-C28:0 fatty acid in the LPS of α-2-subgroup of *Proteobacteria* by Bhat et al, it was found that many of the organisms belonging to this subgroup possess the long chained fatty acid, and in addition can also have 2,3-diamino-2,3-dideoxy-D-glucose as a lipid A backbone sugar(Bhat U.R., 1991). Since this investigation, lipid As from several other organisms have been found to possess this very long chained fatty acid or homologues to *acpXL* or *lpxXL*. These bacteria include a number of

intracellular pathogens that are able to survive with their host cells thus causing chronic infections; e.g. *Brucella abortus* (Bhat, 1994a), *Brucella melentensis* (Basu, 2002), *Bartonella henselae* (Bhat, 1994a), *Rickettsia prowazekii* (Basu, 2002), and *Legionella pneumophila* (Zahringer, 1995), (Zahringer, 1999). However, the biological significance of oxygenated long acyl chains is unclear. Since this long chained fatty acid is double the length of the usual β-hydroxy fatty acids, it may span the entire outer membrane and, thereby, provide extra stability to the membrane that contributes to its ability to withstand a hostile host environment. The location of the functional OH group at the penultimate position of the fatty acid may interact with components of the inner leaflet of the outer membrane, such as phospholipids and proteins, which may contribute to the increased stability of the outer membrane (Bhat U.R., 1991).

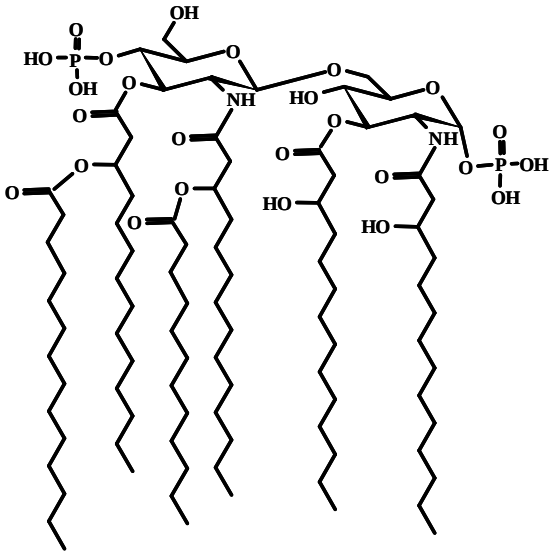
The unique AcpXL required for the synthesis and transfer of 27OH-C28:0 was described by Brozek et al (Brozek, 1996) using an *in vitro* system consisting of a membrane enzyme and the cytosol from *R. leguminosarum*. This combination was able to catalyze the transfer 27OHC28:0 to $(Kdo)_2lipid_{IVA}$, the key lipid A precursor. The presence of the Kdo residues was required for activity. The cytosolic fatty acyl donor was purified using this assay and amino-terminal sequencing of the purified acyl donor showed an exact match with the partially sequenced gene *orf** from *R. leguminosarum*. Upon complete sequencing, it was revealed that *orf** encodes a protein of 92 amino acids. Orf* is a new type of acyl carrier protein, and shows only about 25% homology to the constitutive Acp (AcpP), and the inducible NodF of *R. leguminosarum*. Since the function of Orf* is highly specialized, it was renamed AcpXL (Brozek, 1996). *Rhizobium leguminosarum* membranes contain a Kdo-dependent 27OHC28:0-AcpXL acyltransferase that uses $(Kdo)_2lipid_{IVA}$, the lipid A precursor as its acceptor (Brozek, 1996). This enzyme does not function with 12-16 carbon acyl-Acps derived from *E. coli*, but

rather requires its own dedicated ACP, AcpXL. Basu et al (2002) identified a genomic fragment from *R. leguminosarum* that contains a cluster of 11 ORFs that are involved in the synthesis and incorporation of 27OHC28:0 into lipid A (Basu, 2002). This gene fragment includes structural genes for the 27OHC28:0 acyltransferase (*lpxXL*), the ACP (*acpXL*), and enzymes that are perhaps involved in the biosynthesis of 27OHC28:0. Downstream of *acpXL* are a cluster of seven ORFs transcribed in the same direction (Basu, 2002). Proteins encoded by *orf1*, *orf2*, *orf3*, and *orf4* have significant overall homology to enzymes that are involved in fatty acid or polyketide biosynthesis. Orf1 is homologous to R-3-hydroxyacyl-Acp-dehydratase (FabZ) involved in saturated fatty acid biosynthesis. Orf 2 and 3 are very similar to one another, and belong to a family of condensing enzymes of fatty acid biosynthesis. Perhaps these genes are specifically required for the elongation of the 27OHC28:0 alkyl chain on its acyl carrier protein, AcpXL. Distant sequence similarities were found between Orf5 and various late acyltransferases involved in lipid A biosynthesis. *orf5* was designated as *lpxXL*, and was unequivocally demonstrated to encode the long chain acyltransferase of *R. leguminosarum*, by heterologous expression in *E. coli* (Basu, 2002). BLAST searches of the predicted protein products of Orf6, 7 and 8 did not yield any homology with polypeptides of known function. The ORFs upstream of *acpXL* are Orf114 (no assigned function) and Orf240 (a transcriptional regulator), and have been previously described (Colonna-Romano, 1990), (Brozek, 1996). A detailed map of the region is provided in the next section.

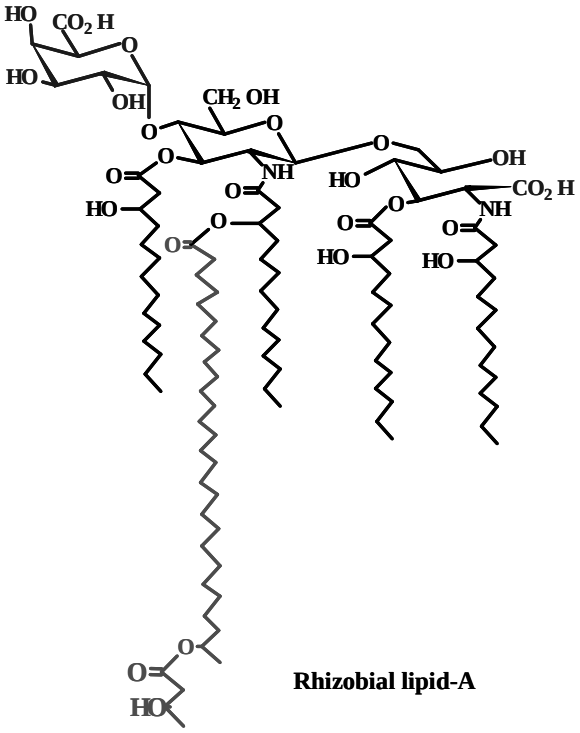
This gene region, *acpXL* through *lpxXL* can also be found in several other genomes. In the genome sequence of *Bradyrhizobium japonicum* 110, which also contains 27OHC28:0 on its lipid A, this region was easily identifiable. In this case, three genes were found in between *acpXL* and *lpxXL*: *bll3808* (1.28 kb) which has sequence similarity to a 3-oxoacyl-(Acp)-

synthase II, *bll3809* (1.20 kb) which is similar to a second 3-oxoacyl-(Acp)-synthase (possibly synthase I), and *bll3810* (0.47 kb) with similarity to a 3-OH-acyl-(Acp)-dehydratase. The organization of these genes is also very similar in other genomes of organisms that possess the 27OHC28:0 on their lipid-A; *e.g*. *S. meliloti* (www.sequence.toulouse.inra.fr), *M. loti* (www.kazusa.or.jp/rhizobase/Mesorhizobium), *R. leguminosarum*(Basu, 2002), (Priefer, 1989), and facultative intracellular pathogens such as *Brucella melitensis* (Que, 2000a) (Stacey et al., submitted). Hence, it is likely that these five genes encode enzymes that are exclusively involved in the synthesis and transfer of the 27OHC28:0.

It has been proposed that this very long chain fatty acyl component may increase the stability of the bacterial membrane. As discussed above, the amount of this residue nearly doubles during the symbiosis of *R. leguminosarum* with peas (Kannenberg, 2001), and this indicates that it is crucial for symbiosis. As stated previously, in peas there is synchronous division of the bacterium and the symbiosome membrane, and this leads to a symbiosome containing a single bacterium. There is probably an intimate interaction between the symbiosome membrane and the bacterial outer membrane during the process of division (Bradley, 1986), and the 27OHC28:0 molecule perhaps plays an important role in stabilizing the bacterial outer membrane during the physical stress of division. The focus of this thesis as stated previously, is on the function of the 27OHC28:0 molecule of *R. leguminosarum* 3841 in symbiosis. In order to determine the function of this component, a *R. leguminosarum* 3841 LPS-mutant strain was constructed in which *acpXL* was inactivated. The construction of this strain, its biochemical and symbiotic phenotype analysis, and the impact of this LPS mutation on the *Rhizobium*-pea symbiosis are described in the next chapters.



***E. coli* lipid-A**



Rhizobial lipid-A

Figure 1.1

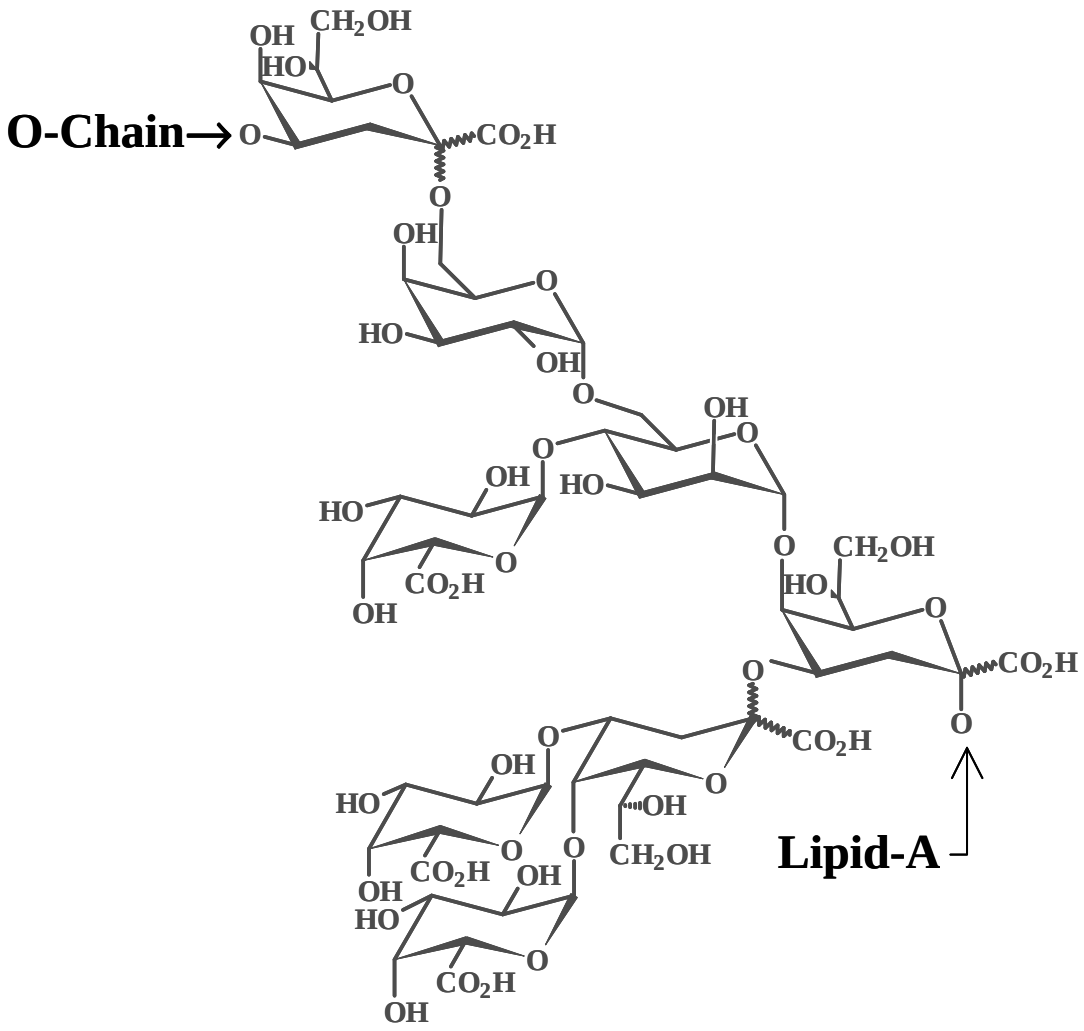


Figure 1.2

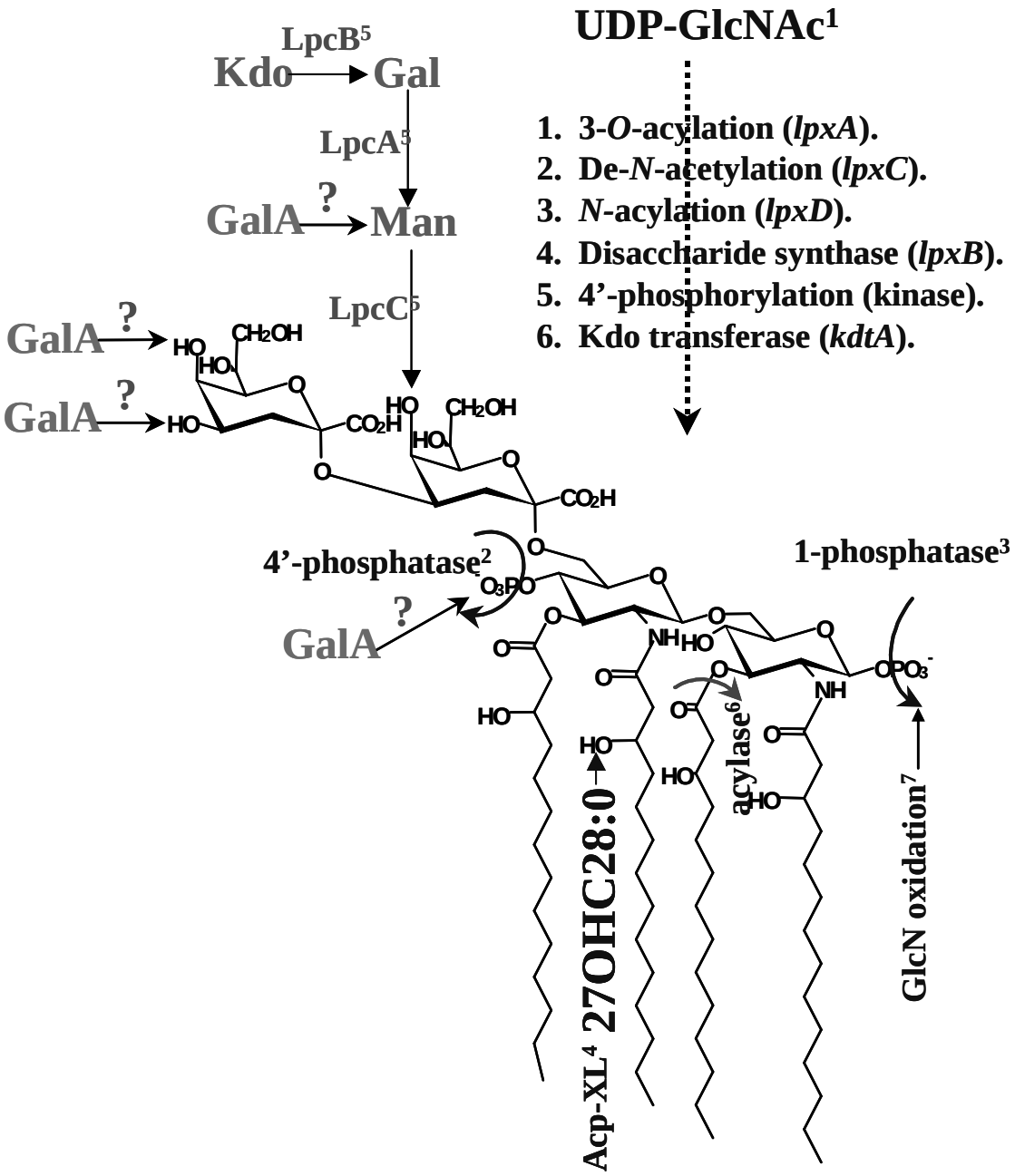

UDP-GlcNAc[1]
1. 3-O-acylation (lpxA).
2. De-N-acetylation (lpxC).
3. N-acylation (lpxD).
4. Disaccharide synthase (lpxB).
5. 4'-phosphorylation (kinase).
6. Kdo transferase (kdtA).
LpcB[5]
Kdo
Gal
LpcA[5]
GalA ?
Man
LpcC[5]
GalA ?
GalA ?
4'-phosphatase[2]
GalA ?
1-phosphatase[3]
acylase[6]
GlcN oxidation[7]
Acp-XL[4] 27OHC28:0

Figure 1.3

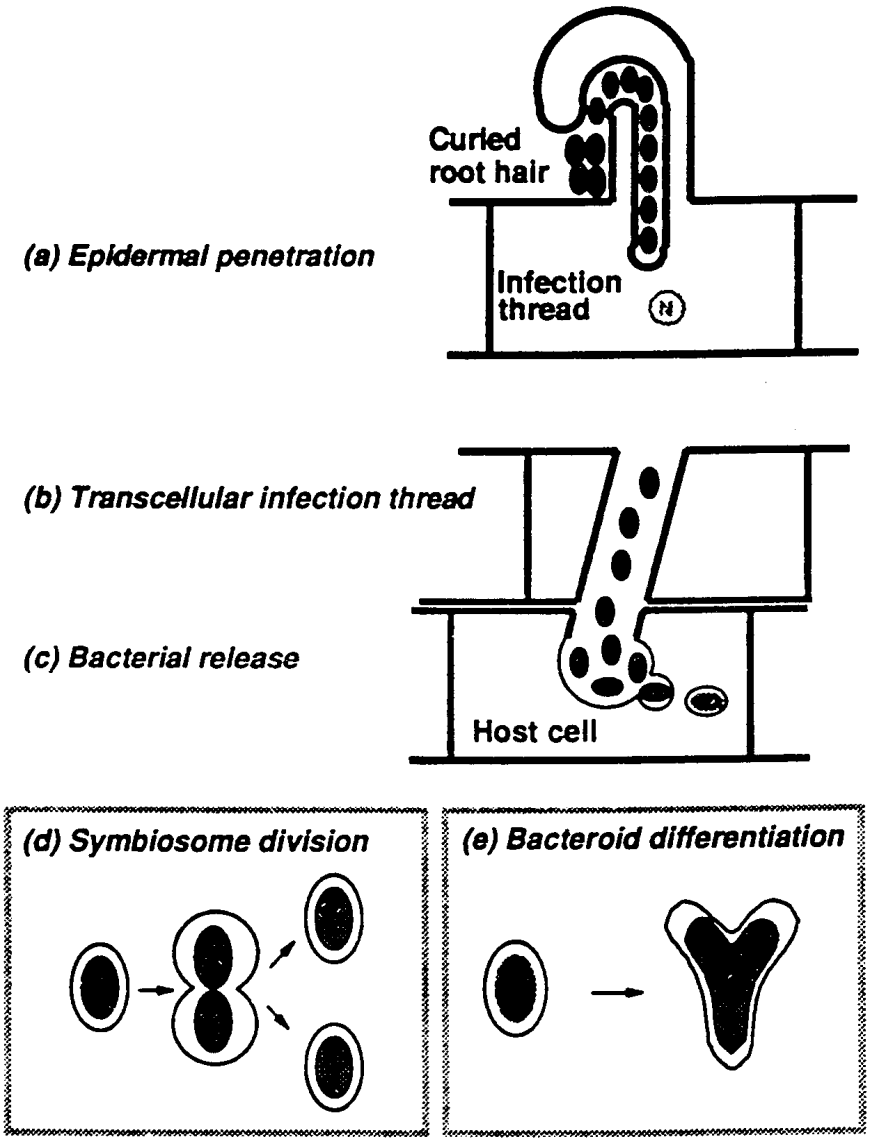

(a) Epidermal penetration
Curled root hair
Infection thread
N
(b) Transcellular infection thread
(c) Bacterial release
Host cell
(d) Symbiosome division
(e) Bacteroid differentiation

Figure 1.4

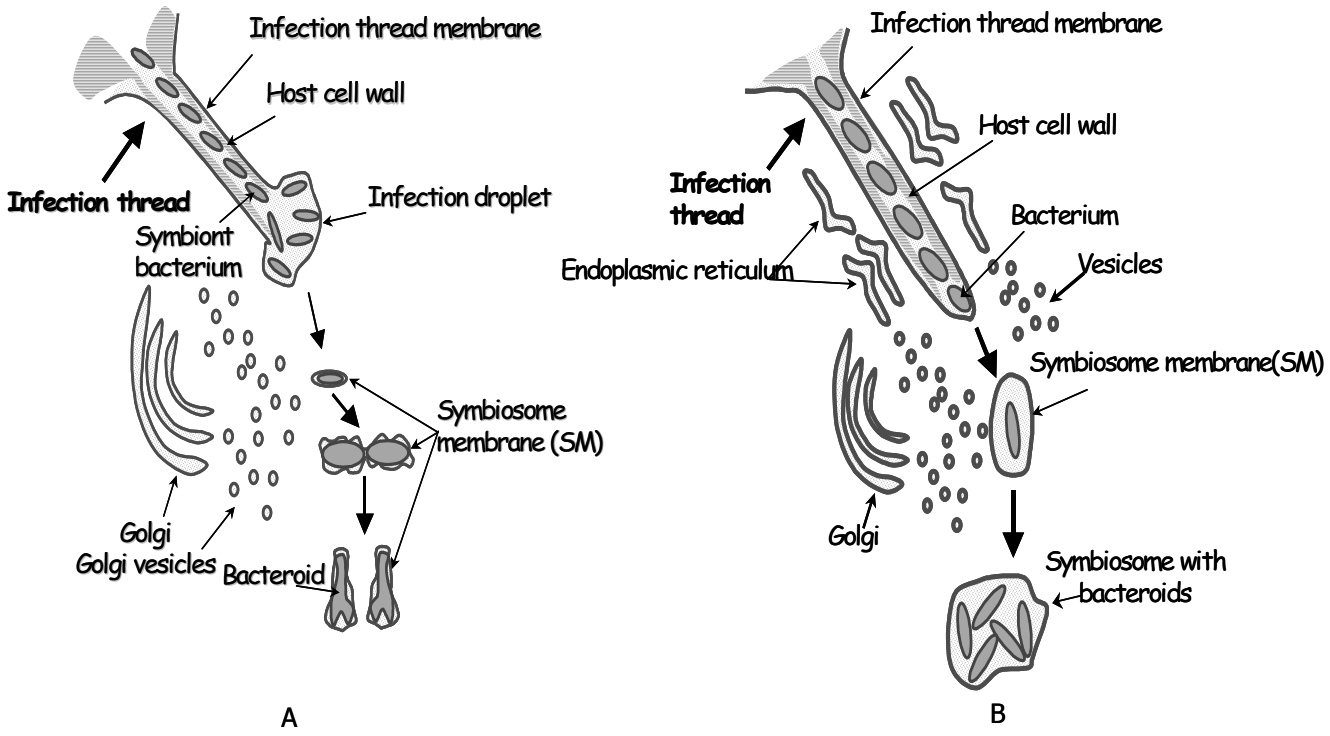

Infection thread membrane
Host cell wall
Infection thread
Infection droplet
Symbiont bacterium
Symbiosome membrane (SM)
Golgi
Golgi vesicles
Bacteroid
A
Infection thread membrane
Host cell wall
Infection thread
Bacterium
Endoplasmic reticulum
Vesicles
Symbiosome membrane(SM)
Golgi
Symbiosome with bacteroids
B

Figure 1.5

Figure Legends

Figure 1.1. Lipid A structure of *Rhizobium etli, Rhizobium leguminosarum* LPS in comparison to that from *E. coli* LPS. Special features of the rhizobial lipid-A include the absence of phosphate groups, an aminogluconate residue, and a C-28 acyl chain.

Figure 1.2. The complete glycosyl sequence of the core region found in *R. leguminosarum* and *R. etli* LPSs.

Figure 1.3. Biosynthesis of rhizobial lipid A.

Figure 1.4. Stages in symbiotic infection in pea.

Figure 1.5. A schematic diagram showing infection of (A) indeterminate, and (B) determinate nodules. (Adapted from: N.J. Brewin. 1998. In: The *Rhizobiaceae*. Ed. By H.P. Spaink, A. Kondorosi, P.J.J.Hooykaas. Kluwer Academic Pub. Pp. 417-429)

CHAPTER 3

A *RHIZOBIUM LEGUMINOSARUM acpXL* MUTANT PRODUCES LIPOPOLYSACCHARIDE LACKING 27-HYDROXYOCTACOSANOIC ACID[1].

[1]Vedam, V, Kannenberg, E.L., Haynes, J.G., Sherrier, D.J., Datta, A., and Carlson, R.W. 2003. *Journal of Bacteriology*. 185: 1841-1850.

Abstract:

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

Introduction

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

The initial stages of symbiosis include root hair deformation and the stimulation of root cortical cell division resulting in nodule formation. These events are induced via an exchange of signal molecules; flavonoids produced by the host plant induce expression of nodulation (*nod*) genes in the *Rhizobium*; for recent reviews see (13, 44). The *nod* gene products produce an acylated chitin oligosaccharide, the Nod factor, which stimulates these initial events. Rhizobia adhere to the emerging

root hairs of the host, and an infection conduit known as the infection thread is formed by invagination of the plasma membrane and deposition of plant extracellular matrix material. Bacteria spread into the nascent nodule tissue though these infection threads. This is followed by intracellular invasion of the root nodule tissue in an endocytotic-like process resulting in an intracellular compartment known as a symbiosome in which the invading *Rhizobium* is surrounded by a plant membrane. In this process, the infection thread penetrates the nodule cortical cell and expands forming an infection droplet that contains a number of symbiont bacteria. Bacteria are released from the droplet into the host cell cytoplasm. With indeterminate nodules, like those formed by pea, the bacteria become singly surrounded by the host-derived membrane known as the symbiosome membrane (SM) via a synchronous bacterial/SM division process. Electron micrographs suggest that during this process there are actual points of contact between the bacterial membrane and the SM (8). Inside the symbiosome, the *Rhizobium* differentiates into a bacterial form known as the bacteroid which produces nitrogenase.

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

The structure of the lipid-A from *R. etli* (*Re*) (formerly *R. leguminosarum* biovar phaseoli) or *Rlv* (5, 39, 40) is shown in Figure 1 together with the structure of *E. coli* lipid-A. The *Rlv* and *Re* lipid-As have identical structures with unique features compared to those of *E. coli* lipid-A. These unique structural features include the replacement of the 4'-phosphate with a galacturonosyl residue, the oxidation of the proximal glucosaminosyl residue to 2-amino-gluconate, the lack of acyloxyacyl lauryl and myristyl residues, and the presence of a very long chain acyloxyacyl moiety, 27OHC28:0. A similar

lipid-A occurs in the LPS from *Rhizobium* species Sin-1 (which nodulates the roots of *Sesbania*) but differs slightly from that of *Rlv* in that it lacks the galacturonic acid residue and, while containing 27OHC28:0, has an altered fatty acylation pattern (22). It has been shown that *Rlv* (and *Re)* contains the same enzymes as *E. coli* to convert UDP-GlcNAc into a common lipid-A precursor known as Kdo_2Lipid-IVA (37), and that *Rlv* has additional enzymes that process this precursor into the unique lipid-A structure shown in Figure 1 (10, 23, 36, 40). In *E. coli* (and in *Salmonella*) the secondary acyloxyacyl lauryl and myristyl fatty acids are added by LpxL and LpxM, respectively (for a review on enteric lipid-A biosynthesis see (41)). In *Rlv* the sole secondary acyloxyacyl residue is 27OHC28:0. This fatty acid residue requires a unique acyl carrier protein, AcpXL(10), and a transferase, LpxXL (1). It is not clear whether the additional amounts of 27OHC28:0 fatty acid residues observed in the lipid-As of *Rlv* bacteroids involve additional biosynthetic enzymes, *e.g.* additional tranferases.

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

Materials and methods

Bacterial strains, plasmids and growth conditions:

Bacterial strains and plasmids used in this work are listed in Table 3.1. All the *E. coli* strains were grown at 37^{o}C, on LB medium. *R. leguminosarum* 3841 was used as the parental strain for this study. All rhizobia were grown on tryptone/yeast extract (TY) medium with added calcium (34) or on Vincent's minimal medium (48). Media were supplemented with the following concentrations of antibiotics where mentioned: ampicillin (Amp, 50 μg ml^{-1}), kanamycin (Kan, 50 μg ml^{-1}), gentamycin (Gm, 40 μg ml^{-1}), and tetracycline (Tet, 10 μg ml^{-1}). The β-galactosidase substrate and inducer, X-Gal and IPTG, respectively, were incorporated into solid Luria broth (LB) medium at 50μg ml^{-1} and 100μg ml^{-1} respectively. Peas were grown on N_2-free Fahraeus medium (35) at 22^{o}C with 14 h of light, 18^{o}C with 6 h of dark, and 55% humidity.

Generation of acpXL::kan mutants and complements:

Plasmid pCS115, containing *acpXL* (formerly known as orf* (12)), was digested using restriction enzymes *HindIII* and *PshAI* to isolate a 3.9 kb fragment containing *acpXL* (327 bp) with flanking regions (about 1.8 kb on either side of *acpXL*). All restriction endonucleases were purchased from New England Biolabs. This fragment was subcloned into cloning vector pBluescript pBST SK(+) that had been predigested with *HindIII* and *SmaI*, yielding pVV1. Plasmid pVV1 was transformed into *E. coli* XL1 Blue. A number of white colony transformants were selected on LB agar plates containing Amp, X-Gal, and IPTG. Plasmid DNA was extracted from the transformants, digested with *HindIII* and *EcoRI*, and the resulting DNA fragments

analyzed by agarose gel electrophoresis to determine if the correct construct was present. Colonies that had the correct construct were preserved in glycerol stocks.

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

A 4.8kb fragment containing the interrupted gene from pVV2 was released by partial digestion with *XhoI* and cloned into suicide plasmid pJQ200uc1 at a unique *XhoI* site in the multiple cloning site of this vector. Plasmid pJ200uc1 carries the conditionally lethal gene, *sacB*, allowing for discrimination between the integration of the vector, and double recombination events (38). The resulting plasmid, pVV4, was transformed into *E. coli* JM109, and transformants were selected on LB agar plates containing Kan. The construction of the pVV4 plasmid is schematically depicted in Figure 2A. Plasmid pVV4 was introduced into *Rlv* 3841 by triparental mating using *E. coli* JM109pVV4 as the donor, *Rlv* 3841 as the recipient, and *E. coli* pRK600 (19) as the helper strain. Transconjugates were selected on Vincent's minimal medium agar plates containing Gm and Kan. Double recombinants were selected on TY agar plates containing Gm, Kan, and 10% sucrose. Two *acpXL::kan* mutants were isolated (*Rlv* 22 and *Rlv* 26), and verified by PCR using primers to *acpXL* , GAGGGGGTTTAAATAGTCA and AGGCCTTGGCCGCTTTGA, and by sequencing the resulting PCR products. All DNA sequencing was done by the Molecular Genetics Instrumentation Facility at the University of Georgia.

A complement of the *acpXL::kan* mutant was constructed to confirm that any observable physiological differences in the mutant were as a result of the *acpXL::kan* mutation. For this purpose, a 2.5 kb fragment containing the parent *acpXL* gene isolated from pVV1 (see above) using the restriction enzyme *KpnI*. This fragment was cloned into pBBR1MCS3, a broad host range vector (38), that had been predigested with *KpnI*. The resulting construct, pVV5 was transformed into *E. coli* JM109 and transformants were selected on LB plates containing Tet. This strain was used as the donor for triparental mating into *Rlv* 22 using *E. coli* pRK600 as the helper strain. Transconjugants were selected on TY plates containing Tet and Kan. Confirmation of *acpXL* (plasmid borne), and *acpXL::kan* (chromosomal) in the complement strains was done by PCR, using primers to *acpXL* (described above) using plasmid, and chromosomal DNA respectively of the complements.

LPS extraction and purification

The wild type and mutant LPSs were first extracted by the TEA/EDTA/ϕ procedure as previously described (43). For each strain, the bacterial pellet, approximately 10 g wet weight, from 8 L of culture was extracted with 40 ml of TEA/EDTA/ϕ (0.25 M EDTA, ethylenediamine tetraacetic acid, containing 5% phenol and tritrated to pH 6.9 with triethylamine, TEA) with constant stirring at 37°C for one hour. The extract was then centrifuged at 10,000 rpm for one hour, and the supernatant was collected, and dialyzed (2000 MWCO, Spectrapor) against deionized water. This material was further purified using polymyxin affinity chromatography using polymyxin B-Sepharose purchased from Pierce Chemical Company as previously described (20, 43, 46). Briefly, after sample application in 50 mM NH_4HCO_3, the column (10 ml bed volume) was sequentially eluted with 30 ml a solution of 0.3 M TEA adjusted to pH 6.4 with

acetic acid with 10% ethylene glycol, followed by 30 ml of a solution of 2.0 M urea in 0.1 M NH_4HCO_3 to elute the non-LPS components. The LPS was eluted from the column with 20 ml of 2.5% DOC in 0.1 M NH_4HCO_3. The LPS fractions were pooled together, and dialyzed extensively (2500 MCWO) against a solution of 50 mM TEA made pH 8.5 with acetic acid and containing 10% ethanol, and then against deionized water, after which they were lyophilized.

Analytical Procedures

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

Lipid-A was isolated from the LPS preparation by mild acid hydrolysis (11). The LPS was dissolved in 1% SDS in 20 mM sodium acetate, adjusted to pH 4.5 with 4 M HCl, and then placed in an ultrasound bath until the sample was dissolved. This solution was then heated at 100 °C for 1h, followed by lyophilization. The SDS was removed by washing the lyophilized residue with a solution of 2:1 deionized H_2O:acidified ethanol (100 μl 4 M HCl in 20 mL 95% ethanol). The residue was collected by centrifugation, washed again with 95% ethanol (non-acidified), and collected by centrifugation (200 ×g for 15 min). The washing and centrifugation steps were repeated. Lastly, the residue was lyophilized to give a white, solid, fluffy lipid-A preparation.

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

Study of physiological properties

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

In order to assess the osmotic tolerance of the mutant, *Rlv* 22, its complement, and *Rlv* 3841 were grown at 30°C on TY/calcium agar containing various concentrations of NaCl; 0.0%,

0.25%, 0.5%, 0.75% and 1.0%. Sensitivity to pH was determined by growth on TY plates with varying pH conditions (7.0, 6.0, 5.5, 5.0, and 4.8), using PIPES (piperazine-N,N'-*bis*[2-ethanesulphonic acid]), and piperazine as buffering agents. PIPES was used at a final concentration of 40 mM to buffer the medium at pH 7, and a final concentration of 40 mM piperazine was used in the medium while adjusting the acidity of the medium to the various lower pH values.

Symbiotic phenotype characterization

The effect of the *Rlv acpXL::kan* mutation on nodulation of pea, *Pisum sativum* bv. Early Alaska, was examined by inoculating pea seedlings with 0.5 mL of late log/early stationary liquid cultures of wild type and mutant strains. The seedlings were grown in Erlenmeyer flasks with defined, nitrogen-free plant agar Fahraeus medium, in growth chambers. The temperature was maintained at 22°C with 14 h of daylight, at 18^{o}C with 10 h of dark, and 55 % humidity. Throughout the growth period, nodule formation was monitored.

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

Results

Characterization of the gene region harboring acpXL.

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

Isolation of Rlv 3841 acpXL::kan *mutants.*

The *acpXL::kan* mutants were isolated as described in the Methods (depicted in Figure 2A). Figure 3 shows the PCR DNA fragments isolated from the parent 3841 strain and the two mutants, *Rlv* 22 and 26 using *acpXL*-specific primers. For comparison reasons, DNA from *Re* CE3 was also included. The *Re* CE3 and *Rlv* 3841 strains had the expected 0.28 kb *acpXL* fragment, while *acpXL*-containing PCR fragments from both mutant strains were approximately 1.56 kb in size which is consistent with the combined size of the *kan* cassette and *acpXL*. The insertion of the *kan*-cassette into the *EcoRI* site of the *acpXL* gene was confirmed for both mutants by sequencing the *acpXL::kan* PCR products. The *kan*-cassette in both mutants showed a parallel orientation to the *acpXL* gene**.** In mutant strains complemented with a plasmid harboring the *acpXL* gene, PCR analysis of the plasmid and chromosomal DNA using the *acpXL*-specific primers gave a 0.28 kb fragment indicative for the wild-type gene in the plasmid

DNA, and a 1.56 kb fragment from the chromosomal DNA indicative for the *acpXL::kan* (data not shown).

Lipopolysaccharide isolation and characterization

We employed two standard protocols for LPS extraction; hot phenol/water, or TEA/EDTA/ϕ. With both methods, the yield of LPS from the mutant was less than that obtained from the *Rlv* 3841 parent, however, the LPS yields from both parent and mutants were increased with TEA/EDTA/ϕ extraction. In the case of the phenol/water extraction, the LPS from the *Rlv* 3841 parent extracted into both the phenol and water phases as previously described (26). However, with the *acpXL::kan* mutants the LPS largely extracted into the water phase with only small amounts found in the phenol phase. This result suggested that the content of hydrophobic LPS in the mutant was much less than that found in the *Rlv* 3841 parent. For chemical analysis, the TEA/EDTA/ϕ extraction method was used since this procedure resulted in increased yields of LPS compared to hot phenol/water extraction. The mutant LPS yield per gram of wet weight bacterial pellet from the TEA/EDTA/ϕ procedure was approximately one-third that obtained from the *Rlv* 3841 parent.

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

presence of the *N*-acylated GlcN residues (*N*-3OHC14:0-, *N*-3OHC16:0-, *N*-3OHC18:1-, and *N*-3OHC18:0-GlcN) is consistent with previous reports (5) showing the heterogeneity of the *N*-acyl substituents and the resistance of the *N*-acyl residues to the methanolysis procedure (2). Neither mutant 22 (Figure 4B), nor mutant 26 have LPS with appreciable levels of 27OHC28:0 or 29OHC30:0. Selective ion scanning *(i.e. m/z* 117) of the mutant LPS indicated the possibility of a slight amount of 27OHC28:0 at less than 1% of the parent LPS level. However, the lipid-A from both mutants have the same level of *N*-acyl GlcN residues as the parent lipid-A. The insert in Figure 4 shows that the GC/MS profiles for the remaining β-hydroxy fatty acids of the parent and mutant lipid-As are essentially identical to one another. The fatty acyl compositions are given in Table 3.2. Other than the lack of 27OHC28:0 and 29OHC30:0, the relative amounts of the remaining fatty acids found in the mutant LPSs are essentially the same to those of the parent LPS. Analysis of the LPS from the complemented mutant showed that its lipid-A was restored in 27OHC28:0 content (data not shown). In addition to the fatty acyl components just described, both the parent and the mutant lipid-A molecules contained galacturonic acid, glucosamine (as indicated above), and 2-aminogluconate.

Lipid-A preparations from the parent and mutant strains were isolated and analyzed by MALDI-TOF mass spectrometry. The results are shown in Figure 5. The mass spectrum of the lipid-A from the parent 3841 strain, Figure 5A, shows two clusters of ions that are consistent with the composition described above and with the previously published *Rlv/Re* lipid-A structures (5, 39, 40). In one cluster, the ions range from *m/z* 1887.6 to 2058.0 with the most intense ion at 1915.4, and in the second cluster, the ions range from *m/z* 1625.7 to 1738.9 with the most intense ion at *m/z* 1653.5. The ion at *m/z* 1915.4 in the first cluster of ions is consistent with the published structure (5, 39, 40) in which the lipid-A has a disaccharide backbone

consisting of a distal glucosminosyl residue β-1,6-linked to a proximal 2-aminogluconic acid residue. This disaccharide is substituted at the 4'-position of the distal glucosamine with an α-galacturonosyl residue, and is fatty acylated with β-hydroxyfatty acyl moieties at the 2, 3, 2', and 3' positions (*e.g.* 3OHC14:0, 3OHC16:0, 3OHC14:0, and 3OHC16:0, respectively) with the 27OHC28:0 moiety present as a secondary acyloxyacyl residue ester linked to the hydroxyl group of the 3'-β–hydroxy fatty acyl residue. The *m/z* 2001.4 ion is the $[M-H]^-$ ion for this structure in which the 27-hydroxyl group of 27OHC28:0 is esterified with β–hydroxybutyrate as previously reported for the *Rlv* lipid A (5, 39, 40). The other ions in this cluster are due to variations in these structures as a result of varying fatty acyl chain lengths. In the second ion cluster, the ion at *m/z* 1653.5 is due to lactonization of the proximal 2-aminogluconate residue and the subsequent elimination of a 3OHC14:0 moiety from the β–position of that residue. The result is a loss of 262 mass units (-244 mass units due to loss of 3OHC14:0 and -18 mass units due to loss of water; 1915 – 262 = 1653) and the formation of a 2,3-unsaturated 2-aminogluconolactone proximal residue; i.e. 2-amino-2,3-dideoxy-D-*erythro*-hex-2-enono-1,5-lactone. This structure was reported to be present in *R. etli* lipid-A (39, 40), and has also been reported as a component in the lipid-A from *Rhizobium* species Sin-1 (a symbiont of *Sesbania*) (22). As previously reported (22, 40), this structure is most likely an artifact of the lipid-A isolation procedure which promotes both lactonization of the 2-aminogluconate residue and the subsequent acid catalyzed β-elimination of the 3-hydroxy fatty acyl moiety (22). The most intense ion in this cluster, *m/z* 1653.5, is due to this structure which lacks a β–hydroxybutyrate group, and the ion at *m/z* 1738.9 is due to the molecule containing the β–hydroxybutyrate group. Again, the other ions in this cluster are due variations of this structure as a result of varying fatty

acyl chain lengths. The structures for the parent *Rlv* 3841 lipid-A molecules are shown in Figure 6A and 6B.

The mass spectrum of *Rlv* mutant 22, Figure 5B, shows two main clusters of ions; one cluster is centered on ion *m/z* 1493.2 and another around the ion at *m/z* 1230.6. A minor third ion cluster is present centered on an ion of *m/z* 1759.5. An identical mass spectrum was observed for the lipid-A from *Rlv* mutant 26 (data not shown). The two main ion clusters are due to lipid-A structures analogous to those of the parent described above but lacking 27OHC28:0 (*i.e.*, less 422 mass units; 1915 – 422 = 1493 and 1653 – 422 = 1231) or lacking both β–hydroxybutyrate and 27OHC28:0 (*i.e.*, less 508 mass units; 2001 – 508 = 1493, and 1739 – 508 = 1231). These structures are shown in Figure 6C and 6D. These results are consistent with the composition analysis showing that the mutant lipid-A lacks 27OHC28:0. The ions in the minor ion cluster, ranging from *m/z* 1732.1 to 1786.0, are approximately 238 mass units larger than corresponding ions in the *m/z* 1493.2 ion cluster; *i.e.* 1493.2 + 238 = 1731.2, 1521.7 + 238 = 1759.7, and 1549.3 + 238 = 1787.3. The additional 238 mass units could be accounted for by the addition of a palmitoyl residue to the mutant lipid-A. Small amounts of palmitic acid are observed in both parent and mutant lipid-A preparations with increased amounts in the mutant lipid-A. However, this fatty acid is normally one of the major components of phospholipids and, therefore, its presence in the lipid-A preparation is usually due to some contamination by phospholipids. Thus, confirmation of palmitic acid as a component of the mutant lipid-A will require enrichment of that minor lipid-A species and further structural characterization.

The LPSs from the mutants and the parent strain were also compared by DOC-PAGE analysis to determine if there were any differences in the LPS banding patterns. The banding patterns for the *Rlv* 3841 parent and *Rlv* mutant 22 (*Rlv* mutant 26 LPS gave the same results as

shown for *Rlv* mutant 22) are shown in Figure 7. Some alterations in both intensities and mobilities of the *Rlv* mutant 22 LPS bands were observed when compared to *Rlv* 3841 LPS. The LPS I banding region of the mutant LPS is missing one band, and the LPS II of the mutant is somewhat greater in intensity with a slightly faster electrophoretic mobility than that of the parent LPS II; a result indicating a slightly lower molecular weight for the mutant LPS II. However, it is somewhat surprising that the mobility of the mutant LPS II, while slightly increased compared to that of the parent, is still so similar since if the mutant lacks 27OHC28:0, its LPS II should have a molecular weight that is 422 mass units less than the parent LPS II. It is possible that an answer to this apparent discrepancy lies in some slight structural difference in the core oligosaccharide of the molecule. Further LPS structural work is in progress.

Physiological properties of acpXL::kan mutants

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

Despite considerable gaps in our understanding of nodule physiological conditions, it is increasingly clear that rhizobia are required to adjust to changes within the nodule microenvironment. Besides the low oxygen conditions of the central nodule tissue and cells,

increased osmolarity is likely to exist in the infection threads and the symbiosomes (31). In addition, acidic conditions are likely to occur within the infection thread and symbiosome microenvironments. The rhizobial symbiont would have to adapt to these changes. Therefore the *acpXL::kan* mutants were examined for their sensitivity to salt and acidic pH values. The growth of the mutant was monitored on solid medium at added salt concentrations of 0.0%, 0.25%, 0.5%, 0.75%, and 1.0%. Its growth was impaired at 0.25% and abolished at 0.5% salt. Figure 8B shows the results at 0.5% salt. Both the parent and the complemented mutant grew well in 0.5% salt. The pH sensitivity was examined by growing the strains on solid medium at pH 7.0, 6.0, 5.5, 5.0, and 4.8. The mutants were unable to grow at pH 5.0 and below, while the parent and complemented mutant grew well at pH 4.8 and above. The results at pH 5.0 are shown in Figure 8C.

Symbiotic phenotype

In order to determine the nodulation characteristics of the mutants, pea plants were either inoculated with the *acpXL::kan* mutant or parent bacteria and nodule development was followed. One-week post-inoculation, the peas inoculated with the parent showed small pinkish nodules. On peas inoculated with *Rlv* mutant 22, at one-week post-inoculation, there were small whitish nodules which were clearly delayed in development compared to those resulting from the parent inoculation. After three weeks the nitrogen-fixing ability of the mutant nodules was compared with that of the parent nodules and no significant differences were observed. The delayed nodule development by the mutant may be due to the fact that the mutant grows more slowly inside the nodule. On the other hand, the delayed nodule development may also be due to the fact that the mutant is severely stressed (*e.g.*, osmotically) in the nodule environment, but is somehow

able to compensate for this defect. A detailed analysis of nodule formation and nodule morphology is under investigation.

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

Discussion

The LPSs from members of the *Rhizobiaceae* and from a number of Gram-negative facultative intracellular pathogens contain 27OHC28:0 in their lipid-As (4), suggesting conserved and important cellular functions for growth and survival in their natural environments. In particular, both members of the *Rhizobiaceae* and the pathogens have the ability to survive within special intracellular compartments of their host cells. There is a great deal of precedence in the literature regarding the ability of bacteria to modify their lipid-A in response to a changing environment, which illustrates the functional importance of lipid-A changes in the adaptation of bacteria to their changing environments. It is known that enteric bacteria modify their lipid-A in response to changes in environmental conditions such as those within the host intracellular "spacious phagosomes", *i.e.* low pH and/or low Mg^{2+} (for reviews see (18, 41)). These modifications, such as the addition of 4-aminoarabinose, phosphoethanolamine, 2-hydroxylation of myristic acid, and the addition of a palmitoyl residue, are mediated by global regulatory gene products PhoP/Q and are important for protecting the bacterium from the host defenses. In symbiotic bacteria, the reported doubling of 27OHC28:0 in the lipid-A of *Rlv* during symbiosis (26) probably reflects one of the ways this bacterium adapts to the changing environment of its host. However, the exact function of this structural modification has not yet been determined.

For other intracellular pathogens, *e.g. Brucella abortus*, which survive within phagosomes and cause chronic infections, it is not known whether the lipid-A 27OHC28:0 component contributes to virulence.

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

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

Analysis of the mutant lipid-A shows that it has the same structure as the parental lipid-A with the major difference being the lack of 27OHC28:0. As stated in the *Introduction*, it has been shown that *Rlv* contains the same enzymes as does *E. coli* that convert UDP-GlcNAc into a

common lipid-A precursor known as Kdo_2Lipid-IV$_A$, that *Rlv* has unique enzymes that process this precursor into its unique lipid-A, and that AcpXL is required (as described in this paper) for acyloxyacylation of Kdo_2Lipid-IV$_A$ with 27OHC28:0. The fact that the *acpXL::kan* mutant is able to produce a lipid-A structure that is the same as that of the parent except for the missing 27OHC28:0 residue shows that the addition of this fatty acid is not required for processing the Kdo_2Lipid-IV$_A$ precursor into the rhizobial structure; *i.e.* 27OHC28:0 acylation is not required for removal of the 4'- and 1-phosphates, addition of the galacturonosyl residue, and oxidation of the proximal glucosaminosyl residue to 2-aminogluconate.

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

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

During symbiosis, the bacterial symbiont must adapt to the environment of its plant host. These environmental changes likely require that the bacteria adjust to changes in osmolarity, acidity, and O_2 tension. In view of these necessary adjustments, it is interesting that the *Rlv acpXL::kan* mutant is able to form nitrogen-fixing nodules even though it is unable to grow at pH 5.0, or in 0.5% NaCl. These results suggest that the mutant, *in planta*, is capable of compensating, at least in part, for the loss of *acpXL*. In a recent review by Geiger and Lopez-Lara (21), the presence of multiple types of Acp in various bacterial species was discussed. Some members of the *Rhizobiaciae* have several specialized types of Acp including AcpXL and NodF (required for the acylation of the acylated chitin oligomers known as Nod factors) (21). Thus, it may be that there is an additional Acp that comes into play in the *in planta* environment. In the case of *S. meliloti*, Geiger and Lopez-Lara report that there is an Acp present on the symbiotic plasmid, and other workers have shown that various long chain ω-1 hydroxy fatty acyl residues (C18 – C26) can be incorporated into the Nod factors and that this incorporation is regulated by *nodD3* (14, 21). Thus, it may be that *Rlv* has an alternative mechanism for the addition of 27OHC28:0 to lipid-A which is host-inducible. The fact that the lipid-A from *Rlv*

3841 isolated from bacteria grown under low O_2 has increased levels of 27OHC28:0 supports the possibility of such an additional and, perhaps, inducible mechanism for 27OHC28:0 acylation.

Further examination of this *Rlv acpXL::kan* mutant may also facilitate understanding the virulence mechanism of several Gram-negative pathogens. As mentioned in the *Introduction*, long chain ω-1 hydroxy or oxo-fatty acyl residues are present in a variety of Gram-negative bacterial species in addition to members of the *Rhizobiaceae*. The presence of these lipid-A long chain ω-1 fatty acyl residues, and/or *acpXL* or *lpxXL* homologs have been reported in a number of Gram-negative facultative intracellular organisms including *Brucella abortus* (4), *Brucella melitensis* (1), *Legionella pneumophila* (54, 55), *Bartonella henselae* (4), and *Rickettsia prowazekii* (1). Thus, the presence of the 27OHC28:0 in the lipid-A of such pathogens may play a role in their ability to survive within the host cell. As with the proposed function for 27OHC28:0 in *Rhizobium*-legume symbiosis, this very long chain fatty acyl component may increase the stability of the bacterial membrane. It is also possible that this fatty acyl residue results in a low endotoxic response in the host. In fact, the *Re* LPSs and the *R.* Sin-1 LPS, which has a lipid-A structure similar to that of *Re*, do not induce cytokines in human macrophage (monomac 6) cells and they inhibit the ability of enteric LPSs to induce cytokines by interfering with endotoxin binding to both lipopolysaccharide binding protein (LBP) and the cellular differentiation protein, CD14 (47). It would be very instructive to characterize the effects *acpXL::kan* mutations on the virulence of these intracellular pathogens.

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Table 3.1. Bacterial strains and plasmids used in this report.

Strain/plasmid	Characteristics	Source
E. coli XL1 Blue	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_{k-}, m_{k+}), *relA*1, *supE*44, *lac*, [F', *proAB*+, $lacl_qZ$ ΔM15, ::Tn10(Tet^R)]	Stratagene Corp.
JM 109	*endA*1, *recA*1, *gyrA*96, *thi*, *hsdR*17 (r_{k-}, m_{k+}), *relA*1, *supE*44, Δ(*lac-proAB*), [F', *traD*36, *proAB*, $lacl_qZ$ ΔM15]	Promega Corp.
DH5α	Φ80d*lacZ* ΔM15, *recA*1, *endA*1, *gyrA*96, *thi*-1, *hsdR*17 (r_{k-}, m_{k+}), *supE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169	Stratagene corp.
Rhizobium leguminosarum bv. viciae		
3841	Strain 300 Str^R, Fix^+	(52)
22	Strain 3841 *acpXL::kan*, Str^R, Kan^R, Fix^+	This study
26	Strain 3841 *acpXL::kan*, Str^R, Kan^R, Fix^+	This study
pBS II SK+	2.96 kb phagemid derived from pUC19, lacZ, Amp^R	Stratagene Corp.
Plasmids		
pCS115	pWKR56 carrying *Hind*III fragment from pRIA76, Tc^R	(12)
pUC4K	source of the *kan* casette , Kan^R	Pharmacia Biotech.
pJQ200uc1	Suicide vector, allows positive selection for integration, Gm^R , sacB	(38)
pRK600	Mobilizing plasmid for pJQ200uc1, pRK2013 Nm^R::Tn*9*, Cm^R.	(19)
pBBR1MCS-3	pBBR1MCS derivative, Tc^R	(30)
pVV1	pBS SK derivative with 3.9kb insert from pCS115 containing *acpXL*	This study
pVV2	pVV1 containing *kan* from pUC4K	This study

pVV4	pVV2 derivative cloned into pJQ200uc1	This study
pVV5	pVV1 derivative cloned into pBBR1MCS-3.	This study

Table 3.2. The relative fatty acid compositions* of the lipid-A from *Rlv* 3841, and its *acpXL::kan* mutant, strain 22.

Sample	3OHC14:0	3OHC15:0	3OHC16:0	3OHC18:0	27OHC28:0
Rlv 3841	40	6.0	13	15	25
Mutant 22	55	7.5	17	20	<1.0

*The compositions are given as relative mole percents. N.d. = none detected. Small amounts of 3OHC17:0, 3OHC18:1, and 29OHC30:0 were also detected but not quantified. Also, small amounts of palmitic (C16:0), stearic (C18:0), and vaccenic (C18:1) acids were present. Mutant 22 lipid-A appeared to have slightly more C16:0 than did the *Rlv* 3841 lipid-A. These straight chain fatty acids are common components of phospholipids which, on occasion, slightly contaminate LPS preparations.

Figure Legends

Figure 3.1. The structures of the lipid-A from *E. coli* and the lipid-A found in *Re* and *Rlv* LPSs. It should be noted that in the case of *Re* and *Rlv*, the lipid-A preparation exists as a mixture of several structures due to variation in both fatty acyl chain length and in the nature of the proximal glucosaminosyl residue (5, 39, 40).

Figure 3.2. (A.) A diagram showing the construction of plasmid pVV4 which contains the *kan* insert within the *acpXL* gene, *acpXL::kan*, and is used to create the *acpXL::kan* mutants of *Rlv* strain 3841, strains 22 and 26. (B.) The arrangement of the genes within the *Rlv* VF39 DNA that is present within cosmid pCS115. The *Rlv* VF39 nucleotide sequence is co-linear to the recently described gene region from *Rlv* strain 3841 (1).

Figure 3.3. An agarose gel showing the PCR *acp-XL* products obtained from *R. etli* (*Re*) CE3, *Rlv* 3841, pCS115, pVV4+, pVV4-, mutant 22, and mutant 26. Plasmids pVV4+ and pVV4- are constructs in which the *kan* insert is placed within *acp-XL* in opposite directions.

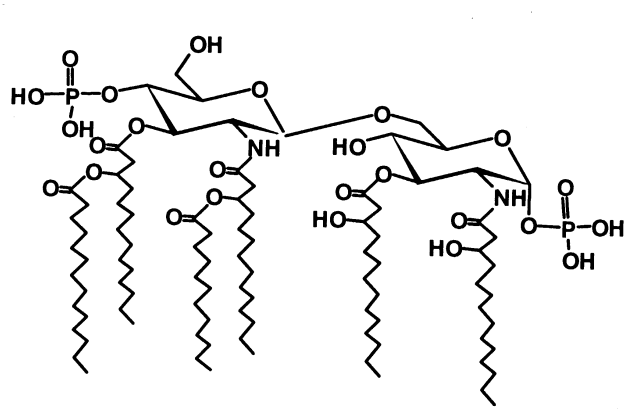
Figure 3.4. The GC-MS profiles showing the fatty acids present in the lipid-A from *Rlv* 3841 (A.) and mutant 22 (B.). The insert shows the β–hydroxy fatty acyl components (scan of the diagnostic *m/z* 175 ion) for the lipid-A from *Rlv* 3841 (top) and mutant 22 (bottom). The peaks are: 1 = the trimethylsilyl (TMS) methyl glycoside of N-3OHC14:0-GlcN, 2 = TMS fatty acid methyl ester of 27OHC28:0, 3 = the TMS methyl glycoside of N-3OHC16:0, 4 = the TMS fatty acid methyl ester of 29OHC30:0, 5 = the TMS methyl glycoside of N-3OHC18:1-GlcN, and 6 = the TMS methyl glycoside of N-3OHC18:0-GlcN; * = contaminating deoxycholate (DOC).

Figure 3.5. The MALDI-TOF MS spectra of the lipid-A from *Rlv* 3841 (A.), and from mutant 22 (B.). The identity of each ion is as described in the text, and as shown in Figure 6.

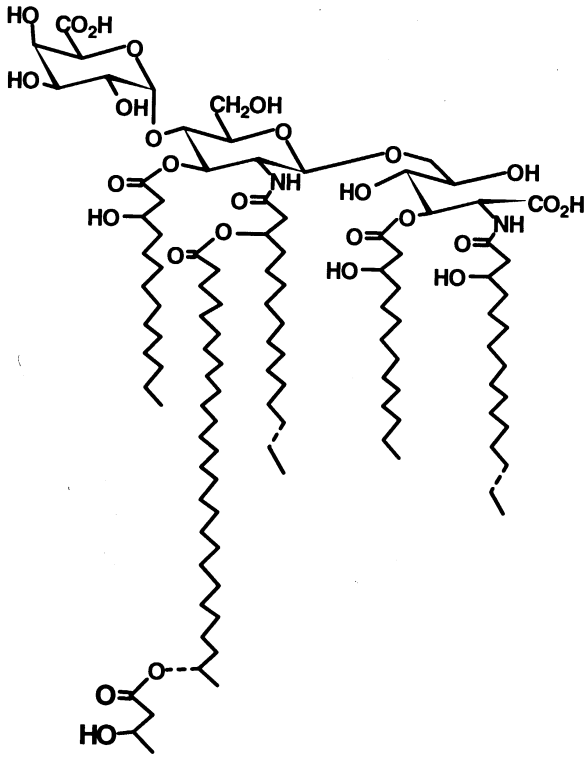
Figure 3.6. The lipid-A structures proposed for the indicated ions observed for the lipid-A from *Rlv* 3841 (A and B), and for the lipid-A from mutant 22 (C and D).

Figure 3.7. The DOC-PAGE profiles of the LPSs from *Rlv* 3841 and mutant 22. The amount of LPS used in each case was 1 μl of a 2 μg/μl solution. The bands in the LPS I region contain lipid-A, core oligosaccharide, and various lengths of O-chain polysaccharide. The band labeled LPS II lacks O-chain polysaccharide.

Figure 3.8. The growth of *Rlv* 3841 (1), mutant 22 (3), and a complement of mutant 22 (2) in normal medium (A), in 0.5% NaCl (B), and at pH 5.0 (C). The media were as described in the Experimental Methods section.



E. coli **lipid-A**



R. etli/leguminosarum **lipid-A**

Figure 3.1

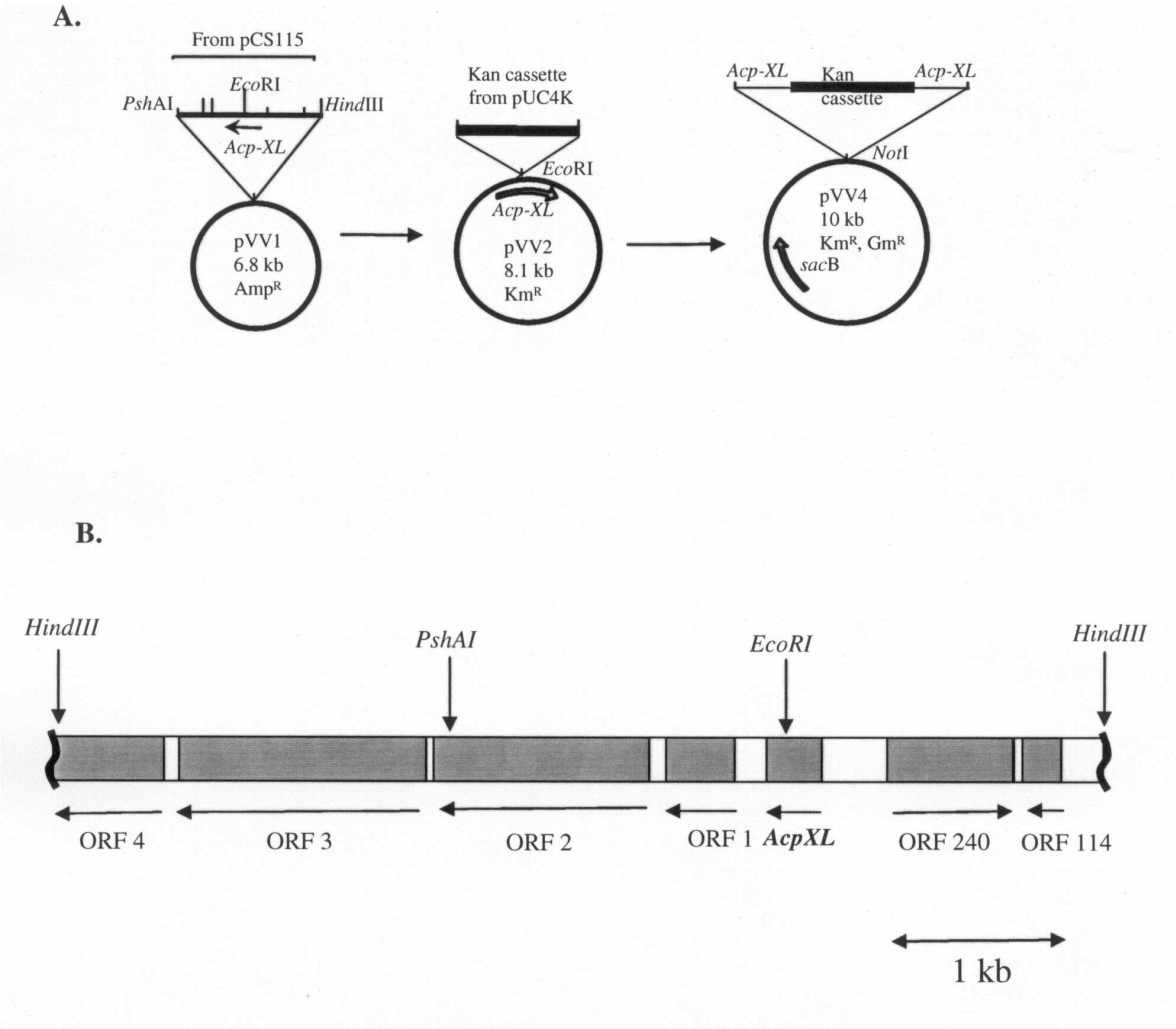

A.
From pCS115
EcoRI
PshAI
HindIII
Acp-XL
pVV1
6.8 kb
Amp^R
Kan cassette
from pUC4K
EcoRI
Acp-XL
pVV2
8.1 kb
Km^R
Acp-XL
Kan
cassette
Acp-XL
NotI
pVV4
10 kb
Km^R, Gm^R
sacB
B.
HindIII
PshAI
EcoRI
HindIII
ORF 4
ORF 3
ORF 2
ORF 1
AcpXL
ORF 240
ORF 114
1 kb

Figure 3.2

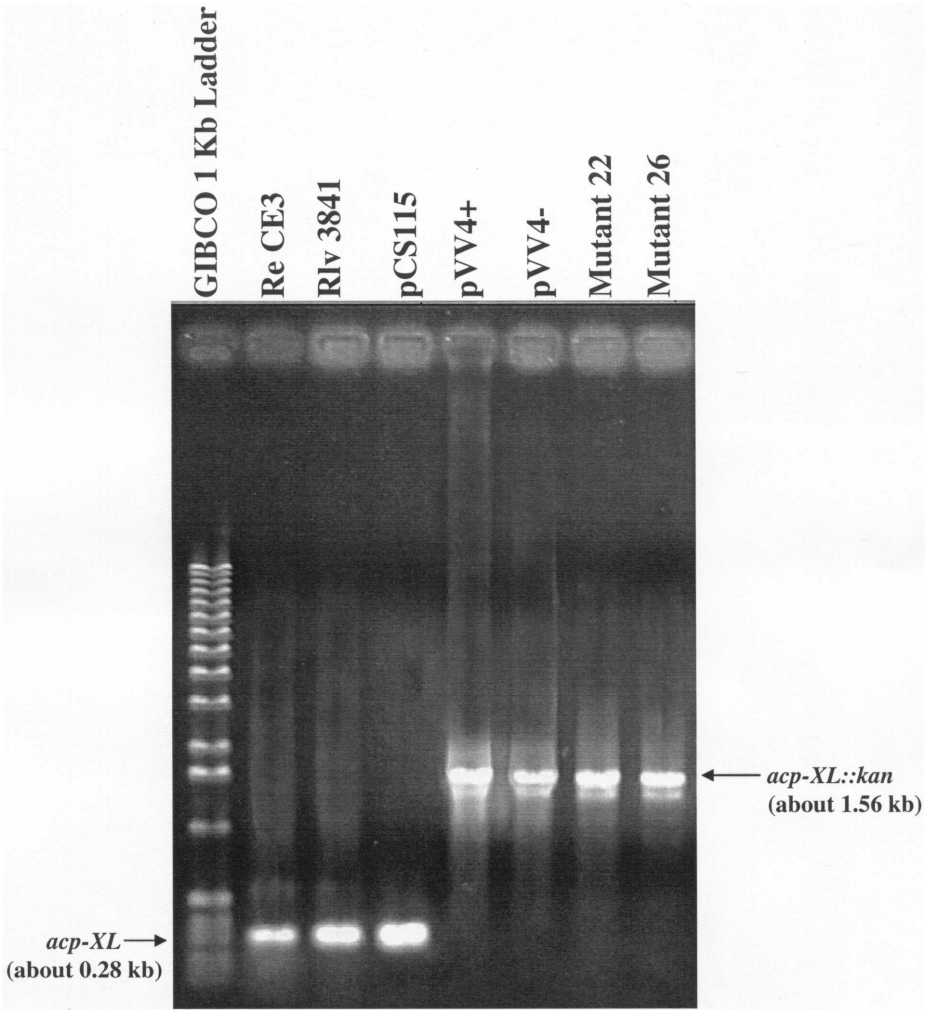

GIBCO 1 Kb Ladder
Re CE3
Rlv 3841
pCS115
pVV4+
pVV4-
Mutant 22
Mutant 26
acp-XL::kan
(about 1.56 kb)
acp-XL
(about 0.28 kb)

Figure 3.3

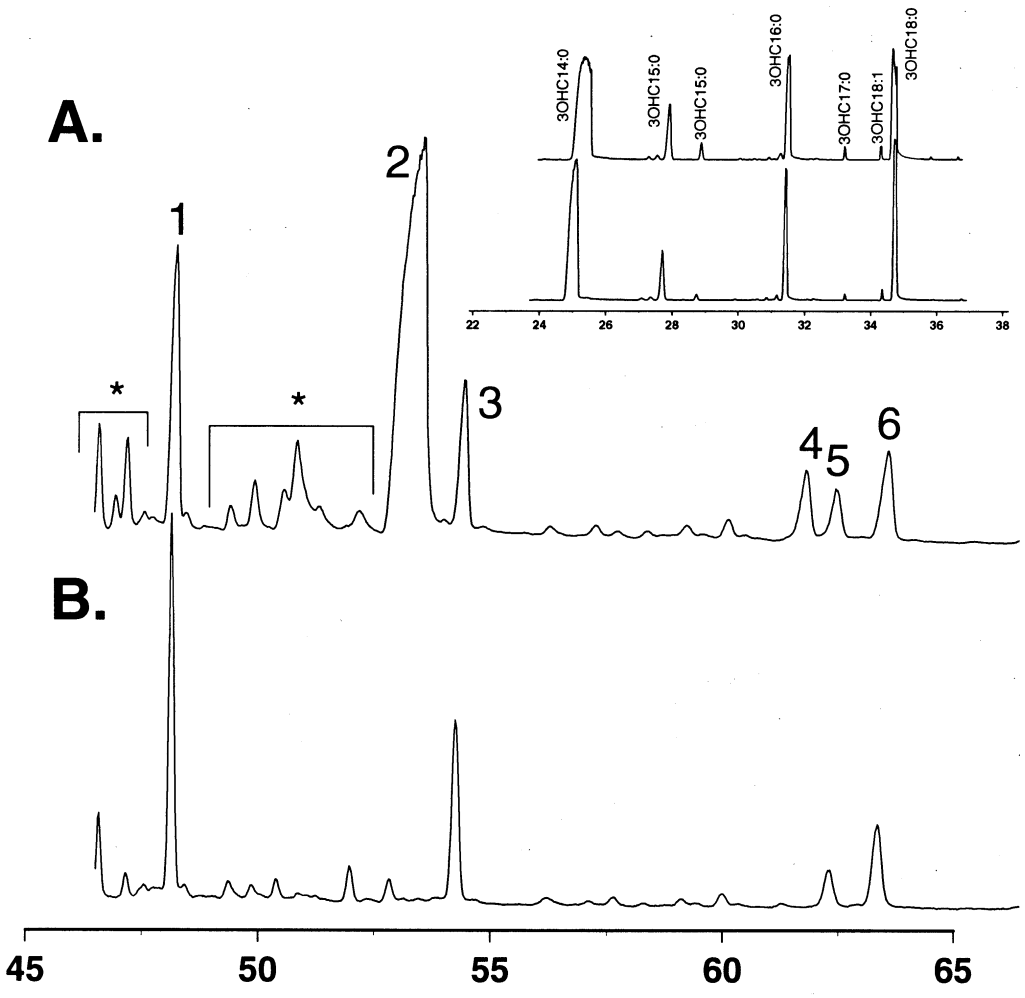

A.
B.
1
2
3
4
5
6
*
*
3OHC14:0
3OHC15:0
3OHC15:0
3OHC16:0
3OHC17:0
3OHC18:1
3OHC18:0
22
24
26
28
30
32
34
36
38
45
50
55
60
65

Figure 3.4

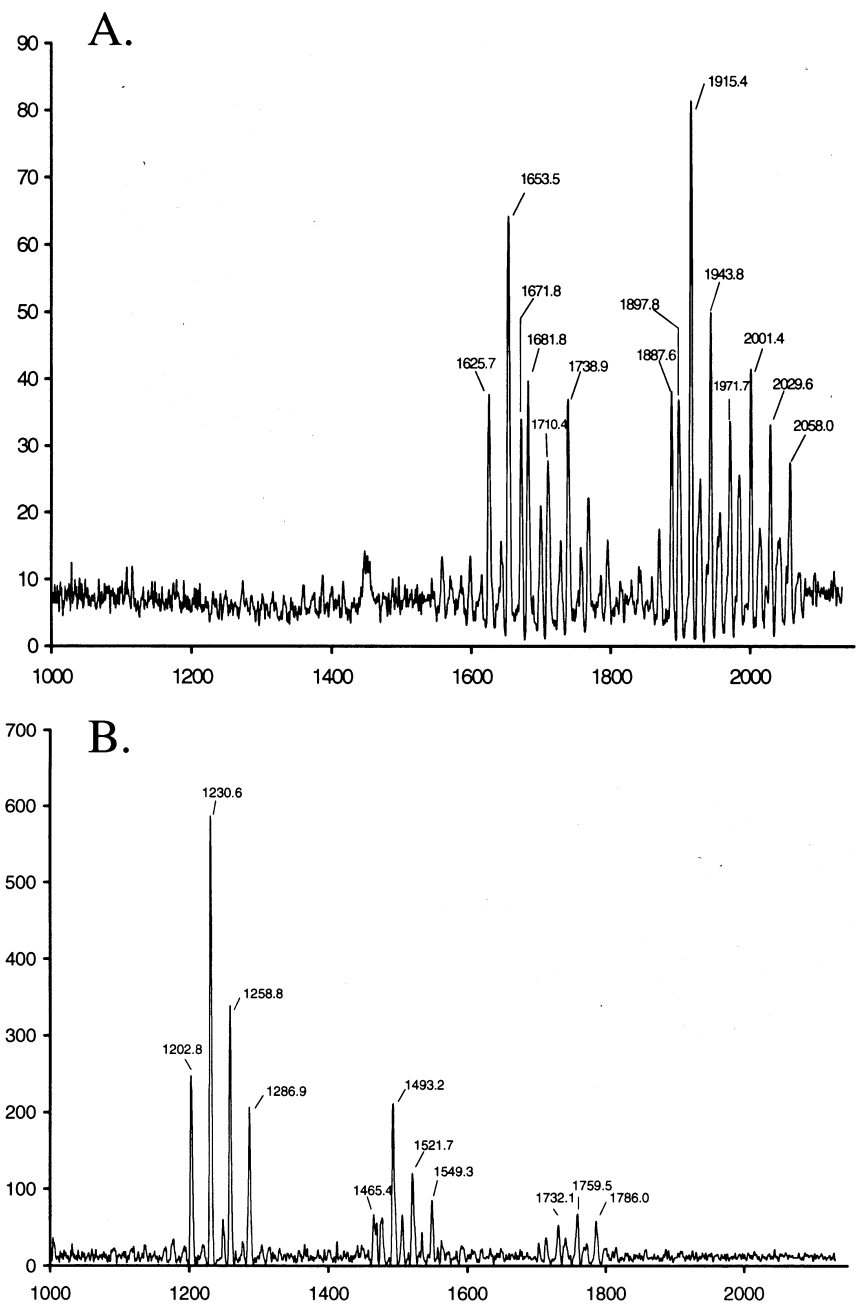

A.
90
80
70
60
50
40
30
20
10
0
1000
1200
1400
1600
1800
2000
1915.4
1653.5
1943.8
1671.8
1897.8
2001.4
1681.8
1887.6
1625.7
1738.9
1971.7
2029.6
1710.4
2058.0
B.
700
600
500
400
300
200
100
0
1000
1200
1400
1600
1800
2000
1230.6
1258.8
1202.8
1286.9
1493.2
1521.7
1549.3
1465.4
1759.5
1732.1
1786.0

Figure 3.5

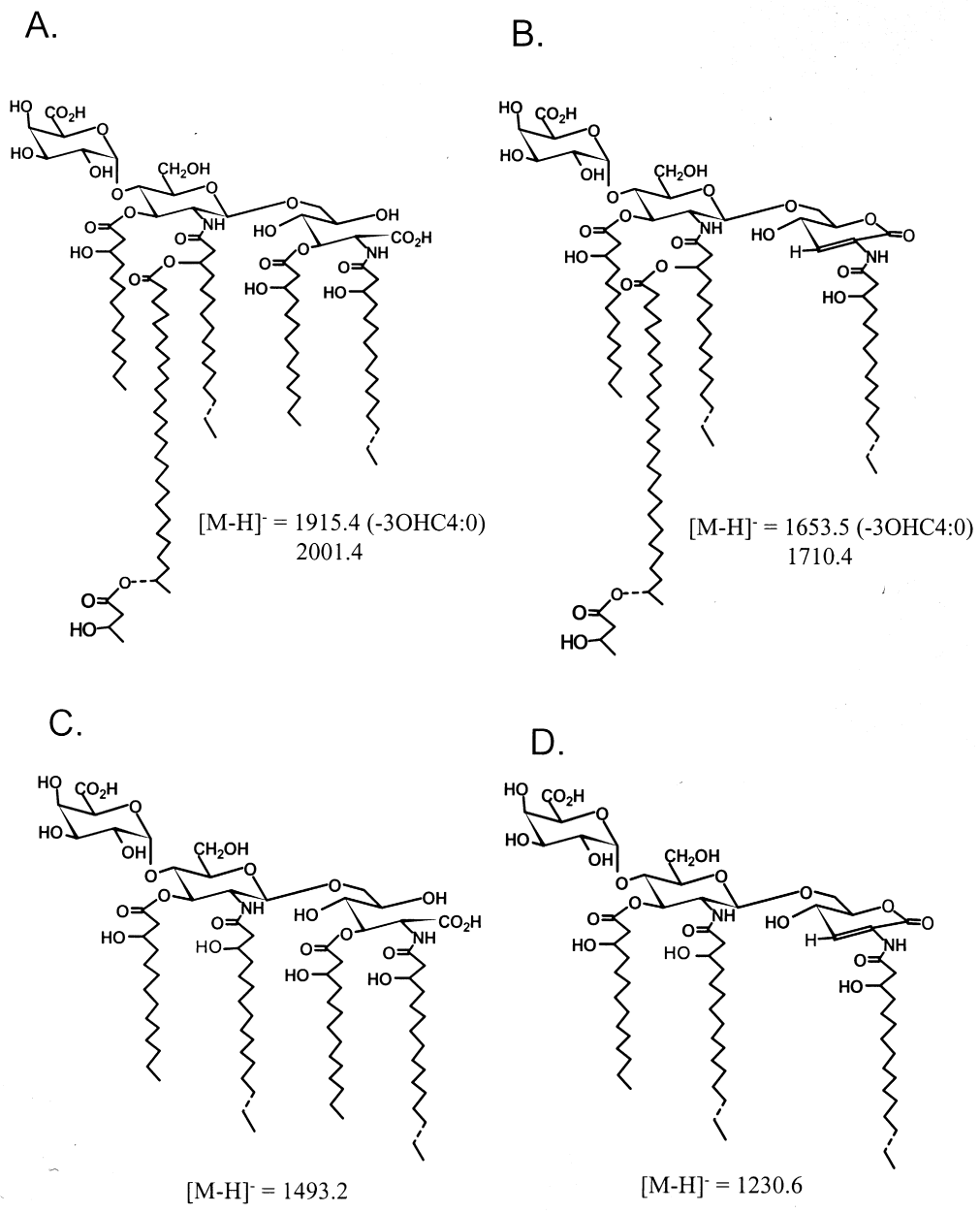

A.
[M-H]- = 1915.4 (-3OHC4:0)
2001.4
B.
[M-H]- = 1653.5 (-3OHC4:0)
1710.4
C.
[M-H]- = 1493.2
D.
[M-H]- = 1230.6

Figure 3.6

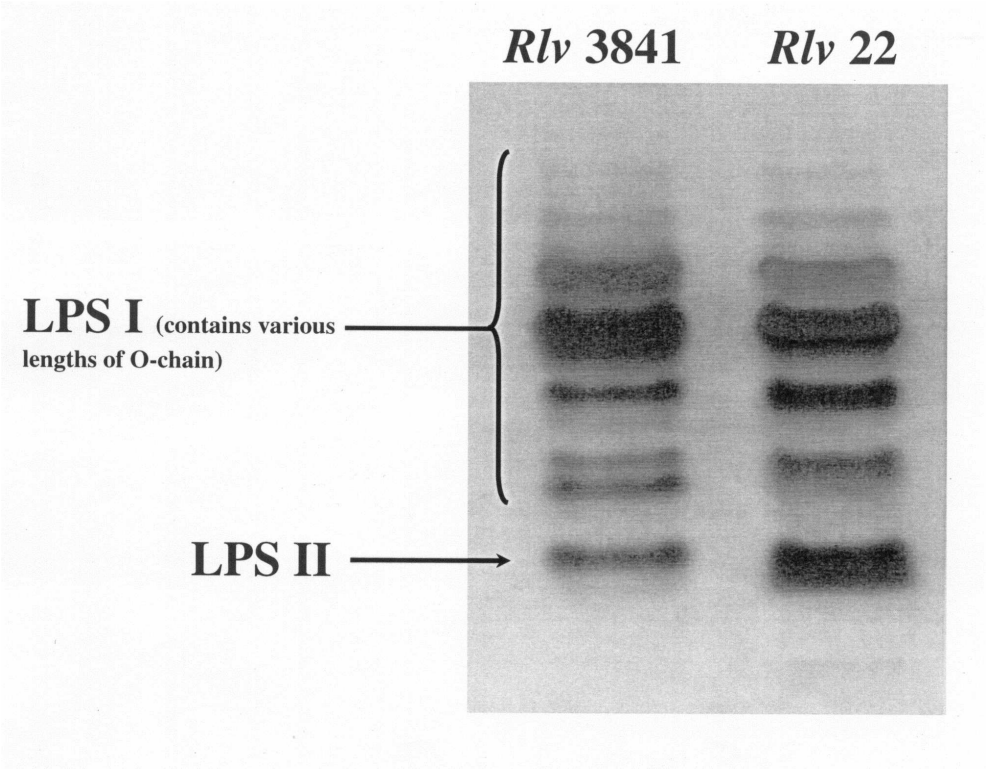

Rlv 3841
Rlv 22
LPS I (contains various lengths of O-chain)
LPS II

Figure 3.7

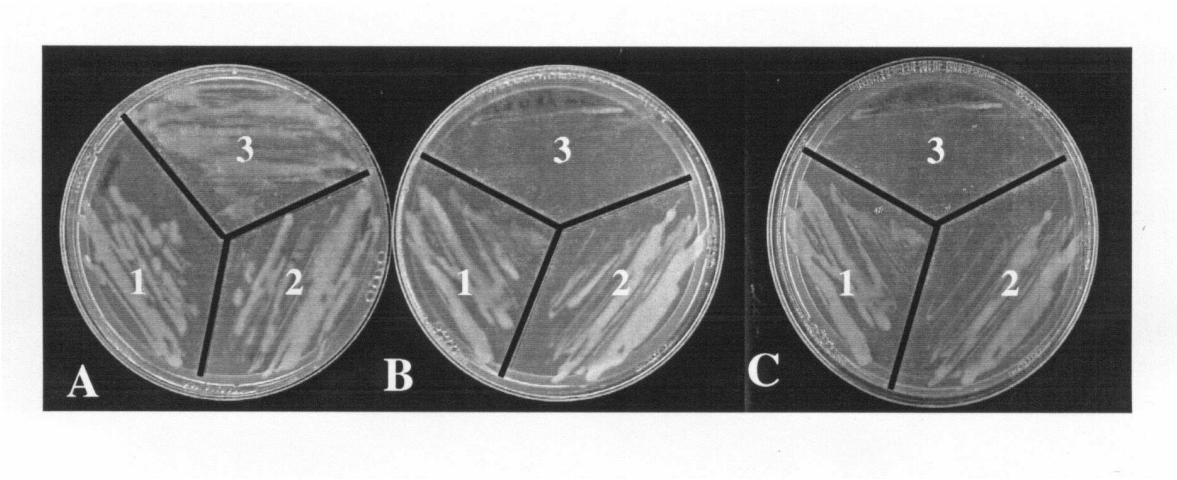


Figure 3.8

CHAPTER 4

A *RHIZOBIUM LEGUMINOSARUM* LIPOPOLYSACCHARIDE LIPID-A MUTANT INDUCES NITROGEN-FIXING NODULES WITH DELAYED AND DEFECTIVE BACTEROID FORMATION[1]

Vedam, V.,[1], Haynes, J.G., Kannenberg, E. L., Carlson, R. W., and Sherrier, D. J. 2004.

Molecular Plant Microbe Interactions. 17:283-291.

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SUMMARY

LPS from pea nodulating strain *Rhizobium leguminosarum* bv. *viciae* 3841, as all other members of the *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.

INTRODUCTION

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 (LPSs) are complex glycolipid molecules that are major constituents of the outer leaflet of the Gram-negative bacterial outer membrane (for a recent review see Raetz and Whitfield 2002). It is well known that LPSs play an important role in determining the virulence of enteric bacterial pathogens (see the review by 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 (EPSs), a more tightly bound acidic, capsular polysaccharide (CPS, or KPS), 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 promote tissue invasion and/or suppress a host defense response (for a review see 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 comprised of 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; Que et al. 2000b). It has

been shown that *E. coli* and *R. leguminosarum* bv. *viciae* (*Rlv*) utilize the same biochemical pathway to catalyze formation of the lipid A precursor molecule Kdo_2lipid-IV_A from UDP-GlcNAc (Price et al, 1994). In later steps of LPS synthesis, the *E.coli* and *Rhizobium* pathways diverge, and *Rhizobium* produces a lipid A that is structurally very different from that of *E. coli* (Bhat et al. 1994; Brozek et al. 1996a; Brozek et al. 1996b; Que et al. 2000b; Que et al. 2000a). 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 Kdo_2lipid-IV_A (Raetz and Whitfield 2002), whereas *R. leguminosarum* puts on a single, extra long fatty acid residue, 27OHC28:0 (Bhat et al. 1994; Que et al. 2000b; Que et al. 2000a). 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 multi-step process wherein rhizobia respond to plant root exudates, and move towards localized regions/sites of the root where they subsequently attach themselves (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 (see reviews by Denarie et al. 1992; Carlson et al. 1994; Long 1996; Schultze and Kondorosi 1998; Spaink 2000; Long 2001; D'Haeze and Holsters 2002). 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 post-meristematic cells and release their bacterial contents into the host cell cytoplasm via an endocytotic-like infection process.

Peas form elongated indeterminate nodules, wherein 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 unwalled 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 symbiosome membrane (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 symbiosome membrane (Kannenberg et al. 1998). All members of the *Rhizobiaceae* that have been examined, with the possible exception of *Azorhizobium caulinodans*, have LPSs 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. 2002). 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 symbiosome membrane 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, grown under controlled environmental conditions and 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, *Rlv* 3841. Nodules formed on the plants inoculated with either the parent or the mutant (Figure 1). Light microscopic analysis of these nodules, showed that the nodule tissue was invaded by bacteria (Figure 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 post-inoculation (Figure 1A). The infection and the nitrogen fixing zones were prominent, contained 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 typical of an indeterminate nodule, but the infection and nitrogen fixing zones were sparsely occupied by the invading bacteria (Figure 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 (Figure 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 days post-inoculation 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 (Figure 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 to the mutant demonstrated statistical differences between the numbers of nodules (Figure 4A). Initially, at day 3, plants inoculated with either parent or mutant produced the same number, 35, of nodules. However, by 5 days post-inoculation, the average number of nodules induced by the mutant was significantly less than by the parent bacteria; 40 nodules/plant vs. 100/nodules per plant, respectively. After 5 days, nodule numbers continued to increase in the mutant-inoculated plants, while in the parent-inoculated plants the nodule numbers remained steady. At 7 days post-inoculation, plants inoculated with the mutant bacteria still had significantly fewer nodules (65 nodules/plant) than in the parent case (110 nodules/plant). By 9 days post-inoculation the nodule counts equalized

between the parent and mutant infected plants, and this trend continued into the last sampling date, 11 days post-inoculation.

In addition to differences in nodule numbers, the size and shape of nodules induced by mutant and wild-type bacteria during the developmental time course 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 mutant were counted and expressed as a percentage of total nodules (Figure 4B). At 3 days post-inoculation, 100% of nodules produced in plants inoculated with either bacterium were unemerged. Two days later, 45% of total nodules on plants inoculated with the mutant were unemerged nodules, whereas only 20% of nodules on plants inoculated with the parent were unemerged. At 7 days post-inoculation, the nodules showed more growth, but the mutant still exhibited statistically fewer emerged nodules (22% of the total nodules remained unemerged) than the parent (10% of total nodules remained unemerged). By 9 days post-inoculation, as observed with nodule numbers, no 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 time point 7 days post-inoculation (Figure 4C). On this sampling date, nodules formed by the mutant measured about 0.6 mm (mean nodule length), compared to 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 days post-inoculation, nodules induced by parent bacteria were beginning to turn pink, while on the same date all mutant-induced nodules remained white. By 9 days post-inoculation, nodules on both sets of plants were pink. To confirm this visual indication that mutant-induced 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 days post-inoculation. However, the mutant nodules consistently had lower levels of activity compared to the parent nodules at all time points (Figure5). The comparison of nitrogenase content was also analyzed by monitoring the amount nitrogenase protein in the nodule during development induced by the parent and mutant stains. Total proteins were extracted from nodules at the different time points, 100 μg protein of each sample was separated by SDS-PAGE, and probed with anti-nitrogenase antibodies (the results are shown in Figure 6). Nitrogenase was not detected in parent or mutant nodules 5 days post-inoculation. The cross-reactive protein was first detected in the parental, but not the mutant, nodule extract 7 days post-inoculation. In contrast a very faint band was detected in mutant nodule extract 9 days post-inoculation, and the protein increased in the mutant nodule extract 11 days post-inoculation, 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 nodules infected with the parent were compared to those infected with the mutant by transmission electron microscopy. Plants were sampled during a developmental time course at 5, 7, 9, and 11 days post-inoculation. At 5 days post-inoculation, 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 post-inoculation, 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 days post-inoculation (Figure 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 (Figure 7A, E). The mutant nodules, in contrast, contained unbranched and abnormally branched, irregularly shaped bacteroids (Figure 7B, C, D, F). There are numerous bulges on the surface of the mutant bacteroids (Figure 7C), unusual branching profiles, and large size (Figure 7F). These features were first detected in nodules at 7 days post-inoculation, occurred more frequently on 9 days post-inoculation, and were very common at 11 days post-inoculation. 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 (Figure 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 symbiosome membrane 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 glycosyl methyl and, possibly, 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 laboratory, 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 comparison to the parent: (i) The mutant was delayed in nodulation as previously reported (Vedam et al. 2003), produced a reduced number of nodules at early post-inoculation time points but caught up with the parent at later time points. (ii) The mutant produced nodules with a delayed onset of nitrogenase production and with reduced nitrogenase at later time points. (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 time points 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 acidic and/or of higher osmolarity, 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 time points. Also the delay in the expression of nitrogenase could be explained in a similar matter. The sensitivity of the mutant to both acidic conditions and increases 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 nine post-inoculation. 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 bi-layer 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 which 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. (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 TY medium at 28°C, as previously described (Vedam et al. 2003). Cultures were supplemented with kanamycin (Kan, 50 $\mu g\, ml^{-1}$), and streptomycin (Str, 5$\mu g\, ml^{-1}$), as appropriate.

Plant nodulation assays: *Pisum sativum* 'Early Alaska' seeds were surface sterilized for 30 sec in ethanol, rinsed four times in sterile water, soaked 3 minutes in 6% sodium hypochlorite (Clorox), and 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 (Difco 214010). The flasks were capped with sterile foam stoppers and 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 replaced to support the shoot. Flasks were wrapped in black plastic and the plants were grown at 21^{o} C, 23% relative humidity, and 78 $\mu mol \cdot s^{-1} \cdot m^{-2}$ light for 14 hours per day.

For inoculation of the plants, parent and mutant bacteria were grown to an optical density of 1.1 at 600 nm, collected by centrifugation, and resuspended in water. Plants, 14 days after sowing, were inoculated with $5x10^{8}$ CFU/plant of either the parent or the mutant.

Nodule counts and measurements

Plants inoculated with parental (*Rlv* 3841) or mutant (*Rlv* 22) bacteria were harvested 3, 5, 7, 9, and 11 days post-inoculation. 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 cleared by placing under vacuum for 15 minutes then soaking at atmospheric pressure for an additional 25 minutes. Root systems were rinsed thoroughly under running water for 2 minutes then allowed to soak in polished water for 20 minutes prior to dissection.

Root systems were scanned systematically under a dissecting microscope and all nodules 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 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 from each time point of the parent or the mutant 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^{o}C, with a 50m by 0.53mm 10-μm, Al_2O_3 – KCl column (Chrompak). The experiment was performed in triplicate.

Light And Electron microscopy--Nodules were collected 3, 5,7, 9, and 11 days post-inoculation for microscopy. Tissues were harvested directly into fixative containing 1% glutaraldehyde (Sigma G5882), 4% formaldehyde in 100mM PIPES buffer, pH 7.0. Larger nodules were cut in half or thirds to aid fixation and infiltration. Tissues were fixed overnight at 4^{o}C with rotation, rinsed three times with room temperature water, post-fixed with 1% OsO4 for 3 hours and were dehydrated in a graded dilution series of acetone. Tissues were infiltrated in Epon 812-Araldite resin and heat cured at 70^{o}C for 48 hours (Mollenhauer 1969).

For light microscopic evaluation, 0.5 µm longitudinal nodule sections were cut with glass or diamond knives (Delaware Diamond Knife) on a Reichert Ultracut E ultramicrotome. Sections were collected onto gelatin-coated glass slides, were counter stained with 0.1% (w/v) basic fuschin, and observed and documented with a Zeiss Axioskop 2 microscope and a Zeiss AxioCam digital camera.

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

Protein Extraction and Western Analysis

Nodules were harvested 5, 7, 9, and 11 days post-inoculation from peas inoculated with the parent or the mutant and homogenized at 4^{o}C in extraction buffer containing 100 mM TRIS, 1 mM EDTA, and 1% (v/v) protease inhibitors (Sigma P9599). Samples were centrifuged at 16.1K x g and supernatant was retained. Proteins were precipitated from the supernatant overnight in 80% acetone at –20^{o}C, and were recovered by centrifugation at 2K x g for 10 min.

Protein content was quantified using the Bio-Rad DC protein assay. Samples were resuspended in Laemmli buffer (Laemmli 1970), and 100 μg of each sample were separated on 12% SDS-PAGE gels using Bio-Rad Mini Protean 3 gel equipment. Separated proteins were transferred to nitrocellulose membrane (Osmonics, Inc) as described (Towbin et al. 1979). Blots were stained with Ponceau S stain (Salinovich and Monterlaro 1986). Protein blots were destained, blocked for 1H in 1% skim milk in TBST, and probed with a universal antibody mixture against dinitrogenase reductase (gift from Dr Paul Ludden, University of California, Berkeley) diluted 1:1000 in 0.1% skim milk in TBST. Blots were rinsed in TBS and incubated with Goat anti-rabbit antibody conjugated to horse radish peroxidase (Sigma). Immunoreactive proteins were detected with enhanced chemiluminescence and documented with X-ray film (Kodak X-OMAT LS).

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 4.1. Light micrographs of nodules at 7 days post-inoculation. (A) Nodule induced by parental strain (3841) and (B) mutant strain (22) of Rhizobium *leguminosarum* bv *viciae*. Regions of the mature nodule include: 1, nodule meristem; 2, prefixation zone; 3, interzone; 4, nitrogen fixation zone. The mature nitrogen fixation zone is not present in most mutant nodules at this developmental stage. Scale bar = 100 μm.

Figure 4.2. Light micrograph of aberrant infection structure in the prefixation zone of a mutant nodule at 7 days post-inoculation. The infection thread (indicated by arrows) contains numerous undifferented bacteroids and lead to cells (arrowhead)with a swollen thread and enlarged invasion structures.

Figure 4.3. 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 = 100μm.

Figure 4.4. Summary of nodule measurement data. Nodules were counted and measured 3, 5, 7, 9, 11 days post-inoculation. (A) Mean number of nodules per plant (n=5), showing statistically fewer nodules in mutant-inoculated plants at 5 and 7 days post-inoculation. (B) Percentage of to 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 days post inoculation. (C) Mean length of all nodules from five plants, showing a difference between parental and mutant nodules at 7 days post-inoculation. Means were separated by Tukey's HSD test, $p \leq 0.05$. Significant differences are indicated by *.

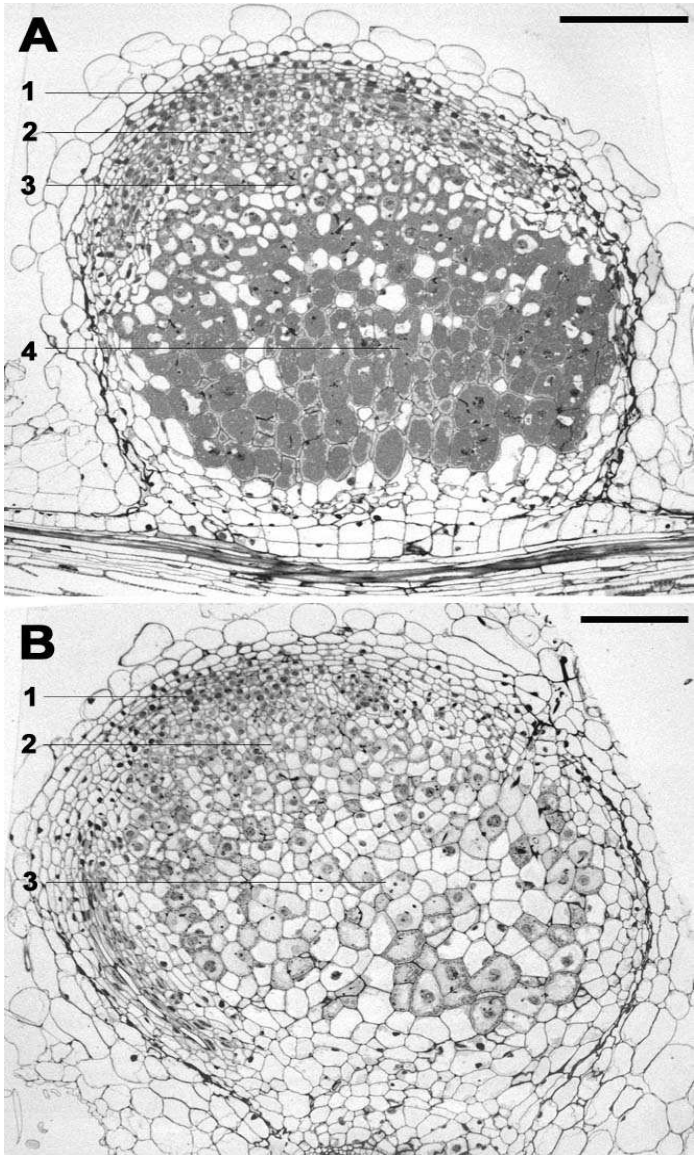
Figure 4.5. Relative nitrogenase activity in mutant versus wildtype nodules.

Figure 4.6. Relative levels of nitrogenase present in nodules the parental strain (3841) or mutant strain (22) of *R. leguminosarum* detected by Western blot analysis. Proteins were extracted 5, 7, 9, and 11 days post-inoculation (dpi). Protein extracts (100 ug in each lane) were separated by SDS-PAGE, transferred to nitrocellulose, probed with anti-nitrogenase antibodies, and visualized using chemiluminescence. A single major reactive band was observed in the range of 31 KDa.

Figure 4.7. Transmission electron micrographs of mutant- and parent-infected nodules.
(A) *Rlv* 3841 bacteroid nine days post-inoculation showing characteristic wild-type branching.
(B) An abnormally branched mutant bacteroids nine days post-inoculation.
(C) Misshapen mutant bacteroid nine days post-inoculation showing numerous bulges Double arrowhead indicate 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 nine days post inoculation. (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 bacteroid contained within one symbiosome unit (indicated by barbed arrowheads). V indicates a vacuole, unusually large for this stage of nodule development. Scale bars = 1µm.

Figure 4.1



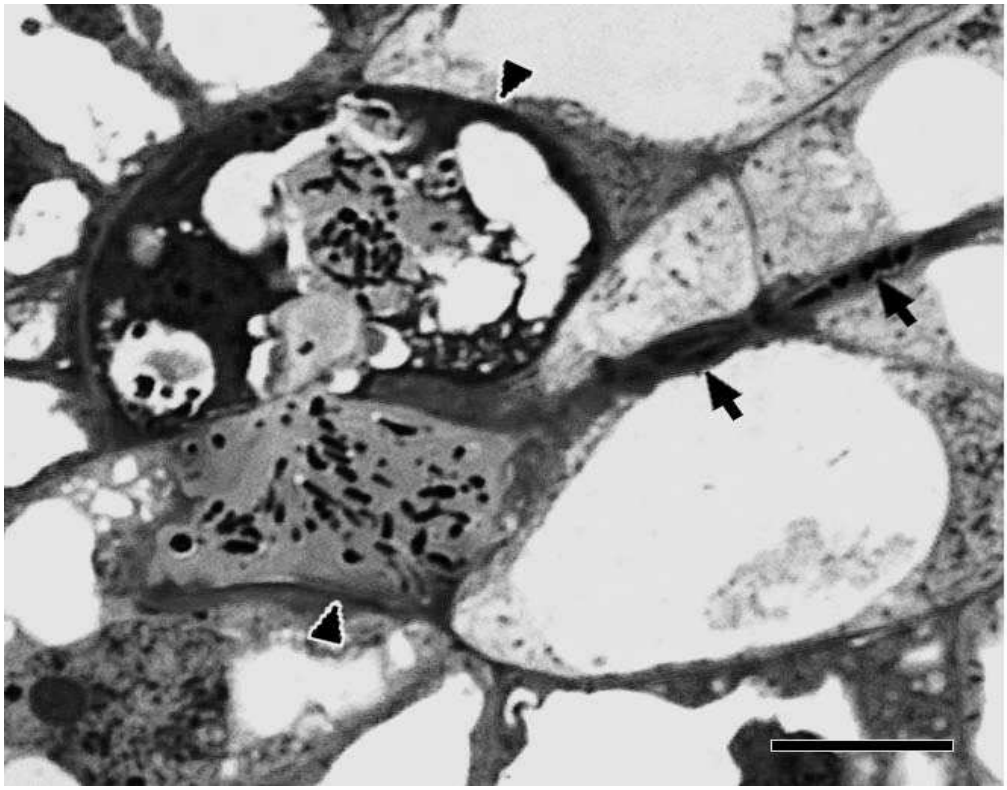


Figure 4.2

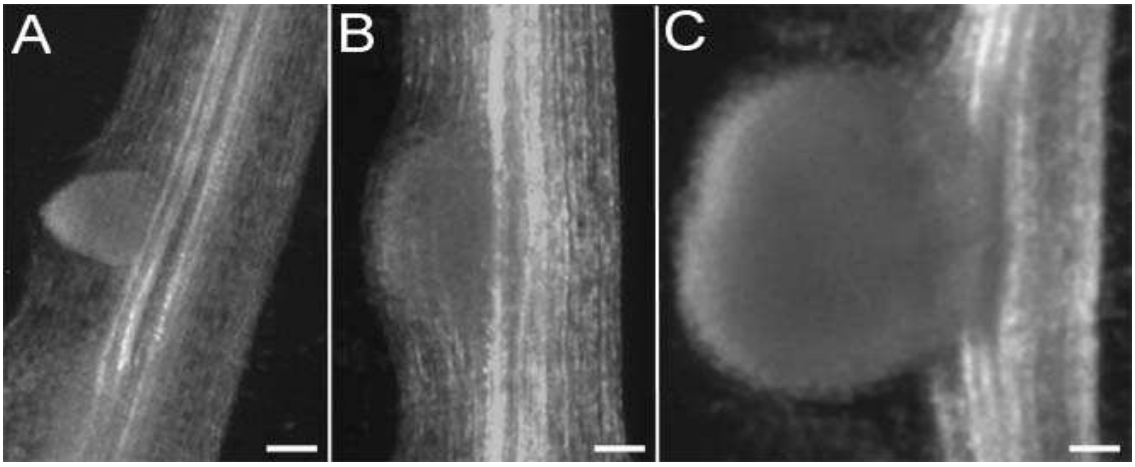


Figure 4.3

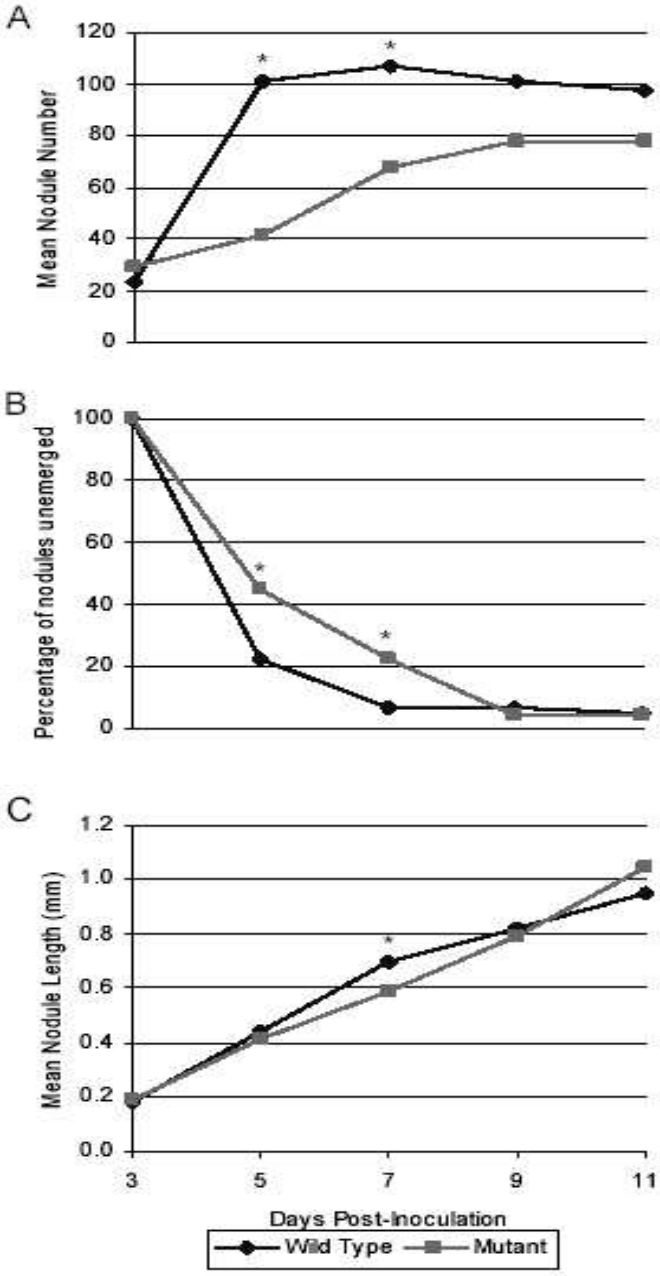

A
Mean Nodule Number
120
100
80
60
40
20
0
*
*
B
Percentage of nodules unemerged
100
80
60
40
20
0
*
*
C
Mean Nodule Length (mm)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
3
5
7
9
11
Days Post-Inoculation
Wild Type
Mutant

Figure 4.4

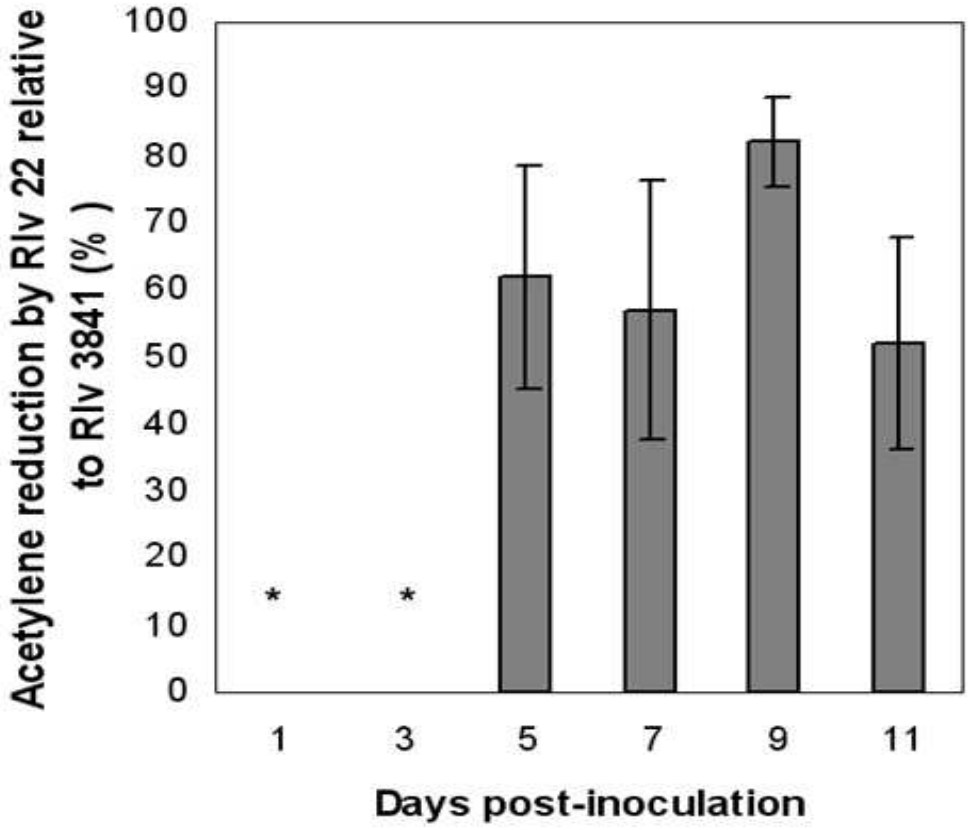

Acetylene reduction by Rlv 22 relative to Rlv 3841 (%)
100
90
80
70
60
50
40
30
20
10
0
*
*
1
3
5
7
9
11
Days post-inoculation

Figure 4.5

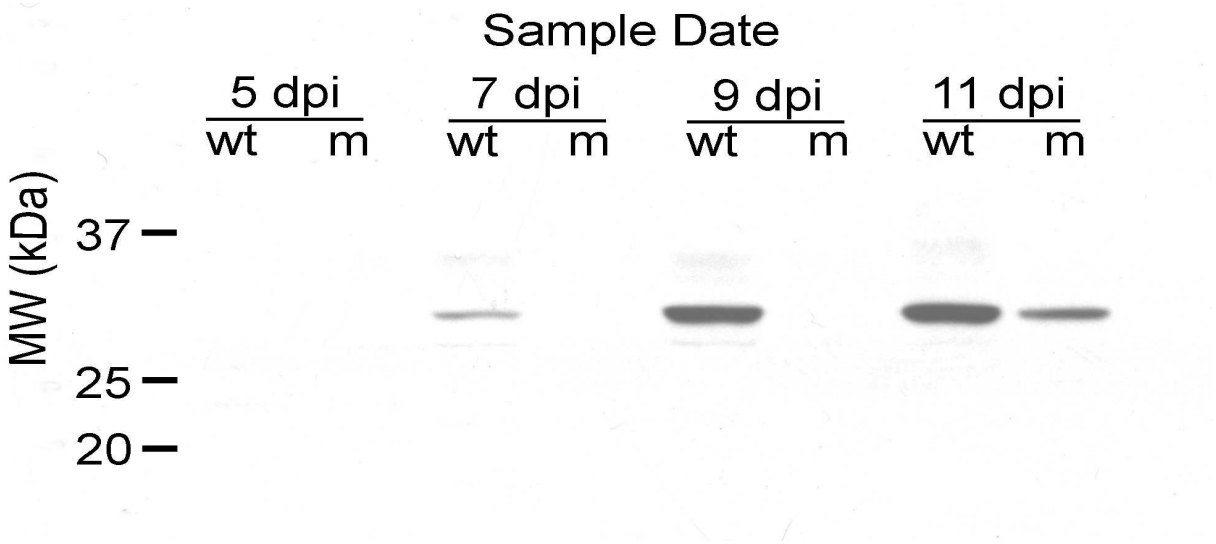

Sample Date
5 dpi
7 dpi
9 dpi
11 dpi
wt m wt m wt m wt m
MW (kDa)
37
25
20

Figure 4.6

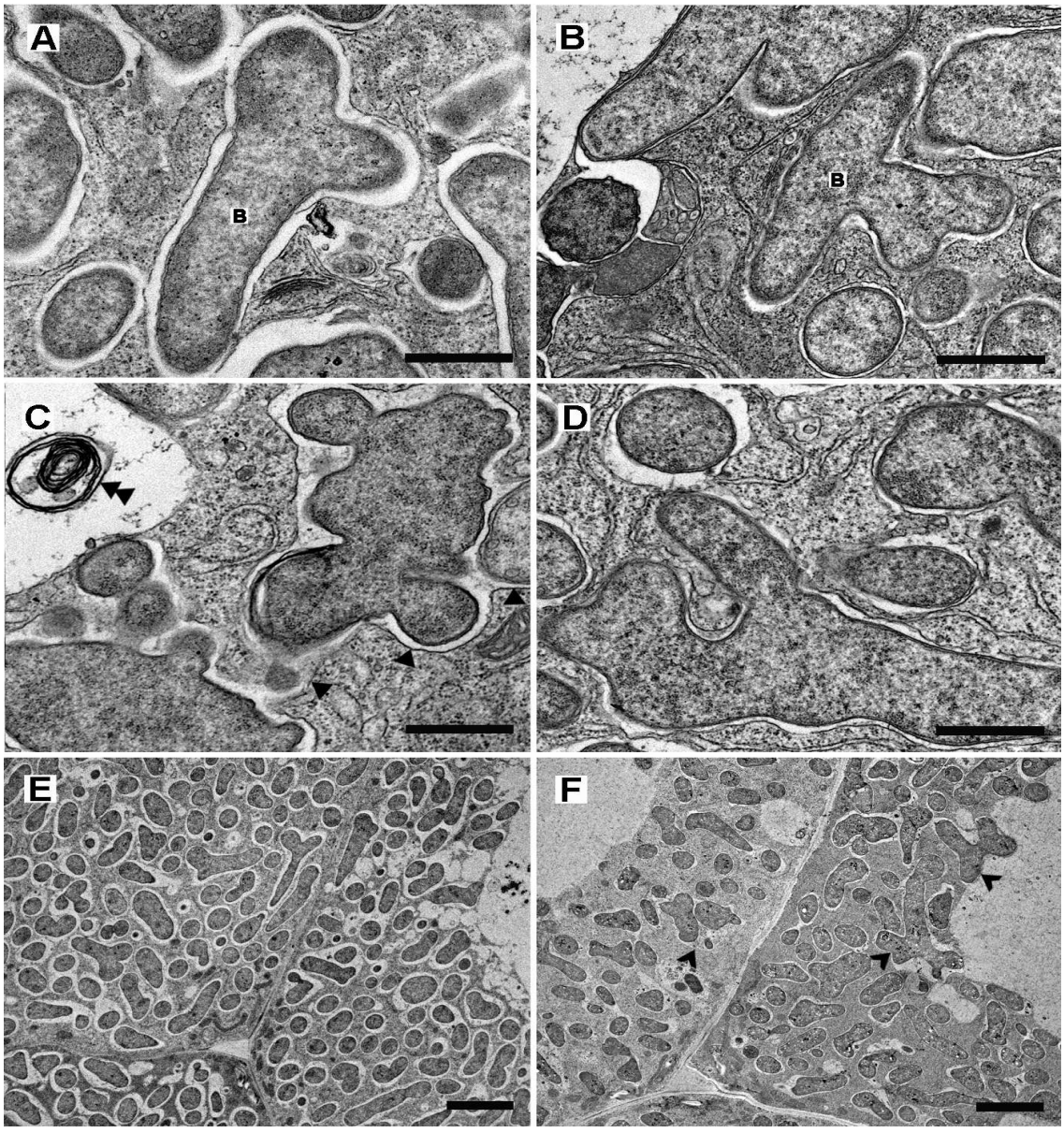


Figure 4.7

CHAPTER 5

A MUTANT IN *acpXL; RHIZOBIUM LEGUMINOSARM* 22, IS CAPABLE OF PARTIALLY RESTORING 27OHC28:0 TO THE LIPID A IN ITS HOST PEA-NODULE ENVIRONMENT[1].

Vedam, V.,[1], Datta, A., Kannenberg, E.L., Haynes, J. G., Sherrier, D. J., and Carlson, R. W. To be submitted.

SUMMARY:

Almost all members of the *Rhizobiaceae* contain a very long chained fatty acid, 27-hydroxyoctacosanoic acid on their lipid As. In previous work, a mutant lacking the specialized acyl carrier protein responsible for transferring the 27-hydroxyoctacosanoic acid residue to the lipid A region was constructed. In studies using this mutant, we have previously shown that despite its altered growth and physiological properties, it was able to infect and invade host plants, although inefficiently, and formed unusual bacteroids, suggesting that this residue is important in the establishment of proper, nitrogen fixing symbiosis, at least in the indeterminate host system. The precise function of this residue in the symbiotic process however, still remains elusive. In this report, we confirm that this residue is essential in the pea-*Rhizobium leguminosarum* 3841 symbiosis. We provide evidence that this fatty acyl molecule is partially restored on the lipid A of LPSs extracted from mutant bacteroids. This suggests that there is perhaps an alternate mechanism for the addition of this residue, when the bacteria are in the nodule environment. Analysis of ex-nodule bacteria from mutant-infected nodules showed restoration of wild type physiological properties (of salt tolerance), despite retention of the original mutation and a lipid A lacking the 27-hydroxyoctacosanoic acid. In this paper, we hypothesize that there is another mechanism for adding on 27-hydroxyoctacosanoic acid to lipid A of *Rlv* 22 bacteroids, which is only switched on *in planta.*

INTRODUCTION:

Rhizobium leguminosarum like most other members belonging to the class collectively called the 'rhizobia' exists either in its free-living state as a heterotroph in the soil, or as an endosymbiont in nodules in the infected host pea plant, where it fixes nitrogen. This endosymbiotic form of the *Rhizobium* is called 'bacteroid'. In contrast to the lab grown/free-living *Rhizobium leguminosarum*, the bacteroid forms are large, can occur as either branched or club-shaped forms, and are individually enclosed by a peribacteroid membrane, which is of plant origin (Newcomb,1979), (Brewin, 1985), Brewin, 1986). The bacteroid cell walls appear thinner, and they may lack the capsular polysaccharide that is present in free-living bacteria. Consequently, bacteroids are more sensitive to osmotic shock and detergents (van Brussel, 1977). Free-living cultures of *R. leguminosarum* have a cell envelope similar to that of other Gram-negative bacteria. Lipopolysaccharide (LPS) is the primary component of the outer leaflet of the outer membrane in *Rhizobium* (Carlson, 1984), and plays a crucial role as a virulence factor in both pathogenic as well as symbiotic gram-negative bacteria. The LPS molecule comprises of three regions: the O-chain polysaccharide, core oligosaccharide, and the lipid A. The lipid region is anchored in the bacterial outer membrane, and the carbohydrate portion projects from the outer surface as the primary immunogenic determinant (Carlson, 1988). Correlating LPS structure to function has been very difficult as LPS is a very complex molecule, and is very heterogeneous in composition. There are pronounced variations in LPS structures from strain to strain, and even within a given strain there are different sizes and compositions of the LPS macromolecules (Carlson, 1984). Interestingly, there are marked differences in the size, distribution and antigenic properties between LPSs from free-living cultures with those from nitrogen-fixing bacteroids (Brewin, 1986), (VandenBosch, 1989).

The lipid A from *Rhizobium leguminosarum* has a glycosyl residue backbone, and is acylated with various hydroxylated fatty acids of different chain lengths (Bhat, 1994). One of these fatty acids is a very unique, very long chained fatty acid, 27-hydroxyoctacosanoic acid (27OHC28:0) (Hollingsworth, 1989). During the formation of symbiotic nodules, rhizobia are put under considerable stress, as the process is very demanding, and the bacteria are faced with a number of different extra- and intracellular plant environments (Kannenberg, 2001). Initially, rhizobia are contained extracellularly within the infection thread environment, but are finally enveloped by the cytoplasmic membrane of the root nodule cells, and are released into a symbiosome which is a specialized, organelle that is present in the interior of nodule cells (Brewin, 1998). In pea nodules, rhizobia multiply synchronously with the plant-derived peribacteroid, and eventually develop into mature, pleiomorphic, nitrogen-fixing bacteroids (Kannenberg, 1998), (Brewin, 1998).

Inside the symbiosome compartment, the *Rhizobium* must be able to adapt to the low oxygen, and acidic environment. Hence, the symbiosome environment is stressful to the rhizobia, and they need to be equipped with specialized metabolic and structural features that help them function in the changing environments that they face during the process of symbiosis (Brewin, 1991). Since the primary contact between the *Rhizobium* and its environment occurs at the rhizobial cell surface, it is easy to envision that the bacterial surface molecules are involved intimately in the infection process. It has been shown that rhizobial LPS undergoes structural modifications during the formation of bacteroids, and there are composition differences between the bacterial and bacteroidal LPSs (Brewin, 1986), (Bhat, 1992). Such differences in structure have also been detected using LPS-specific monoclonal antibodies (Kannenberg, 1998), (VandenBosch, 1989). Variation in LPS structures have been studied in free-living rhizobia in

response to environmental conditions such as oxygen, pH, carbon sources, and plant-derived compounds (Kannenberg, 1989), (Bhat, 1992), (Noel, 2000a). Such studies have shown that cues from the environment play an extremely important role in LPS expression, and lead to the hypothesis that LPS expression in the soil and inside the root nodule of the legume is probably controlled to a large extent by the microenvironmental conditions. LPS modifications that are induced by physiological conditions such as low oxygen and low pH can be simulated in lab grown rhizobia by altering the growth conditions appropriately (Kannenberg, 1998). Inside pea nodules, rhizobia face two very different environmental conditions; those in the infection thread, and those in the symbiosome (Brewin, 1991). In the symbiosomes, the mature bacteroids are not encapsulated, and this allows for direct contact between the bacterial cell surface and the plant membrane. Additionally, as previously mentioned, bacteroids face low oxygen conditions and likely acidic conditions in the symbiosomes (Brewin, 1991). In a study by Kannenberg and Carlson (2001), *Rhizobium leguminosarum* 3841 was cultured under different growth conditions, and the LPS structural modifications were analyzed chemically and immunochemically (Kannenberg, 2001). It was observed that the LPS extracted from nodule bacteria was much more hydrophobic that the LPS from free-living rhizobia (Kannenberg, 2001). Further, a doubling of 27OHC28:0 in the lipid A of *Rlv* 3841 grown under low oxygen conditions has been reported (Kannenberg, 2001) indicating that similar conditions of low oxygen within the nodule may cause a doubling of this residue in the bacteroid lipid A. This increase may be required for the increased stability and barrier properties of the bacteroid within the symbiosome compartment, as conceptually, this fatty acid residue has the capability to span the entire lipid bilayer of the bacterial outer membrane (Kannenberg, 2001). Such an increase in lipid A fatty acylation has also been reported for *Salmonella* where also the lipid A acylation pattern is

perhaps important in providing outer membrane barrier properties (Guo, 1998). Other LPS structural modifications contributing to the increase in hydrophobicity include changes in O-chain carbohydrate composition, methylation, and O-acetylation. The expression of a hydrophobic LPS in bacteroids implies that the molecule could be part of a (non-specific) hydrophobic interaction. Hydrophobic patches on the bacteroid surface could be involved in non-specific plant-bacterium attachment by mediating close proximity and adhesion at the plant-bacterium interface (Kannenberg, 2001). In pea symbiosomes, there is indication of increased intimacy between the plant-bacterium interface (Brewin, 1991), (Brewin, 1998). This fact, coupled together with the increased levels of hydrophobicity observed in the LPS of invading bacteria and bacteroids suggests that the hydrophobic regions of the mature bacteroids "lock" into similar areas/patches on the plant cell (Kannenberg, 2001). This type of interaction would mean that a number of functions such as the endocytotic process, synchronous cell division within symbiosomes, protection of the bacteroids and many more would be affected (Kannenberg, 2001).

An alteration in the hydrophobic nature/content of the LPS would mean that the finely tuned machinery leading to the intimate plant-bacterium interaction is disrupted, thereby resulting in a dysfunctional symbiosome compartment. In this paper, we present the results from a study that was performed with a previously constructed *Rhizobium leguminosarum* 3841 lipid A mutant lacking 27OHC28:0 (Vedam, 2003). A partial characterization of the requirement and role of this fatty acid in the *Rhizobium leguminosarum*-pea symbiosis is presented. In this study, bacteroidal LPS (lipid A) from both the parent and mutant strains were analyzed. Previously, it had been difficult to obtain sufficient LPS material from bacteroids to perform biochemical analyses (Carlson, 1984). However, we were able to generate sufficient amounts of bacteroidal

LPS by mass growing peas using an aeroponics growth system and inoculating roots with rhizobial cultures in a controlled environment. From the nodules that were generated by this method, we were able to prepare sufficient bacteroid material, and extract LPS for biochemical analysis.

Chemical communication between rhizobia and the plant host is extremely crucial in the development of the rhizobial-legume symbiosis (Brewin, 1991). Bacterial nod genes are induced by flavonoid compounds that are produced by the plant. The induced nod genes direct synthesis of the lipochitooligosaccharide Nod factors (Carlson, 1994), (Long, 1996), (Reuhs, 1994), (Noel, 1996). Structural changes occur in the LPS during the symbiotic infection process, and also in the presence of root compounds (Noel, 1996). Since it is possible that there is an induction of LPS modification in the presence of Nod gene inducing compounds, it was of interest to determine whether cultures (wildtype and mutant) exposed to hesperitin, a nod-gene inducing compound would trigger changes in LPS structure. Therefore, both the parent and its mutant lacking 27OHC28:0 were grown in the presence of hesperitin and their LPSs isolated and analyzed.

In order to exclude the possibility of a genetic reversion, we tested nodule extracted mutant bacteria for retention of the original *acpXL::kan* mutation. We also analyzed a few ex-nodule isolates for their physiological phenotype, particularly their growth response to the presence of NaCl in the medium, which was a condition used in previous work. Additionally, LPS from the isolates was prepared, and analyzed chemically for the 27OHC28:0.

MATERIALS AND METHODS:

Bacterial strains and culture conditions

The strains used are listed in Table 1. Strains were cultured on solid or liquid tryptone yeast extract (TY) medium (Difco laboratories), as previously described (Vedam, 2003). Cultures were supplemented with kanamycin (Km, 50 $\mu g\, ml^{-1}$), and streptomycin (Str, 5$\mu g\, ml^{-1}$), as appropriate. The osmotic tolerance of ex-nodule isolates was studied by growing two strains on TY+0.5% NaCl at 30ºC, along with the appropriate controls.

To analyze changes in structure of LPS in the presence of a Nod gene inducer, cultures were supplemented with Hesperitin as appropriate.

Isolation of ex-nodule strains

Root nodules from several pea plants inoculated with *Rlv* 22 were surface sterilized with 95% ethanol. Nodules were crushed to extract the bacteria. The extracted bacteria were plated on TY medium, and single colonies with distinctly different morphologies were selected and tested for their resistance to Km, and NaCl. Bacterial colonies that were resistant to both Km and NaCl were preserved for further physiological and biochemical analyses. The two ex-nodule strains isolated were verified by PCR with primers to *acpXL* (GAGGGGGTTTAAATAGTCA and AGGCTTGGCCGCTTTGA) as previously described by Vedam et al, (Vedam, 2003).

Growth condition of peas

Peas (*Pisum sativum* bv. Early Alaska) were surface sterilized, and allowed to germinate in the dark for 24h. Pea seedlings were sown in ‘caissons’ and supplied with Lullien’s nutrient solution [devoid of ammonia]. Caissons provide controlled conditions for the growth of peas. After allowing one week for germination and stabilization of the pea seedlings, the peas were inoculated with either *Rlv* 3841 or *Rlv* 22. The pea plants were allowed to nodulate for three

weeks, and the caissons were periodically refreshed every week with nutrient solution. At the end of the growth period, the nodules were harvested for preparation of bacteroid material.

Extraction of bacteroids from pea nodules

Nodules were harvested from pea plants inoculated with either *Rlv* 3841 or *Rlv* 22 into an ice cold solution of 0.5M sucrose in 50mM Tris-HCl pH 7.4, + 10mM protease inhibitors[2]. Nodules (and approximately equal volume of PVPP [polyvinylpolypyrrolidone]) were ground and the nodule homogenate filtered and centrifuged at 11,000 rpm for a minute. The pellet containing the symbiosomes was resuspended in a solution of 0.5M sucrose in 50mM Tris-HCl pH 7.4, 10mM protease inhibitors[2], and overlayed on a solution of 1.5M sucrose in 50mM Tris-HCl pH 7.4, 10mM protease inhibitors[2] and centrifuged at 8000 rpm for 30 seconds. The top phase and the interphase were collected, and centrifuged at 10,000 rpm for 90 seconds. The pellet was resuspended in a solution of 0.5M sucrose in 50mM Tris-HCl pH 7.4, 10mM protease inhibitors[2], and centrifuged at 10,000 rpm for a minute, resuspended in 500 µL 50mM Tris-HCl pH 7.4, 10mM protease inhibitors[2], and then vortexed to disrupt the peribacteroid membranes. After centrifugation at 10,000 rpm for a minute, the pellet containing bacteroids was collected and stored at –80ºC.

Lipopolysaccharide extraction and purification

LPSs were extracted by the triethylamine (TEA)/ethylene diamine tetraacetic acid (EDTA)/phenol (Φ) procedure as previously described (Ridley, 2000).

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[2] Proteinase inhibitor cocktail:p-amino benzamidine @ 10mg/mL:50uL/mL, APMSF 1mg/mL : 50uL/mL, phosphoramidon 5mg/mL : 5uL/mL, E64 10mg/mL: 1ul/mL, pepstatin 2mg/mL:1uL/mL.

Briefly, for each strain the LPS was extracted from the bacterial pellet using 3x volume of TEA/EDTA/Φ (0.25M EDTA, 5% phenol, titrated to pH 6.9 with TEA with constant stirring for 1 h at 37ºC. The extract was centrifuged at 10,000 rpm for 1 h and the supernatant was collected and dialyzed (10,000 MWCO; Spectrapor) against deionized water. The bacterial and bacteroidal LPSs were lyophilized for analysis. The LPS isolated by this procedure from the Nod gene induced cultures were subject to further purification by polymyxin-agarose affinity chromatography (Ridley, 2000), (Forsberg, 1998).

Analytical procedures

Lipid A was isolated from the LPS preparations by mild acid hydrolysis (Caroff, 1988). Briefly, the LPS was dissolved in 1% SDS in 20 mM sodium acetate, adjusted to pH 4.5 with 4 M HCl, and then placed in an ultrasound bath until the sample was dissolved. This solution was then heated at 100 °C for 1h, followed by lyophilization.

The SDS was removed by washing the lyophilized residue with a solution of 2:1 deionized H_2O:acidified ethanol (100 μl 4 M HCl in 20 mL 95% ethanol).

The residue was collected by centrifugation, washed again with 95% ethanol (non-acidified), and collected by centrifugation (200 ×g for 15 min). The washing and centrifugation steps were repeated. Lastly, the residue was lyophilized to give a white, solid, fluffy lipid-A preparation.

Sugar composition was determined by GC-MS analysis of alditol acetates and trimethylsilyl methyl glycosides as described by York et al (York, 1985).

Matrix-assisted laser desorption ionization (mass spectrometry) (MALDI-TOF/MS) was performed using a Hewlett Packard MALDI-time of-flight (TOF) spectrometer system in the negative-ion reflectron mode with a 337 nm nitrogen laser, operating at a 20–kV extraction voltage, and with time-delayed extraction.

Approximately 2 µl of a 1 mg/ml lipid-A solution in chloroform: methanol (3: 1, v/v) was mixed with 1 µl of trihydroxyacetophenone matrix (THAP) solution (~ 93.5 mg of THAP/1 ml of methanol) and applied to the probe for mass analysis. Spectra were calibrated externally using *E. coli* lipid-A (Sigma).

HPAEC analysis of core oligosaccharide

Oligosaccharides derived from the mild acid hydrolysis (1% acetic acid at 100^{o}C for 1 h) of the LPS from *Rlv* 3841, *Rlv* 22, *Rlv* 3841+hesperitin, and *Rlv* 22 + hesperitin were subjected to High Performance Anion-exchange chromatography as described by Reuhs et al (Reuhs, 1994) and Carlson et al. (Carlson, 1995).

RESULTS:

Physiological properties of Ex-nodule strains from Rlv 22- infected pea plants:

In order to rule out the possibility of a genetic reversion of the *acpXL* mutation in planta, bacteria extracted from nodules infected by *Rlv* 22 were tested periodically for resistance to kanamycin, the antibiotic marker cassette introduced into the *acpXL* in the original construct (Vedam, 2003). Approximately a total of 500 colonies were screened (at different time points in the course of all of the studies) and there was no loss of the resistance marker. In addition, chromosomal DNA from a few of these colonies (ca. 12) were tested by PCR, using primers to the *acpXL*. All twelve of the colonies tested by PCR showed that the mutation was still intact. Further, chromosomal DNA from two of these colonies was analyzed by Southern hybridization using amplified *acpXL* as the labeled probe, and results from this experiment showed that the nodule recovered bacteria retained the intact mutation (data not shown). The ex-nodule strains were isolated as described in the Methods section above. Previously, the original *acpXL* mutant was examined for its ability to

adapt to different physiological stresses such as those within nodules. The mutant was tested for its ability to grow in increased osmotic conditions, and decreased pH, and it was established that its growth was abolished at 0.5% NaCl, and at a pH value of 5.0 (Vedam, 2003). Sixteen ex-nodule isolates were grown in the same test conditions as the original mutant. It was observed that all of the ex-nodule isolates were able to grow in the presence of 0.5% NaCl in the medium (Fig. 1). PCR was performed on the genomic DNA of two ex-nodule isolates, using primers specific to *acpXL*. The PCR product of the mutated gene region from the original mutant *Rlv* 22 was 1.56 kb in size, consistent with the combined size of the *kan* cassette and *acpXL*, and was the same for the ex-nodule isolates EN2, and EN4 (Fig. 2).

Lipopolysaccharide extraction and characterization:

From our previous work, it was established that the LPS yields from both parent and mutant were increased with TEA/EDTA/ϕ extraction. Hence, we employed this method for the extraction of LPS from bacteroids. In order to determine if the mutation in *acpXL* resulted in bacteroids with LPS that lacked 27OHC28:0, LPS was prepared using the above mentioned method from the bacteroids extracted from pea root nodules formed by *Rlv* 3841, and *Rlv* 22.

The lipid A from LPS preparations of parent and mutant bacteroids were analyzed by MALDI-TOF mass spectrometry. The results are shown in Fig. 3. The mass spectrum of the lipid A from the parent strain *Rlv* 3841 (Fig. 3A) shows two clusters of ions, one ranging from m/z 1887.6 to 2058.0 with the most intense ion at m/z 1914.0, and the other ranging from m/z 1625.7 to 1738.9 with the most intense ion at m/z 1652.0. The ion at m/z 1914.0 is consistent with the published structure (Vedam, 2003), in which the lipid A has a disaccharide backbone consisting of a distal glucosaminosyl residue β-1,6 linked to a proximal 2-aminogluconate residue. The 4’

of the distal glucosamine is substituted with a α-galacturonosyl residue, and the glucosamine/2-aminogluconate disaccharide is acylated with β-hydroxy fatty acids at the 2, 3, 2' and 3' positions. The 27OHC28:0 molecule is present as a secondary acyloxyacyl residue, and is ester linked to the hydroxy group of the 3'- β-hydroxy fatty acid residue. The ions of m/z 2001.4 and above are due to molecules in which the 27-OH group of the 27OHC28:0 molecule is esterified with β-hydroxybutyrate. This is consistent with previous reports of the *Rlv* bv. viciae lipid A structure (Bhat, 1994), (Que, 2000b), (Que, 2000a). Other ions in this cluster are due to variations in the structure due to different acyl chain lengths. In the second ion cluster, the most intense ion is *m/z* 1652.0 and is due to a structure caused by the elimination of the β-hydroxy fatty acyl residue from the 3-position of the 2-aminogluconate residue; a reaction which may be an artefact of the lipid A isolation procedure (Vedam, 2003), (Jeyaretnam, 2002). The structures corresponding to the wildtype lipid As are shown as structures I and II in the lower panel of Fig. 3. The mass spectrum of mutant strain *Rlv* 22 (Fig. 3C) also shows two main ion clusters, one centered around ion *m/z* 1493.0, and the other around ion *m/z* 1230.0. A third, minor cluster is centered on ion m/z 1758.0. The two main ion clusters are due to structures similar to those described in the parent strain, only devoid of the 27OHC28:0, and β-hydroxybutyrate. These structures are shown as III and IV, and are identical to the previously reported structure for the mutant lipid A (Vedam, 2003). We had also reported that the minor cluster of ions surrounding *m/z* 1758.0 was due to the addition of a palmitic acid residue. These ions were observed again in the current study.

Figure 3B and 3D shows the mass spectra of the bacteroid lipid A from *Rl*v 3841 and *Rlv* 22, respectively. The mass spectrum of *Rlv* 3841 bacteroid lipid A is identical to the parent, lab-grown lipid A structure with two major ion clusters (Fig. 3A). The spectrum of *Rlv* 22

bacteroid lipid A is shown in Fig. 3D. This mass spectrum shows four major ion clusters. Three of the clusters are identical to those observed in the lipid-A from laboratory-grown *Rlv* 22 (Fig. 3C). The fourth ion cluster is centered around *m/z* 1914.0, and is identical to the second ion cluster from the lab and bacteroid *Rlv* 3841 lipid A spectra. This indicates that the 27OHC28:0 moiety is present in this mutant bacteroid lipid A preparation. Also present in the *Rlv* 22 bacteroid lipid A preparation are structures which lack 27OHC28:0 but contain an added palmitoyl residue, which was observed in the lab grown lipid A preparation. In summary the lipid-A preparation from the *Rlv* 3841 bacteroids appears to have the same structures as from laboratory grown cells while the *Rlv* 22 mutant bacteroid lipid A preparation consists appears to be partially restored in the addition of 27OHC28:0.

In order to determine whether the two ex-nodule isolates EN2 and EN4, which have regained the parental level of salt tolerance and have retained the original *acpXL* mutation in the chromosome, contain LPSs that lack the 27OHC28:0 residue, lipid A was prepared from these two strains and analyzed by MALDI-TOF/MS. The results are shown in Fig. 4. The mass spectrum of the lipid A from *Rlv* 22 was as described above (See Fig. 4A and Fig.3C). The mass spectra of the lipid As from EN2 and EN4 were identical to one another, and to that of the mass spectrum of the lipid A from *Rlv* 22 i.e. they all lack the 27OHC28:0 residue. The minor difference in the spectra of the two ex-nodule isolated lipid A preparations was that the third, minor ion cluster centered around *m/z* 1786.0 was significantly less intense compared to the lipid A of the mutant preparation. As discussed previously, the ions in this minor cluster are approximately 238 mass units larger than the corresponding ions in the *m/z* 1493.0 cluster, and are probably due to the addition of a palmitoyl residue (Vedam, 2003). In order to study whether the LPS produced by *Rlv* 22 is altered by growth in the in the presence of the *R. leguminosarum*

nod gene inducer, lipid A from a culture of *Rlv* 22 grown in a medium with added hesperitin was analyzed by MALDI-TOF/MS. The results are shown in Fig. 5. The mass spectrum of *Rlv* 3841 grown in the presence of hesperitin is very similar to that of the spectrum from the lab-grown *Rlv* 3841 without the addition of hesperitin (structures I, and II are identical, corresponding to the two major ion clusters found in spectrum from the lab-grown *Rlv* 3841). An additional minor ion cluster is observed around *m/z* 1493.0, and corresponds to structure III (see Fig. 3), which lacks 27OHC28:0. The mass spectrum of the lipid A from *Rlv* 22 grown in the presence of hesperitin is identical to that from *Rlv* 22 grown in the absence of hesperitin (Figure 5B). These results show that the nod gene inducer does not influence the addition of 27OHC28:0 to the lipid A in either the parent *Rlv* 3841 strain or in this *acpXL* mutant, *Rlv* 22. In order to assess the alterations to the carbohydrate portion of the LPS produced by growth in the presence of the nod gene inducer, hesperitin, the carbohydrates were released by mild acid hydrolysis of the LPSs and analyzed directly by HPAEC to observe changes to the core oligosaccharides. In addition, the O-chain polysaccharides were further purified by gel-filtration chromatography using Bio-Gel P2 and their glycosyl residue compositions determined. The HPAEC results are shown in Figs. 6A, B, C, and D respectively, and the structures corresponding to 1, 2, 3 and 4 are shown in Fig. 7. These peaks correspond to monomeric Kdo, GalA, a tetrasaccharide core component, and a trisaccharide core component, that are commonly produced mild acid hydrolysis of *R. leguminosarum* and *R. etli* LPSs. The results reveal that there is little to no difference in these components between *Rlv* 3841 and *Rlv* 22 LPSs and between LPSs grown in the presence or absence of the nod gene inducer, hesperitin. The glycosyl composition results for the O-chain polysaccharides are summarized in Table 5.1, and the different sugars in the O-chain oligosaccharides are presented as relative mole % (Table 5.2). These data clearly show that there

are no significant differences in either the sugar composition of the O-polysaccharides from *Rlv* 3841 when compared to *Rlv* 22, and also *Rlv* 3841+hesperitin compared to *Rlv* 22 + hesperitin.

DISCUSSION:

Lipopolysaccharides constitute an important group of the rhizobial cell-surface molecules, and play a crucial role in the establishment of a normal, nitrogen-fixing symbiosis (Spaink, 2000). The lipid A is the hydrophobic anchor of the LPSs into the outer membranes of Gram-negative bacteria, and is substituted with an unusually long fatty acyl moiety, the 27-hydroxyoctacosanoic acid (27OHC28:0) in most members of the Rhizobiaceae (Bhat, 1991). A specialized acyl carrier protein, AcpXL is required as the acyl donor in the transfer of 27OHC28:0 to $(Kdo)_2$-lipid IV_A, a precursor during the biosynthesis of lipid A (Brozek, 1996), (Geiger, 2002). This reaction is catalyzed by a special C28 acyltransferase leading to a piggy-back acylation (Brozek, 1996) of the 3-hydroxy fatty acyl residue at the 2' position of $(Kdo)_2$-lipid IV_A (Que, 2000a). Mass spectrometry of the purified AcpXL revealed that the major species was 27OHC28:0, and the other mass peaks suggested AcpXL acylation with other (ω-1)-hydroxylated fatty acyl residues, namely C18-C26. Until recently, there were no mutants in either the C28 acyltransferase or the *acpXL*, and hence no description was available of the relevance of the 27-hydroxyoctacosanoic acid substitution in the process of rhizobial symbiosis. In our previous work ((Vedam, 2003), (Vedam, 2004)), we have shown that mutating the *acpXL* results in an LPS that is devoid of 27OHC28:0, and that nodules infected by this mutant are much less occupied compared to wild type infected nodules. Further, mature mutant bacteroids are irregularly shaped and a large proportion of symbiosome membranes enclose multiple, aberrant bacteroids.

During symbiosis, the bacterial symbiont is subject to a number of physiological stresses due to the changing micro-environment in the plant, and hence must be able to adapt to the plant host environment. The conditions likely include changing pH, O_2 tension and osmolarity. As mentioned above, in our previous reports, we have shown that despite these stresses, the *acpXL* mutant is able to invade, nodulate, and fix nitrogen in its pea host even though nodulation was delayed and many severely mis-shaped bacteroids were observed together with some normal looking bacteroids (Vedam, 2003), (Vedam, 2004). The ability of this mutant to form nitrogen-fixing nodules, even with these phenotypic alterations led to the hypothesis that, in planta, the *Rlv* 22 mutant is capable of compensating, at least in part, for the loss of 27OHC28:0. The work presented in this report provides data supporting salt tolerance of ex-nodule *Rlv* 22 isolates, and for the host-specific induction of an alternative mechanism, independent of *acpXL*, for the partial restoration of 27OHC28:0 to the bacteroid LPS.

In this report, we show that the bacteroid LPS from the *acpXL* mutant strain contains a mixture of lipid A structures with, and without 27OHC28:0. One of the first questions following this discovery was whether or not the mutant had genetically reverted in planta. In order to check for this, several hundreds of mutant nodule bacterial colonies were screened through the course of all of our studies. All of the colonies recovered from mutant infected nodules retained the kanamycin resistance marker, and of those tested by PCR and Southern hybridization, all retained the intact *acpXL:: kan* mutation in their chromosome. This indicates that rather than genetic reversion of the mutation, a host-dependent alternative mechanism is induced that enables the partial restoration of the capability of *Rlv* 22 to add 27OHC28:0 to the bacteroid LPS.

In order to investigate the salt tolerance of ex-nodule bacteria, sixteen random isolates were obtained from nodules infected with *Rlv* 22. All of the ex-nodule isolates from nodules infected by *Rlv* 22 were restored to their original parental levels of resistance to NaCl. These ex-nodule isolates were also kanamycin resistant, and when examined by PCR using primers to *acpXL*, contained the intact kan cassette within *acpXL*. Thus, passage through the host plant had altered the salt sensitivity of *Rlv* 22. To determine if the return of salt tolerance was related to partial restoration of 27OHC28:0 to the LPS, the lipid As from two of the ex-nodule isolates, EN2 and EN4, were isolated and analyzed, and were shown to lack 27OHC28; i.e. the EN2 and EN4 lipid As were structurally almost identical to the original mutant lipid A. This indicates that suppression of salt sensitivity does not select for an alternative mechanism to add 27OHC28:0 to the lipid-A. Hence, there is at least a partial in planta circumvention of the *acpXL* mutation, which involves the stimulation of an alternate mechanism for the addition of 27OHC28:0 and is achieved either by physiological conditions, or by other signals within the nodule. However, induction of this alternate mechanism is in addition to, not commensurate with, the selection of a suppressor of salt sensititivity. We also show that induction of the alternate mechanism for 27OHC28:0 synthesis does not involve the *R. leguminosarum* nod gene inducer, hesperitin, since there is no significant difference between the lipid A composition from cultures of *Rlv* 22 or *Rlv* 3841 grown in the presence of hesperitin with those grown in the absence of hesperitin.

A doubling of 27OHC28:0 in the lipid A of *Rlv* 3841 grown under low oxygen conditions has been reported (Kannenberg, 2001) which indicated that low oxygen conditions within the nodule may cause similar changes to the bacteroid lipid A. However, it was found that the lipid A from *Rlv* 3841 bacteroids consisted of the same structures as the lipid A from laboratory-grown cells. This would indicate that the increase in hydrophobicity in the LPS of bacteroids

(Kannenberg, 2001) must largely be due to the reported alterations in the carbohydrate portion of the LPS. Analysis of the carbohydrate portion of the bacteroid LPS is currently being pursued.

The mechanism by which the *Rlv* 22 mutant bacteroids circumvent the mutation to *acpXL* and permit partial restoration of 27OHC28:0 to the lipid A is not yet understood. However, it is known that some of the more 'complex' bacteria possess multiple ACPs that perform different functions. Apart from the known, four major ACPs in rhizobia, genomics predicts even more ACPs (Geiger, 2002). The completed sequence of S. meliloti indicates that there are at least two novel ACPs. One of these is located on the Sym plasmid (it is located in a cluster of four genes, all closely linked, perhaps belonging to one operon), and the other in the chromosome. The *M. loti* genome also contains an operon similar to the one identified in *S. meliloti* (Geiger, 2002). This opens up the possibility that perhaps many rhizobia possess this same operon, and if so, it will be imperative to study the gene product, and its function. It may be possible that one of these newly identified ACPs also exists in the *Rlv* system, and comes into play in planta thereby partially compensating for the lack of the 27OHC28:0 residue during symbiosis. It maybe that there are specific plant signals within the nodule that in response to physiological conditions that are responsible for switching on an alternate mechanism for the addition of 27OHC28:0 to the lipid A of mutant bacteroids. Identification of these signals can be highly complicated, and will be the subject of further investigation.

We have previously shown that the *Rlv* 22 mutant is defective in symbiosis in that nodulation is delayed, misshaped bacteroids are formed, and the synchronous division between the bacteroid and symbiosome membranes is disrupted (Vedam 2003), (Vedam, 2004). Nevertheless that work also showed that nitrogen fixation still occurred indicating that the mutant was able to adapt in some manner to the in planta conditions and overcome its sensitivity to changes in pH and

osmolarity. The work we describe here shows that passage of this mutant through the plant selects for bacteria that are now returned to a parental level of salt tolerance, and that mutant bacteroids are able to partially restore the addition of 27OHC28:0 to their lipid A. Thus, the presence of 27OHC28:0 on the lipid A is mostly likely an essential function for symbiotic infection and/or bacteroid development in pea. In order to fully understand the function of 27OHC28:0 in the symbiotic process, it will be essential to construct a mutant that is completely incapable of adding this residue to the lipid A region. This may be achieved by deletion of the entire *acpXL-lpxXL* gene region. In the event that this deletion results in a pleiotropic mutant phenotype, a double mutant in *acpXL* and *lpxXL* can be constructed, and its symbiotic phenotype studied. In summary, although the work presented here does not give us a complete picture as to the function of 27OHC28:0, it serves as the first step in the elucidation of the role 27OHC28:0, and shows that this residue is very important in the pea-*Rlv* symbiosis. Only further genetic, and biochemical work will help unravel this complex structure-function issue.

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Table 5.1. Bacterial strains used in this report

Strain	Characteristics	Reference/source
R. *leguminosarum* bv. *viciae* 3841	Strain 300 Str^r Fix^+	Wood et al, (Wood, 1989)
R. *leguminosarum* bv. *viciae* 22	Strain 3841 *acpXL::kan* Str^r Km^r Fix^+	Vedam et al, (Vedam, 2003)
R. *leguminosarum* bv. *viciae* 22 EN2*	Strain 22 Str^r Km^r $NaCl^r$	This study
R. *leguminosarum* bv. *viciae* 22 EN4*	Strain 22 Str^r Km^r $NaCl^r$	This study

*EN2, EN4: Ex-nodule isolate #s 2 and 4.

Table 5.2. O-polysaccharide composition table for Rlv3841 & Rlv22

Relative mol%

Sugar	**Rlv3841-lab**	**Rlv3841-Hesp**	**Rlv22-lab**	**Rlv22-Hesp**
Me-6-D-Talose	8.0	9.1	5.7	7.1
Fucose	1.0	0.8	1.0	0.8
Xylose	0.8	0.7	1.1	0.7
Galactose	11	11	10	10
Mannose	8.2	7.8	8.0	7.7
Kdo	6.0	5.8	4.8	4.6
GalA	13	13	14	15
QuiNAc	52	52	55	54

FIGURE LEGENDS

Fig.5.1. Growth of mutant *Rlv* 22 (sector 1), wild type *Rlv* 3841 (sector 2), and ex-nodule isolates EN2 (sector 3) and EN4 (sector 4) in normal medium with 0.5% NaCl (A), and medium with 0.5% NaCl supplemented with kanamycin (B). Media are described in Materials and Methods.

Fig.5.2. Agarose gel showing the *acpXL* (using primers VV) and 16SrDNA (using primers VM) products obtained for

R. leguminosarum 3841 (*Rlv* 3841), *R. leguminosarum* 22 (*Rlv* 22), and Ex-nodule isolate 2 (EN 2).

Fig. 5.3 (i). MALDI-TOF/MS spectra of lipid A from *Rlv* 3841 lab culture (A), *Rlv* 3841 bacteroid (B), *Rlv* 22 lab culture (C), and *Rlv* 22 bacteroid (D).

Figure 5.3 (ii) The identity of each of the proposed structures for the indicated ions (I, II, III, and IV) is shown, and is the same for all the figures.

Fig. 5.4. MALDI-TOF/MS spectra of lipid A from *Rlv* 22 lab culture (A), Ex-nodule isolate EN2 (B), and Ex-nodule isolate EN4 (C).

Fig. 5.5. MALDI-TOF/MS spectra of lipid A from *Rlv* 3841 lab culture, grown in the presence of Nod gene inducer hesperitin (A), and *Rlv* 22 lab culture, grown in the presence of Nod gene inducer hesperitin (B).

Fig. 5.6. Analysis of the LPS core oligosaccharide of Rlv 3841 (A), Rlv 3841 grown in the presence of Nod gene inducer, hesperitin (B), Rlv 22 (C), and Rlv 22 grown in the presence of Nod gene inducer, hesperitin (D). The peaks correspond to monomeric Kdo, GalA, a tetrasaccharide core component, and a trisaccharide core component that are commonly

produced mild acid hydrolysis of *R. leguminosarum* and *R. etli* LPSs. The structures corresponding to these peaks are shown in the panel below.

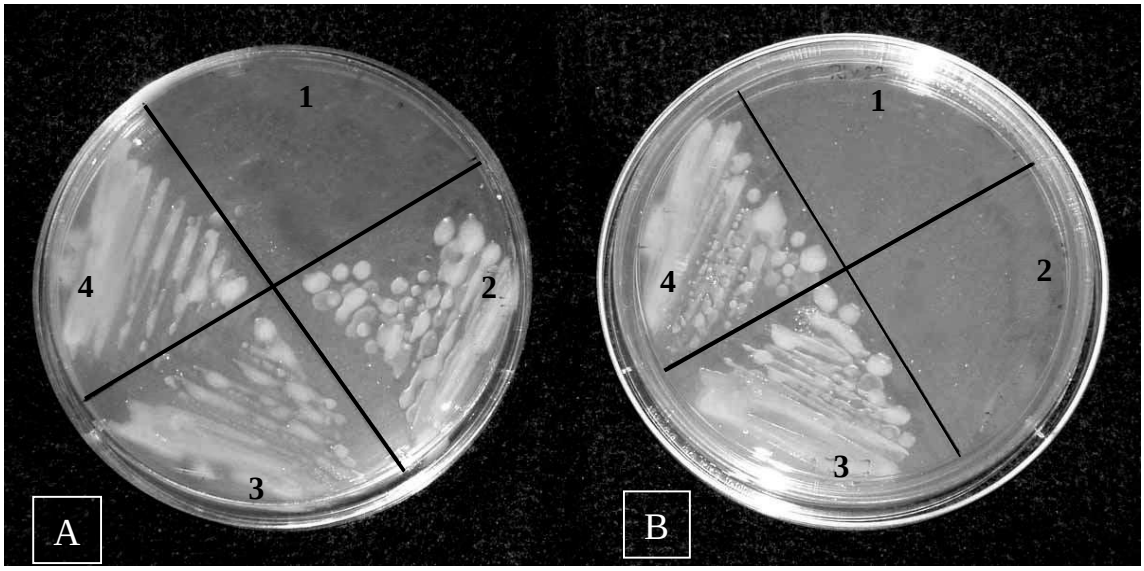


Figure. 5.1

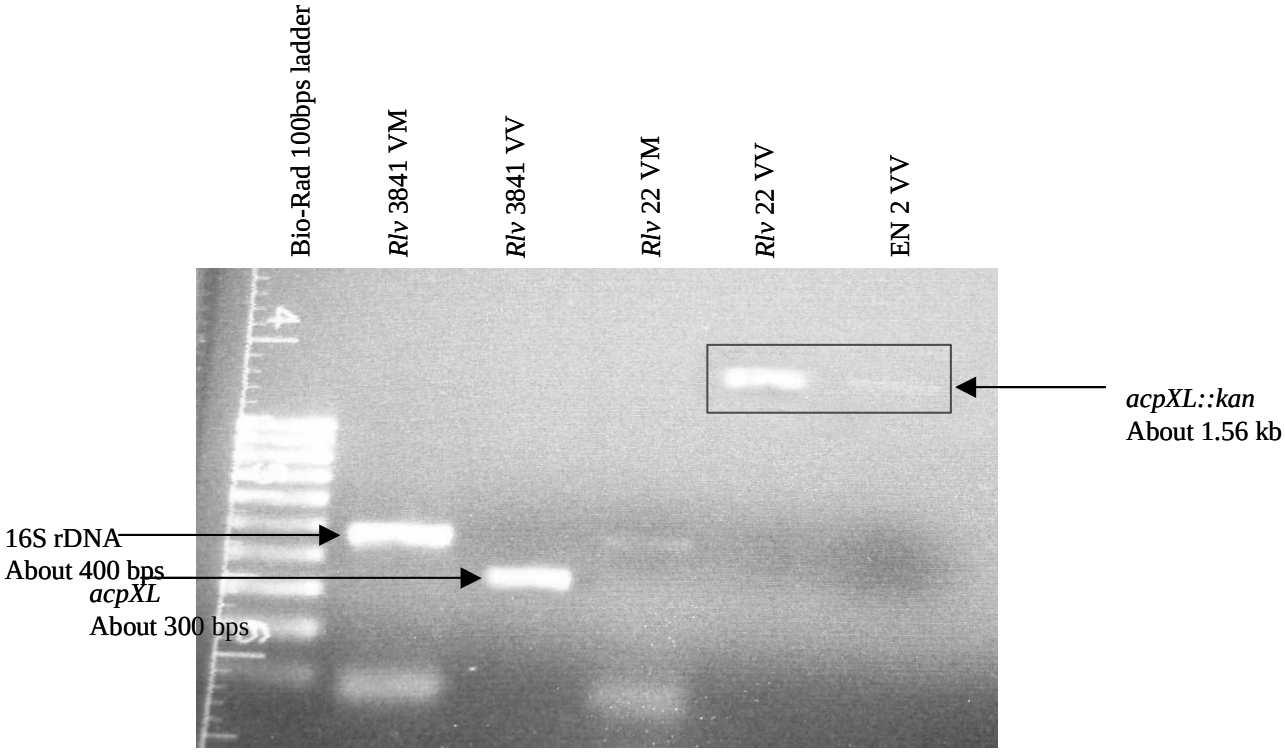

Bio-Rad 100bps ladder
Rlv 3841 VM
Rlv 3841 VV
Rlv 22 VM
Rlv 22 VV
EN 2 VV
acpXL::kan
About 1.56 kb
16S rDNA
About 400 bps
acpXL
About 300 bps

Figure. 5.2

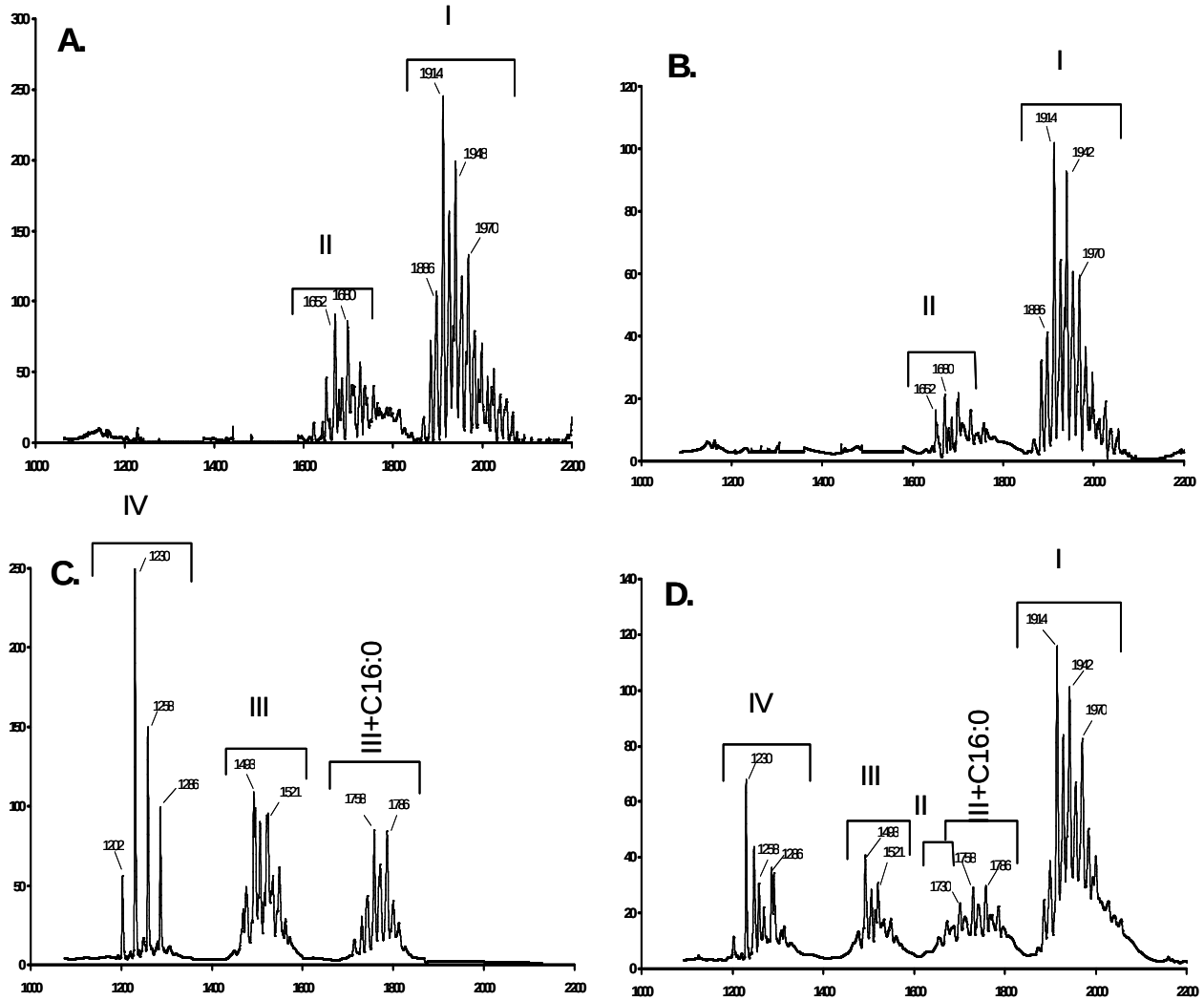
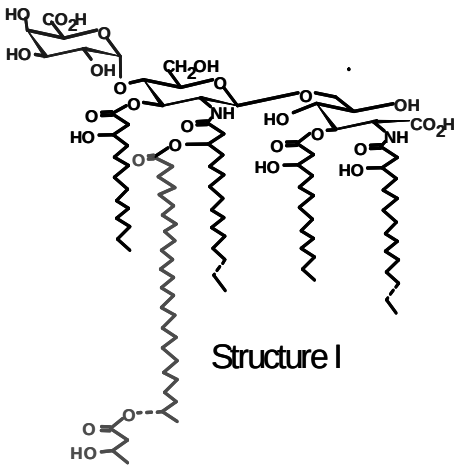
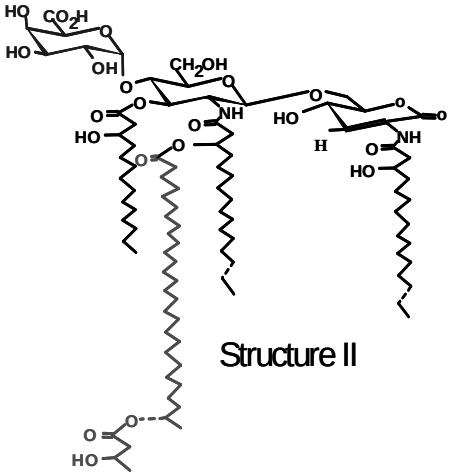

A.
I
II
1914
1942
1970
1886
1652
1680
B.
I
II
1914
1942
1970
1886
1680
1652
C.
IV
III
III+C16:0
1230
1258
1286
1488
1521
1758
1786
1202
D.
I
IV
III
II
III+C16:0
1914
1942
1970
1230
1488
1521
1258
1286
1758
1786
1730

Figure 5.3(i)



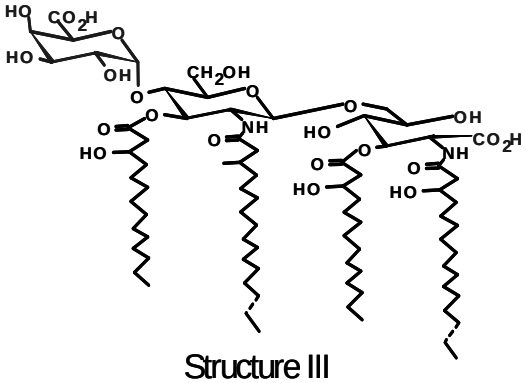
Structure I

$[M-H]^-$ = 1915.4 (-3OHC4:0)
2001.4 (+3OHC4:0)



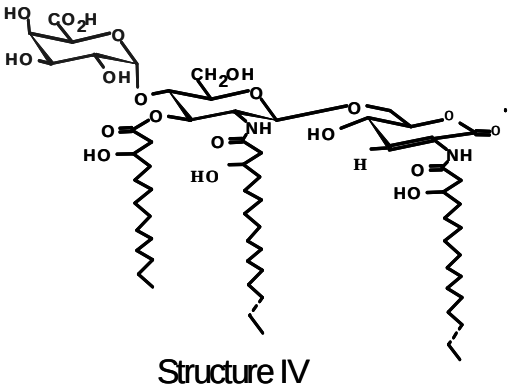
Structure II

$[M-H]^-$ = 1653.5 (-3OHC4:0)
1738.4 (+3OHC4:0)



Structure III

$[M-H]^-$ = 1493.2



Structure IV

$[M-H]^-$ = 1230.6

Figure 5.3 (ii)

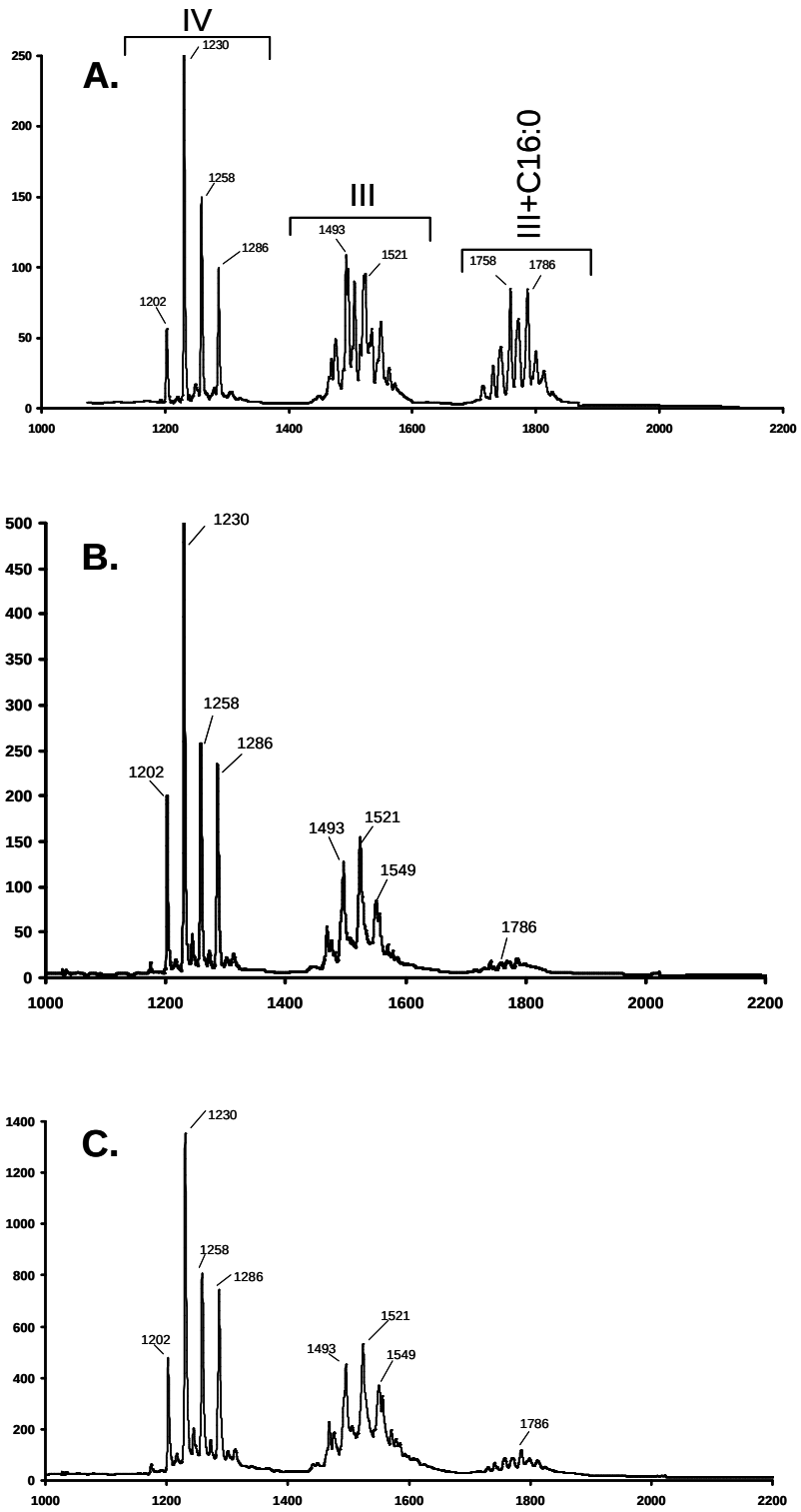

A.
IV
III
III+C16:0
1230
1258
1286
1202
1493
1521
1758
1786
B.
1230
1258
1286
1202
1493
1521
1549
1786
C.
1230
1258
1286
1202
1493
1521
1549
1786

Figure 5.4

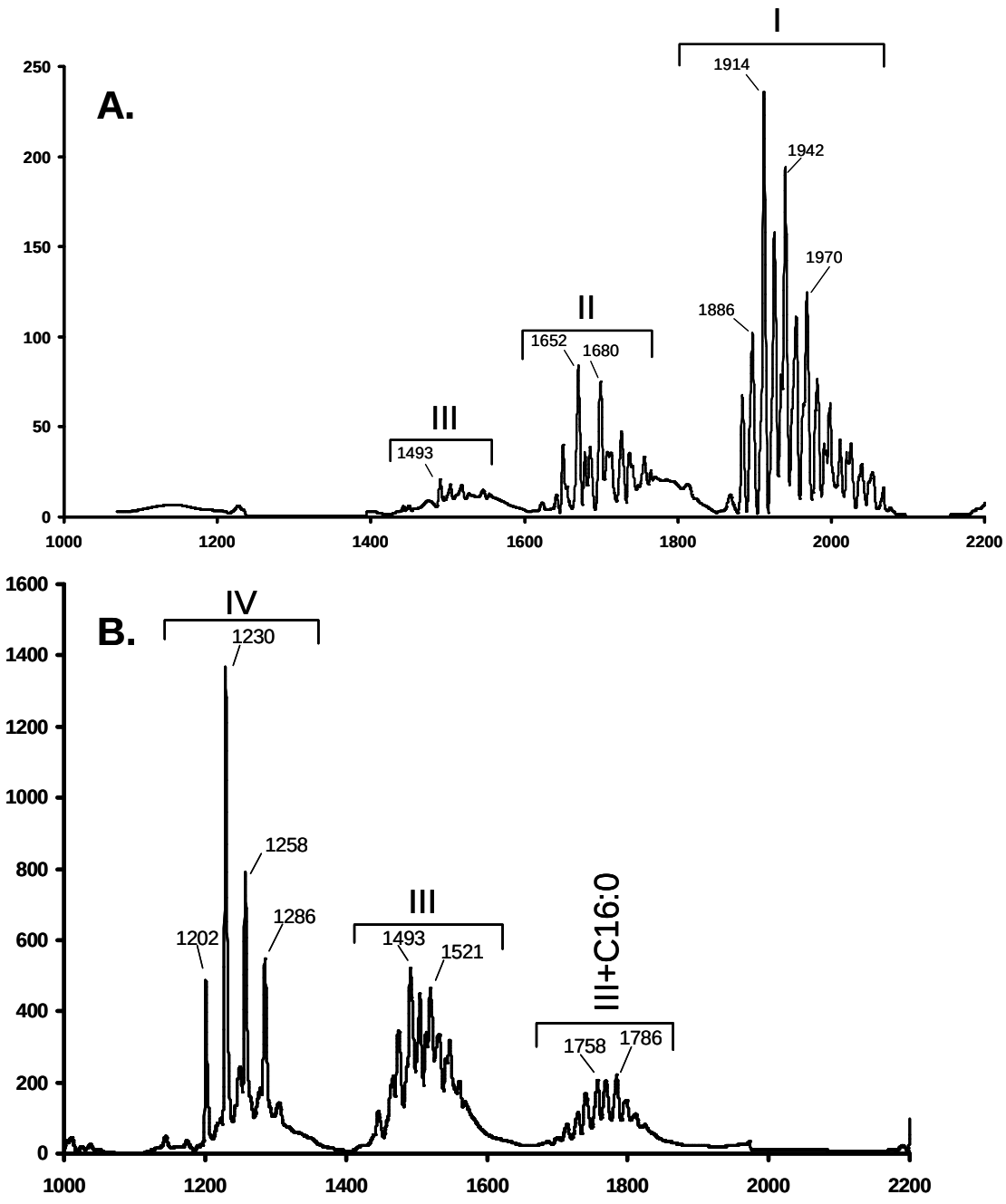

A.
I
1914
1942
1970
1886
II
1652
1680
III
1493
250
200
150
100
50
0
1000
1200
1400
1600
1800
2000
2200
B.
IV
1230
1258
1286
1202
III
1493
1521
III+C16:0
1758
1786
1600
1400
1200
1000
800
600
400
200
0
1000
1200
1400
1600
1800
2000
2200

Figure 5.5

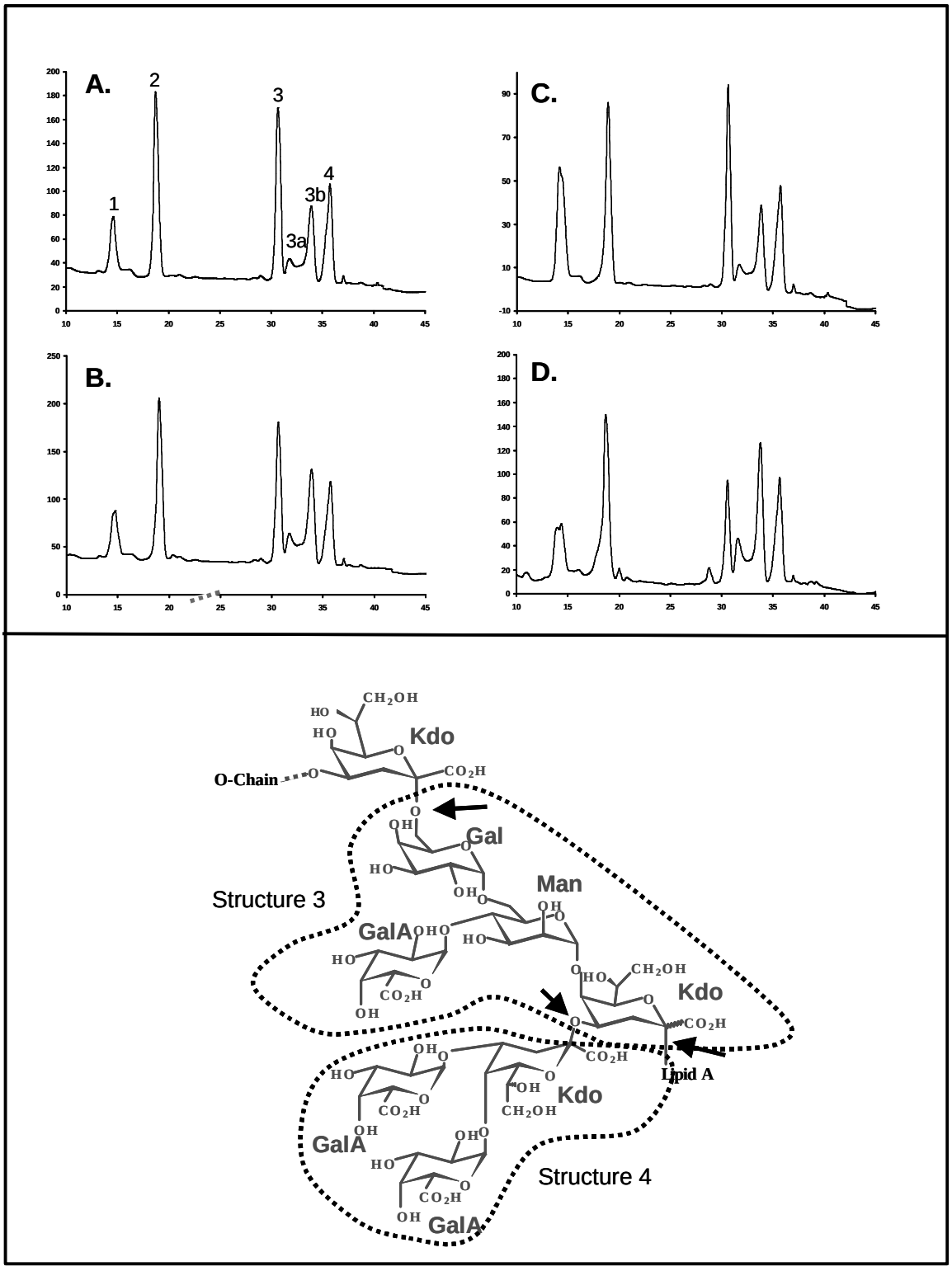

A.
1
2
3
3a
3b
4
B.
C.
D.
O-Chain
Kdo
Gal
Man
GalA
Structure 3
Kdo
Lipid A
Kdo
GalA
GalA
Structure 4

Figure 5.6

CHAPTER 6

SUMMARY AND CONCLUSIONS:

The lipopolysaccharide molecule perhaps plays a pivotal role in the establishment of proper, nitrogen-fixing symbiosis. The correct LPS structure is essential for root hair infection, nodule invasion, for avoiding host defense responses (suppression of host defense responses during these infections) and for physiological adaptation to the endophytic microenvironment. Noel and coworkers proposed that infection requires structure-specific interactions between the bacterial LPS and receptors on the plant cell plasma membrane (Noel, 2000b). Its role has been explained variously in different systems. One of the proposed roles of the LPS molecule has been the suppression of a host response (Fraysse, 2003), (Perotto, 1994), (Albus, 2001). Another role has been postulated for the rhizobial LPS, based on the observation that nodule bacteria produce more predominantly smooth, hydrophobic LPSs (Kannenberg, 2001). Due to the highly hydrophobic surface, the LPS perhaps allows the bacterium to adopt an optimal degree of interaction for close contact with the host plant surfaces, as the plant cell membranes are also hydrophobic (Kannenberg, 2001), (Fraysse, 2003). While the LPS is perhaps crucial in the inhibition of host responses, it is perhaps also involved in allowing the bacterium to adapt to the endosymbiotic conditions (Fraysse, 2003). The function of LPS also appears different for indeterminate and determinate nodules. As infection starts, the presence of O-antigen becomes critical, especially on determinate hosts, because LPS I appears to be required for formation of stable narrow infection threads. This may be related to the direct contact of the bacteria with the host plasma membrane in these narrow structures (Fraysse, 2003). LPS I is not required for

formation of broad infection threads present in indeterminate nodules. In these latter nodules, LPS mutants disturb nodulation at a later stage (Kijne, 1992). Several features of the aberrant nodule development caused by O-antigen mutants suggest that deleterious host defense responses occur throughout the invasion process and may be the principal cause of the symbiotic failure (Perotto, 1994).

Lipid A anchors the LPS molecule to the outer phospholipid layer of the microbial membrane through its numerous fatty acids, hence acting as an anchor molecule (Fraysse, 2003). Recently, progress is being made in understanding the role of this molecule in symbiosis. The lipid As from the members of the family *Rhizobiaceae* and many intracellular pathogens contain 27-hydroxyoctacosanoic acid (27OHC28:0). The conserved nature of this residue suggests that it may be important for the growth and survival of these organisms in their natural environments. Interestingly, the length of this fatty acid could enable its insertion into the phospholipid bilayer, hence perhaps facilitating phagocytosis of the bacteroid by the plant (Fraysse, 2003). A doubling of this fatty acid residue in the lipid A of nitrogen-fixing bacteria within root nodules has been reported, indicating that it is important in the symbiotic process. Considering that the long chained fatty acid is twice the length of a normal acyl chain in the rhizobial lipid A, and that it can span the entire lipid bilayer, this 27OHC28:0 residue could play a crucial role in stabilizing the membrane, especially during the symbiotic process where the bacterium is subject to tremendous environmental stresses. However, the precise function of this residue in symbiosis is not known. The research reported in this thesis focuses on the construction, and characterization of a mutant that is lacking in the 27OHC28:0 residue in the lipid A of *Rhizobium leguminosarum* 3841. The work presented herein provides a partial understanding of the requirement of this

residue in the pea-symbiosis, and raises some very important questions that will require further work in order to fully understand the significance of this unusual residue.

The construction of an *acpXL* minus *R. leguminosarum* strain was described in detail in Chapter 3. Chemical analysis of the lipid A of the mutant strain (*Rlv* 22) confirmed the lack of the 27OHC28:0 molecule, but detailed structural analyses of the LPS revealed that apart from the missing 27OHC28:0 residue, the remainder of the LPS of the mutant strain (*Rlv* 22) was identical to that from the parent strain (*Rlv* 3841). Interestingly, *Rlv* 22 displayed different physiological properties when compared to *Rlv* 3841. For instance, it was unable to grow in the presence of NaCl (0.5%) in the medium, and in acidic conditions (pH5.0). This showed that the osmotic properties of *Rlv* 22 were altered as a result of the LPS mutation, suggesting that *Rlv* 22 would be challenged by the environmental stresses encountered during symbiosis. Hence, it was first thought that a mutant lacking this long chained fatty acid residue would be incapable of fixing nitrogen for its host plant. However, it was found that the mutant is able to invade and nodulate the host plant, and even form nitrogen-fixing nodules (Chapters 3& 4).

In order to assess/compare the efficiency of infection and invasion of host pea plants by *Rlv* 3841 and *Rlv* 22, a time-course experiment was designed as reported in Chapter 4. Results from this experiment showed that the efficiency of infection, invasion and nitrogen fixation were significantly reduced in those plants infected by *Rlv* 22 when compared to plants infected by *Rlv* 3841. In addition, it was noticed that the mutant bacteroids did not mature into the characteristic branched and ‘Y’ shapes. Instead, they were larger, very irregularly shaped, and often multiple bacteroids were enclosed within a single symbiosome membrane. This points to a breakdown in the process of synchronous division, thereby leading to the formation of irregularly shaped, enlarged, multiple bacteroids within a single symbiosome membrane. LPS I appears to be

essential at the stage of bacterial contact with the plasma membrane, i.e. during endocytotic uptake from the infection thread matrix. So far, the mechanisms of involvement of LPS are not clear. However, if uptake of rhizobia proceeds by a `membrane-zippering' mechanism, as suggested for receptor-mediated phagocytosis by macrophages, the distribution of endocytotic factors on the rhizobial surface may determine whether a bacterium is completely endocytosed or not (Lerouge, 2002). A model in which LPS play a role in attachment to the cytoplasmic membrane has some precedence in animal systems where the involvement of LPS in cell adherence and invasion is under discussion (Lerouge, 2002). Whether or not the lipid A portion of LPS also plays an important role in symbiosis is unknown. Que and coworkers, who recently re-evaluated the structure of lipid A in *R. etli* CE3 (Que, 2000a), (Que, 2000b), favor the view that the unusual structure of lipid A in *R. etli* somehow facilitates symbiosis by masking a potential plant “immunostimulatory” activity that might be associated with more conventional (phosphate-containing) lipid A structures. Perhaps, the plant does not reject the *R. etli* endosymbiont because *R. etli* lipid A is not recognized by the innate-like immune system of the plant. In this scenario, the plant could still respond to infections by Gram-negative pathogens that possess a conventional phosphate-containing lipid A moiety, while retaining the nitrogen-fixing endosymbiont (Lerouge, 2002).

Synchronous division probably involves a strong adhesion between the O-chain polysaccharide of the LPS and the symbiosome membrane. This force of adhesion is perhaps counter-stabilized by the 27OHC28:0 which then functions by providing a stabilizing force on the bacterial membrane. Thus, it can be envisioned that the 27OHC28:0 residue provides an anchoring force for the lipid A to the bacterial outer membrane. As a result of the mutation in *acpXL*, this force is reduced thereby causing many disruptions in the areas of adhesions between

the bacterial outer membrane and the symbiosome membrane. Thus, the division of the symbiosome compartment (and the enclosed bacteroid) would be impaired, hence resulting in the large, and very irregularly shaped bacteroids, which was the phenotype observed in our study, as discussed in Chapter 4.

Chapter 5 describes the detailed chemical analysis of LPS from both wild type, and mutant bacteroids. MALDI-TOF analysis of the *Rlv* 22 bacteroid LPS revealed the presence of two populations of lipid A molecules: one lacking the 27OHC28:0, and another population similar to wild type containing the 27OHC28:0 residue. In order to exclude the possibility of reversion, a several nodules were screened for the retention of antibiotic resistance marker. Bacteria extracted from nodules were tested for their resistance to kanamycin (introduced in the original mutation), and there were no cases of loss of the resistance marker. Additionally, an optimal number of ex-nodule bacteria were further tested by PCR to confirm the mutation.

In Chapter 5, we also describe a more detailed analysis of *Rlv* 22 isolates from host nodules (ex-nodule bacteria). These ex-nodule bacterial isolates were examined for their sensitivity to salt in comparison with the *Rlv* 3841 parent as well as with the original Rlv 22 mutant which is sensitive to 0.5% NaCl. All of the ex-nodule isolates tested behaved like wild type, i.e. they showed no sensitivity to 0.5% NaCl and behaved like the original wild-type strain. These strains were again confirmed to be kanamycin resistant, and PCR analysis also confirmed the original mutation to be intact in these strains. Chemical analysis of the lipid A of these strains showed that the lipid-A of the mutant ex-nodule isolates had the same structure as the original *Rlv* 22 mutant lipid A; namely, it completely lacked the 27OHC28:0 residue. These results suggest that once within the nodule, the mutant is tolerant to salt, and that *in planta*, the partial addition of 27OHC28:0 to the lipid-A also occurs. The fact that such strains, under laboratory

conditions, can grow in 0.5% NaCl but not add 27OHC28:0 to the lipid-A suggests (a.) that the mechanism for salt tolerance is not directly related to the presence of 27OHC28:0 on the lipid-A, and (b.) that the addition of 27OHC28:0 to the mutant lipid-A within the nodule depends on activation of as yet unknown genes by *in planta* environmental conditions and/or signal molecules.

The partial restoration of the ability to add 27OHC28:0 to the LPS of the mutant inside the nodule by a mechanism alternative to *acpXL* suggests that the 27OHC28:0 residue is essential for symbiotic infection. Certain bacterial species are known to possess multiple ACPs, which perform specialized functions. It is conceivable that there is more than one ACP, which can function in the transfer of the 27OHC28:0 to the lipid A in *Rhizobium*. Most likely, this second ACP is only turned on when the rhizobia are inside the root nodule, for there were no detectable amounts of the 27OHC28:0 in the lipid A of LPSs extracted from lab grown cultures.

A more thorough understanding of the role of 27OHC28:0 in symbiosis will be possible only from the study of a mutant that entirely lacks this residue. This could perhaps be achieved by the construction of a double mutant lacking in both *acpXL* as well as *lpxXL*, or by deleting the *acpXL- lpxXL* gene region. Future directions for this project include the complete structural analysis of the bacteroid LPS, and identifying the (proposed) alternate Acp, by constructing and characterizing the above-described double mutant.

As described previously, in the determinate root nodule system, there is intimate contact between the bacterial membrane and the symbiosome membrane, and this is important during the formation of infection threads. To test the proposed model for the function of 27OHC28:0, future work will involve the construction of an *acpXL* mutant in a determinate system (e.g; *R. etli* - bean) and testing the symbiotic phenotype of such a mutation on host plants. As previously

discussed, literature suggests that LPS mutants of determinate host systems disturb symbiosis at an early stage. It will hence be interesting to see whether the *acpXL* mutation in such a system will affect the symbiosis at the early infection stage, perhaps during thread formation.

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